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The secret of Mediterranean food: How "omic" sciences, biochemistry and human physiology can be applied to exploit the secrets of Mediterranean foods

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Review

From Achille Bertelli onward: more than 100 years of research and production of dietary supplements based on natural molecules typical of the Mediterranean diet

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Keywords

Achille Bertelli • Catramina Bertelli • Aerostave • Natural molecules • Mediterranean diet

Summary

Achille Bertelli was an aeronautics pioneer and an innovative entrepreneur of the pharmaceutical industry. After graduating in Chemistry in Italy, he moved to the United States of America where he opened a chemical-pharmaceutical laboratory in San Francisco in 1879, and later moved back to Italy where he opened a chemical and pharmaceutical industry in Milan (1886). The "A. Bertelli" pharmaceutical company developed the famous cough pills "Catramina Bertelli", as well as new cosmetics and perfumes. Apart from his chemical experience, Achille Bertelli was a passionate aeronautics expert. He wrote many essays on this topic and devoted himself to aeronautical experiments by designing the apparatus "Autovol", "Aerocurvo", "Autovol no. 2", "Autovol no. 3", and "Aerostave", which are considered the prototypes of the helicopter. Achille Bertelli was also the president of the Electric Company of Salò, which installed an electrical system that served the lighting in many cities on Lake Garda (Italy). Finally, Achille Bertelli also participated in the Italian revival after the First World War, especially by supporting the agricultural revival. Throughout his life, Achille Bertelli teamed with several famous people from all over Italy, such as Gabriele D'Annunzio, Cesare Lombroso and Cordero di Montezemolo. Today, Achille Bertelli's interest for natural molecules, his ideas, and his entrepreneurial approach are carried forward by his descendant, Matteo Bertelli.

Achille Bertelli's biography

Achille Bertelli was born in Brescia on January 6th, 1855 [1, 2]. He was a great aeronautics pioneer and an important and innovative entrepreneur. In 1875 he graduated in Chemistry at the University of Pavia and a few years later he decided to move to North America in search of new stimuli for the opening of his own business. This journey led him to open a chemical-pharmaceutical laboratory in San Francisco (1879). This experience gave him the possibility to acquire decisive skills and knowledge that led him to become a point of reference for the other experts in this field.

After his North American experience, in 1884, he decided to return to Italy. He moved to Milan, where he opened a factory of chemical and pharmaceutical products, which he managed to advertise on a large scale thanks to an innovative approach, implemented in collaboration with his friend Gabriele d'Annunzio, one of the most important Italian poets of the 20th Century [3]. Later, in 1898, his factory was transformed into the company of pharmaceutical chemicals "A. Bertelli", of which he was president. Achille Bertelli's success in those years was due to the fact that his products were purely based on natural extracts, for example the catramin-based cough pills called "Catramina Bertelli", which were much appreciated by the elite of the contemporary society, including scientists and intellectuals [3]. More specifically, the product "Catramina Bertelli" was presented also with the help of University professors, such as Cesare Lombroso, a Sephardi Jewish scholar and a dear friend of Achille Bertelli [3].

But the activities of an entrepreneur were not enough for Bertelli's active mind: he was also very interested in the aeronautical field [3]. He wrote many essays on this topic and devoted himself to aeronautical experiments by designing a first apparatus called "Autovol", patented in 1902. Two years later, in Paris, he set up and tested the "Aerocurvo", followed by "Autovol no. 2" (which remained at the project stage) and by "Autovol no. 3", considered the prototype of the helicopter [2, 4].

Dissatisfied with the results of these previous experiments, between 1905 and 1906 Achille Bertelli teamed with Cordero di Montezemolo to design and build a sort of helicopter "with mixed support, static and dynamic, called 'aerostave' (Fig. 1) [2, 4, 5]. In the same period, he patented an emulsion pump for extracting water from deep veins, which was widely employed by the army [2, 6].

Among the countless sectors in which Achille Bertelli worked, it is important to mention the electrical branch: when he was president of the Electric Company of Salò, together with its friend Senator Giuseppe Zanardelli, he installed an electrical system that served the lighting in the towns of Toscolano, Maderno, Salò, and Desenzano, thus bringing countless benefits to the population of that area on the shores of Lake Garda [3, 8].

Fig. 1. Bertelli's "aerostave" [7].

Dr. Achille Bertelli did not stop even during and after the First World War: he organized several welfare initiatives to relieve the Italian population from the economic problems derived from the war, especially in the field of agricultural revival, of which he was a very competent and passionate supporter. His intense and profound life came to an end on July 24th, 1925 [2, 9].

Pharmaceutical factory

In 1886, after his experience in North America, Achille Bertelli decided to return to Italy to open his innovative Chemical Pharmaceutical Laboratory Achille Bertelli & C., which was founded in Milan, near the church of San Babila [1].
What brought Achille Bertelli to establish his own chemical-pharmaceutical company were cough pills. These tablets were called "Catramina Bertelli" and for some decades this product was widely advertised, especially in newspapers and magazines (Fig. 2). The success of "Catramina Bertelli" at the end of the Nineteenth century led the company (which in the meantime had become a joint stock company, with a capital of 1,250,000 lire) to move to a larger factory in via Paolo Frisi, 26 (section of the road now called "via Maiocchi"), which was completely destroyed by the bombings that struck Milan during the Second World War [1].
In addition to pharmaceutical specialties, Dr. Bertelli also dealt with cosmetics: his company also produced a line of creams, mouthwashes and perfumes [1].
In order to promote these products, he commissioned the production of beautiful, scented calendars, which also attracted refined collectors. Such innovative marketing campaign, which had never been carried out before, led to a great success of these pills. Indeed, Dr. Bertelli proved to be a forerunner of modern advertising techniques.

A century later - From Achille to Matteo Bertelli

More than a hundred years have passed from Achille Bertelli to his descendant Matteo Bertelli, but history has not changed: just like the man who inspired him, Dr Matteo Bertelli brought innovations to the medical-scientific field.
Matteo Bertelli was born in Desenzano del Garda on November 16, 1973. He graduated in Medicine in 1998 at the University of Brescia, specialized in Medical Genetics in 2002 and completed a PhD in Medical Biotechnology in Siena in 2008.
In 2020 Matteo relaunched the logo of A. Bertelli's company, which made the history of Italy in the field of chemical-pharmaceutical production. Like his ancestor Achille, Matteo is interested in natural molecules and set up a biotechnology company to create products especially aimed at the well-being of the respiratory tract and oral cavity.
Matteo intends to follow in his ancestor's footsteps, carrying forward the ideas and entrepreneurial approach of Achille Bertelli, in the broader context of a new Rinascimento, where Italian science and business regain the world stage.
As Achille Bertelli did, Matteo intends to go beyond the Italian borders, reaching the United States of America. His primary goal is to bring to the USA a series of dietary supplements based on plant extracts that originate from typical ingredients of the Mediterranean diet. Moreover, he plans to develop research laboratories in which DNA and RNA analysis can be carried out, as he is doing in Italy, as well as employ mass spectrometry to improve the discovery of active molecules in natural products.

Fig. 2. Advertisement for "Catramina Bertelli" pills [10].

To conclude, the interest on natural molecules and on the Mediterranean diet guided Achille Bertelli's research and enterprise, and his efforts inspired generations of scientists, including Matteo Bertelli.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; VM, KD, SG, GB: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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Review

Foods of the Mediterranean diet: tomato, olives, chili pepper, wheat flour and wheat germ

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Keywords

Mediterranean diet • Flavonoids • Wheat germ • Chili pepper • Tomatoes • Olives

Summary

Mediterranean people, which follows a diet rich in minimally-processed plant-based foods, are believed to live longer and healthier lives than many other populations in the Western world. Epidemiological and clinical data suggest that the Mediterranean diet has beneficial effects for several chronic diseases, such as cardiovascular diseases, obesity, cancer and diabetes. Although the mechanisms of action of the Mediterranean diet are not completely clear, the synergistic effects of a number of its components and their bioactive phytochemicals exert antioxidant, anti-inflammatory, anti-microbial and anti-cancer effects. The Mediterranean diet includes daily consumption of whole cereals, fruit, vegetables and legumes in moderate proportions, weekly consumption of white meat in low to moderate proportions and occasionally sweets and chocolates in small amounts. Since olive oil is the main lipids source, it has special significance for health. Healthy fruit and vegetables, rich in phytochemicals, are a major proportion of this diet and contribute to the overall nutritional value and bioactivity of its components. Here we review the nutritional and health benefits of wheat germ, tomatoes, olives and chili pepper, items at the base of Mediterranean diet food pyramid that provides beneficial molecules, such as polyphenols, vitamins and flavonoids, and exert anti-inflammatory, anti-microbial and anti-oxidative actions.

Introduction

The Mediterranean diet is currently considered one of the healthiest diets in the world [1-3]. It features daily intake of nuts, vegetables, fruit, olives, olive oil, legumes and whole grains. Rich in antioxidants, fiber, vitamins, minerals, phytosterols, probiotics, omega 3 and omega 6 fatty acids, it has value for human health and wellbeing [1-3]. *Mediterranean diet* is a term coined for the dietary pattern of the people of the southern European countries in 1960s. This population was observed to have a high adult life expectancy, and low rate of diet-related chronic diseases and of certain cancers. The food pattern is typically represented in the form of a pyramid [1]. Indeed, regular consumption of whole grains, vegetables, fruit and legumes with plenty of olive oil and moderate weekly consumption of white meat and low consumption of red meat were the main features of their diet. Although it is still unclear why this diet affords health benefits, epidemiological and clinical studies have produced much evidence that it has lipid-lowering effects, scavenges free radicals thereby reducing oxidative stress, suppresses inflammation and platelet aggregation, restricts specific amino acids thereby inhibiting nutrient-sensing pathways, modulates and regulates growth factors and hormones involved in oncogenesis, and shapes the gut microbiota and corresponding metabolites, thereby influencing overall metabolic health [1-5]. Further research is needed to define the specific effects Mediterranean diet's nutrients, and how their modification can modulate the microbiome, energy intake and expenditure, thus influencing cells, tissue and organ health during aging.

In addition, the Mediterranean diet is abundant in fresh fruit and vegetables and relies heavily on olive oil as lipid source. Low to moderate consumption of dairy products, red meat, fish, seafood, poultry and eggs [1-3] is another feature. Various studies report that portion-controlled consumption of the components of the diet is beneficial in preventing non-communicable diseases, including chronic and inflammatory diseases such as obesity [4-6], cardiovascular disease [7], diabetes [8, 9], metabolic syndrome [4] and cancer [10] and has a positive impact on autoimmune diseases [11, 12]. The diet has been observed to reduce inflammation and enhance mobility and vitality in patients with rheumatoid arthritis [12] and to decrease the risk of other autoimmune diseases including multiple sclerosis [11]. Among the several foods typical of the Mediterranean diet, wheat, tomatoes, chili pepper and olives proved to have high beneficial effects by many studies. Thus, in this review we focused on the beneficial properties of these specific foods.

Whole grain cereals

Since ancient times, cereals have remained the major staple food across the globe. Cereals are an important

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content of every diet, providing carbohydrates, dietary fiber and bioactive molecules with antioxidant, anti-cancer and anti-thrombotic action [13, 14]. Cereals and their derivatives, such as bread and pasta, contribute 55-60% of the total calories of the Mediterranean diet and are therefore indicated at the base of the food pyramid [15]. Unlike refined cereals, whole grain cereals maintain bran, germ, thus containing several bioactive molecules that promote health and mitigate metabolic disorders [16].

Whole wheat

Wheat is not only an important cash crop but also one of the most widely consumed staple foods in the world. Besides being a major source of starch, dietary fiber and energy, wheat provides substantial amounts of protein (60-70% of which is gluten), B vitamins, carotenoids, phenolic acids, benzoxazinoids, tocopherols, alkyl-resorcinol, phytosterols, biogenic amines and lignans [13-15]. The high fiber content of whole wheat helps digestion and bowel movements, thereby decreasing the risk of intestinal cancer [17]. Phenolic compounds, vitamin E and carotenoids with antioxidant properties help scavenge free radicals, thereby protecting against their deleterious effects.
Wheat polyphenols and antioxidants prevent or decrease the development of several chronic diseases, among which colon and breast cancer, type 2 diabetes and cardiovascular diseases [18-21]. Different varieties of bread wheat and wild-type wheat have been explored for their chemical constituents and bioactive compounds. Some bioactive compounds, such as phenols, antioxidants and vitamin E, are present in almost all types of wheat, however wild-type wheat contains more bioactive compounds. For instance, a comparative study of *Triticum monococcum* ssp. *Monococcum*, *Triticum aestivum* (bread wheat) and *Triticum durum* Desf. (durum wheat) revealed that the first had significantly higher total phenolic, ferulic acid and p-coumaric acid contents than bread and durum wheat, indicating its antioxidative activities and potential health benefits in reducing and preventing cardiovascular disease, diabetes and cancer, in addition to its valuable nutritional properties [22].
The main antioxidants of wheat grain are terpenoids and phenolic acids, such as hydroxycinnamic acid derivatives. They are not present in white wheat flour, while they are concentrated mainly in the bran and germ of wheat [21]. Examples are synapic and p-coumaric acids and dehydrodimers and dehydrotrimers of ferulic acid [23]. The phenols are bound to the cell wall of the bran by ester bonds. The highest antioxidant activity occurs in the aleurone layer of the wheat grain [24]. Other antioxidants in wheat bran are flavonoids, carotenoids (mainly lutein) and lignans [25, 26]. In addition to this, wheat contain many vitamins. Indeed, wheat is rich of vitamin B1 (thiamin), B2 (riboflavin), B3 (niacin), B6 (pyridoxine) and B9 (folate) [27].

Wheat germ

Wheat germ is usually removed with the milling process, although it is rich in nutrients [17]. Indeed, what germ contains a high amount of proteins and carbohydrates, as well as water and lipids. Moreover, as well as whole wheat, wheat germ contains bioactive molecules such as antioxidants (e.g., flavonoids, polyphenols, tocopherols, tocotrienol i.e. vitamin E), carotenoids, plant sterols and biogenic amines [18] (Tab. I).
Biogenic amines have several beneficial effects for human health, especially on fat metabolism, blood pressure and neurotransmitters regulation, but they can cause food poisoning if assumed in excess [19, 20]. Examples are putrescine, spermine, spermidine and histamine. Indeed, they are used as industrial food quality parameters in the Chemical Quality Index [19]. Several research studies proved that these polyamines have an antioxidant activity, preventing damages to cellular membrane and nucleic acids [18]. Wheat germ is rich in polyamine, especially if compared to other foodstuffs [21].
Another significant bioactive compound of wheat is gamma-oryzanol, a phenolic compound with antioxidant activity. Moreover, it participates in lipid metabolism, lowering lipid uptake and improving blood lipid levels [22]. Gamma-oryzanol have been tested in various diseases, such as diabetes, hyperlipidemia and cancer, for its beneficial effects [23-27].

Vegetables and fruit

Bran cereal fiber, fruit, vegetables and tea have a special place in the Mediterranean diet as they inhibit the onset and progress of cardiovascular disease and cancer by virtue of their antioxidant, anti-genotoxic, anti-inflammatory properties.

Tomatoes

Tomatoes both raw and cooked are extensively consumed in the Mediterranean region. Tomatoes are low

Tab. I. Classification of bioactive phytochemicals in whole wheat and wheat germ.

Classification	Bioactive compounds	Bioactivity	References
Polyamines	Putrescine, spermine, and spermidine	Healthy metabolic function, antioxidants	[18, 19]
Phenolic acids	Hydroxy benzoic acid and hydroxy cinnamic acid derivatives e.g., γ-oryzanol, ferulic acid	Antioxidants, lower lipid absorption, anti-cancer, anti-inflammatory	[22]
Tocopherol	Alpha tocopherols and tocotrienols	Cardioprotective	[23]
Carotenoids	Carotenoids, lutein	Anti-cancer, anti-proliferative	[24]
Phenolic lipids	Alkylresorcinols such as 5-alkenylresorcinol	Antioxidant, anti-genotoxic and cytostatic activities	[22]

in fats, rich in vitamins A and C, folate, potassium, carotenoids and polyphenols [28]. Dietary consumption of ripe red tomatoes (rich in polyphenols such as lycopene, flavanones and flavones and carotenoids such as phytoene, β-carotene and lycopene) has beneficial chemoprotective, anti-inflammatory, anti-genotoxic and anti-proliferative effects [29]. Tomato has been studied for its biologically active compounds (Tab. II).

Rich in phytochemicals with anti-proliferative, anti-mutagenic, anti-cancer, anti-inflammatory, and anti-oxidative properties, tomatoes inhibit the onset and progression of chronic diseases, such as cardiovascular disease and cancer. For instance, lycopene and vitamins C and E reduce oxidative stress and the risk of chronic diseases [34, 35]. Specifically, blood concentrations of lycopene are reported to be inversely correlated to the incidence of heart disease [37]. Tomato intake is also inversely correlated with atherosclerosis by virtue of its anti-inflammatory properties [38]. Lipophilic compounds in tomatoes modulate LDLs and the corresponding atherogenic processes in endothelial cells, thereby reducing the risk of cardiovascular disease and atherosclerosis [39].

Tomato products have shown protective effects against lung and prostate cancer [40, 41]. This may be because polyphenols and carotenoids prevent tumorigenesis by impeding the initiation and progression of cancer [34]. Moreover, flavonoids such as quercetin foster chromatin remodeling, thereby inhibiting epigenetic changes that promote cancer [34].

Because tomatoes are rich in carotenoids, tomato consumption promotes intercellular signaling pathways, modulates immune reactions, regulates the cell cycle, induces apoptosis, and interacts with many physiological systems, thereby playing an important role in protecting against chronic diseases [42]. One of the most studied carotenoids of tomato is lycopene. It is stably released during cooking when the plant cell wall is disrupted by heating [43].

In the Mediterranean diet, absorption of lycopene, a non-polar compound, is favored by olive oil. Since lycopene has many useful properties, it has been extensively studied. Figure 1 summarizes some interesting studies on lycopene bioactivity.

Olives

Olives trees, olives and olive oils have special significance in the Mediterranean region, providing an important food and nutrient source for the indigenous populations of the region. The fruit of the olive tree is processed to obtain table olives which are consumed daily in a moderate amount. Olives contain nutritional and non-nutritional components such as carbohydrates, proteins, lipids, minerals, water and phenols [62]. Phenolic compounds occur in almost all parts of olives in different concentrations. The major phenolic compounds are acids, alcohols, flavonoids and secoiridoids [63, 62]. The hydroxytyrosol derivatives, ligstroside, oleuropein and verbascoside are the most abundant biologically active phenolic alcohols of the olive fruit along with tyrosols [63]. They are produced by hydrolysis of oleuropein in the unripe fruit, removing the typical bitter taste of untreated olives [64, 65]. Besides being precursors of hydroxytyrosol, ligstroside and verbascoside, oleuropein has cardioprotective, anti-hypertensive, antioxidant and anti-inflammatory properties [66]. Hydroxytyrosol has been proposed for the treatment of diseases, such as lymphedema and COVID-19 [67-71]. Olive fruits are also rich in flavonoids such as quercetin-3-rutinoside, apigenin-7-rutinoside, apigenin-7-glucoside, luteolin-7-glucoside and luteolin-5-glucoside [72].

Since the phenolic compounds of olive fruits are high in hydroxytyrosol and flavonoids, they have anti-carcinogenic, anti-proliferative and antioxidant activity [73]. Hydroxytyrosol has been shown to scavenge free radicals and activate glutathione reductase and superoxide dismutase [74, 75]. It also has anti-inflammatory properties through inhibition of prostaglandin E, nitric oxide and inflammatory cytokines release [76]. It plays a cardioprotective role by reducing oxidative stress [19, 64], and has cancer preventing properties, significantly suppressing tumor proliferation in skin and breast tissue [77]. Hydroxytyrosol, oleuropein, ligstroside and verbascoside are also useful in the treatment of osteoarthritis [78], in neuroprotection and for wound healing (Fig. 2) [79].

Chili pepper

Epidemiological data regarding chili pepper, a significant constituent of the Mediterranean diet, is still scarce. Chili pepper (*Capsicum annuum*) belongs to the highly diverse and globally distributed genus *Capsicum* of the Solanaceae family, in which domesticated species such as *C. baccatum*, *C. pubescens*, *C. chinense* and *C. frutescens* are also present. Although chili pepper is best known for its pungent flavor, it has significant amounts of carotenoids, provitamin A, vi-

Tab. II. Bioactive compounds of tomato and their health effects.

Classification	Bioactive compounds	Bioactivity	References
Phenolic acids	Caffeic, chlorogenic, synapic, p-coumaric and ferulic acids	Reduction of oxidative stress	[30, 31]
Flavonoids	Quercetin, rutin, kaempferol, chlorogenic acid and naringenin	Antioxidant, anti-cancer	[32, 33]
Carotenoids	Lycopene, α-carotene, β-carotene, γ-carotene, δ-carotene, phytoene, phytofluene, neurosporene and lutein	Antioxidant	[34]
Vitamins	A, B, C and E	Antioxidants, radical scavenging	[35]
Glycoalkaloids	α-tomatine and dihydroxy tomatine	Toxic to pathogens	[36]

Fig. 1. Health benefits of lycopene.

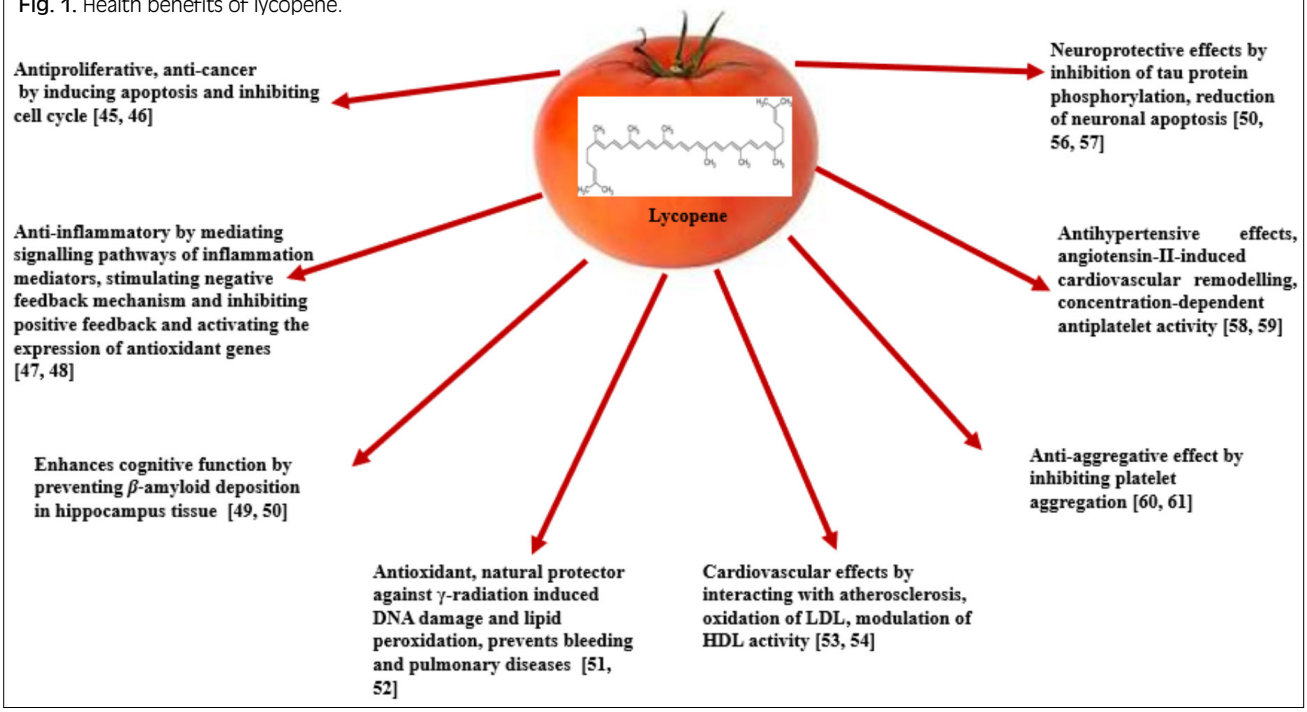
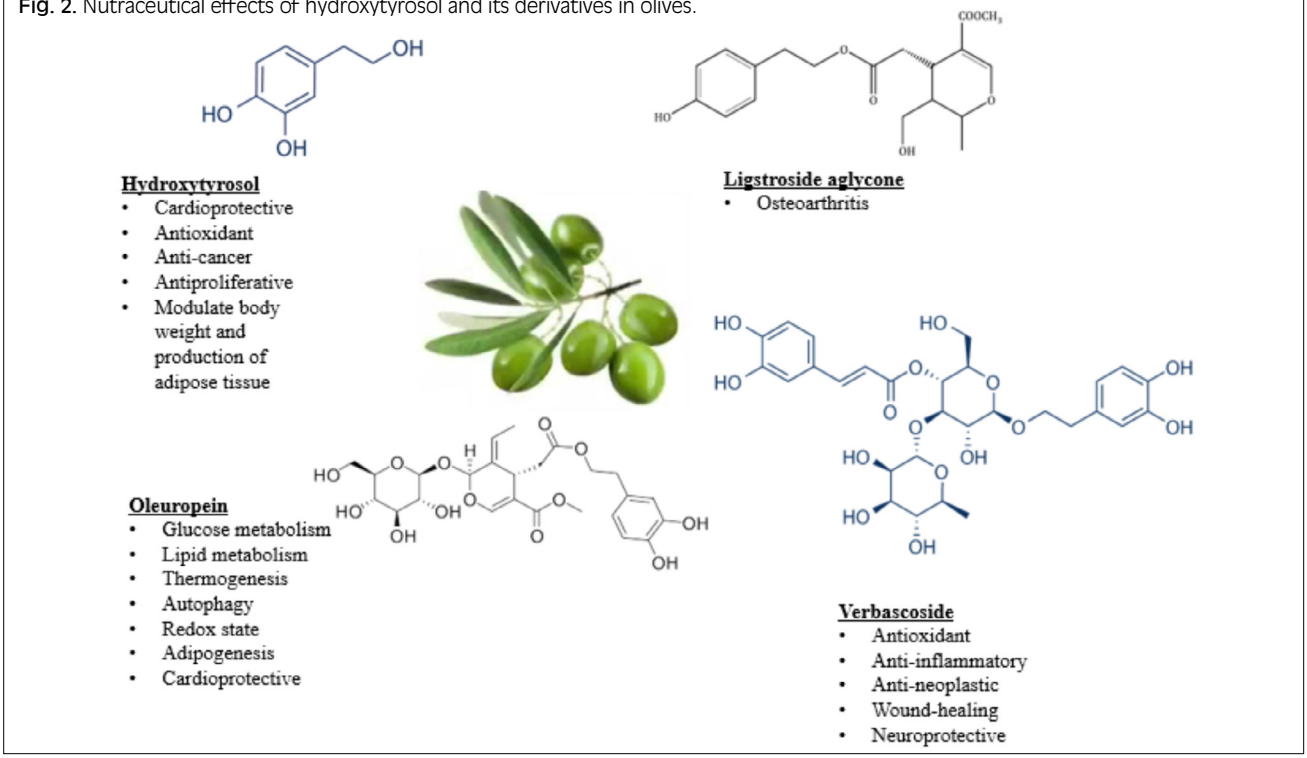


Fig. 2. Nutraceutical effects of hydroxytyrosol and its derivatives in olives.



tamins K, E and C and phenolic compounds such as capsaicinoids, quercetin and luteolin (Fig. 3), which act synergistically to confer antioxidant, anti-inflammatory, anti-cancer, anti-microbial, antiseptic and immunomodulatory properties [80-82]. This makes chili pepper particularly useful in scavenging free radicals and promoting good health.

Chili pepper have been used for the treatment and prevention of several conditions, such as toothache, wound healing, sore throats, cough, parasitic infections and rheumatism. Creams containing capsaicin and capsicum are commercially available for intractable pain and HIV-linked neuropathy [82]. Antioxidant carotenoids (α- and β- carotenes), vitamin C and pro-vitamin A in capsicum peppers support immune function and combat inflammation, thereby easing rheumatism, asthma attacks and arthritis [83]. Chili pepper has been studied for its biologically active compounds (Tab. III).

Tab. III. Biologically active metabolites of chili pepper.

Biological activity	Metabolites	Target cell/organism/disorder	Mechanism	References
Anti-proliferative	Quercetin	Glioblastoma multiforme	Regulates several proteins participating in cell signal transduction	[84]
Antimicrobial	Capsaicin	*Bacillus, Micrococcus sp., E. coli, Pseudomonas sp., Citrobacter sp., Salmonella typhimurium, Pseudomonas aeruginosa*	Affects membrane stability	[85, 86]
Antiviral	Capsaicin	Guinea pig cutaneous herpes simplex virus	Disrupts virus-neuron connections	[87]
Insecticidal	*Capsicum frutescens* extracts	*A. aegypti* mosquitoes	Extract acts as repellent	[88]
Anthelmintic and larvicidal	*Capsicum annuum* leaf extract essential oils	Cercaria of *Schistosoma mansoni*	Kills larvae	[89]
Cardiovascular effects	Capsaicin (10-300 µg/kg)	Mongrel dogs	Temporary increase in mean systemic blood pressure	[90]
Anti-inflammatory	Carotenoids	Egg albumin-induced inflammation of rat hind paw	Reduces inflammation	[91]
	Capsaicin	Osteoarthritis	Pain relief	[92]

Conclusion

The Mediterranean diet is rich in fresh fruit, vegetables and cereals that not only are economical but a healthy choice for a long life. These ingredients at the base of the pyramid are recommended for daily consumption. Rich in phytochemicals such as vitamins, carotenoids, flavonoids and phenols, these dietary items may be responsible for the beneficial effects of the Mediterranean diet. Indeed, the Mediterranean population is believed to live longer and healthier than many other population in the Western world, with low incidence of non-communicable diseases such as obesity, cardiovascular diseases and cancer. It therefore stands to reason that the Mediterranean diet holds promise for healthy people with a healthy lifestyle and also for people with chronic health conditions.

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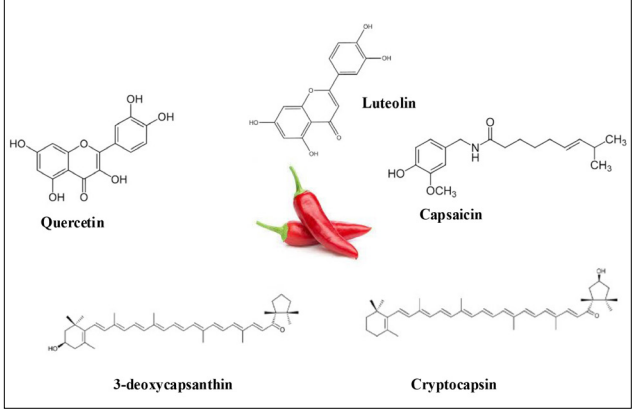
Fig. 3. Major phytochemicals of chili pepper.



Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; ZN, Kristjana D, Kevin D, BA, VV, GM, AI: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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Review

Foods of the Mediterranean diet: garlic and Mediterranean legumes

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Keywords

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Summary

The Mediterranean diet is a dietary regime derived from the one followed by the ancient civilizations of the Mediterranean region. It is characterized by many healthy constituents, among which are cereals, legumes, fruits, vegetables, olives, and white meat. Many studies suggest that this dietary regime is the key to obtaining a healthy and long life, like that of the Mediterranean peoples. Despite its popularity among health professionals, this diet is still confined to a certain geographical area of the world. Due to globalization and the modern busy lifestyle, this cultural diet is losing ground even in its home region, with more and more people embracing the so-called Western diet. An awareness of health benefits of the individual components of the Mediterranean diet will therefore draw attention from all over the world to this healthy and affordable dietary pattern, which can not only improve the overall health, but also reduce the risk of developing chronic and infectious diseases. In this regard, garlic and Mediterranean legumes present a huge repertoire of phytochemicals having both nutritive and nutraceutical properties, which therefore should be included in our daily dietary routines in moderate proportions. This narrative review aims at summarizing the principal components and health benefits of the Mediterranean diet, in particular of garlic and legumes.

Introduction

Food is one of the most important attractions of our lives. The choice, quality, and quantity of food determines and defines our mental and physical health. Research has shown that the individual taste perception, encoded by taste genes, becomes a guiding force in the selection of food to satisfy our dietary demands. In addition, variations in the taste genes result in different taste perceptions, resulting in varied food choices and to the associated diet-related metabolic syndromes and diseases, such as cardiovascular problems, cancer diabetes, and obesity [1]. On the other hand, the chemical compounds from food activate various taste receptors, altered by genetic polymorphism in taste genes, indicating a complex relation between taste perception and preferences influenced by genetic and environmental factors [2]. This partially justifies why different geographical areas of the world have different food traditions and their corresponding lifestyles and diseases. People living in different parts of the world have developed dietary habits overtime that have influenced the expression of taste genes and coined taste perception and food preferences. For instance, the Mediterranean region is known for the healthy dietary pattern of its populations and the corresponding healthy lifestyle, with a significantly lowered risk of metabolic disorders and associated diseases. The term Mediterranean diet (Med Diet) therefore represents the healthy food choices of the Mediterranean peoples that gained considerable fame a few decades ago, when numerous large-scale clinical studies reported enhanced cardiac protection by reduced atherosclerosis in the populations used to this dietary regime [3]. Subsequent trials supported the notion that the Med Diet indeed proved beneficial also in lowering the risk of other pathophysiological conditions, including metabolic syndromes, neurodegenerative diseases, ocular diseases, type 2 diabetes mellitus, obesity, and cancer [4]. Currently, the Med Diet is considered among the healthiest dietary regimes of the world and it is equally favoured by medical practitioners and nutritionist; however, the implementation of this diet on a global scale to provide health benefits in geographically diverse populations is challenged by numerous socio-economic and cultural factors [5]. In addition to the daily consumption of fruits, vegetables, cereals, and legumes, this diet is also characterized by the inclusion of healthy spices: not only they add flavour and aroma to the foods, but also provide healthy nutrients and phytochemicals. These spices include but are not limited to basil, bay leaf, fennel, cloves, cumin, ginger, turmeric, garlic, oregano, rosemary, mint, parsley, thyme, and sage, and are used in different combinations and proportion in the different areas of the Mediterranean region. These spices not only enhance the culinary essence of the food, but they also make it nutrient-dense because of their antioxidant, anti-cancer, antimicrobial, and anti-inflammatory contents, thus playing a major role in promoting a healthy lifestyle [6]. In this review we will discuss about two important components of the

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Med Diet, i.e. garlic and legumes: not only they confer taste, but they also provide several health benefits to this dietary regime.

Garlic

Garlic (*Allium sativum*) is an essential ingredient of almost all Mediterranean dishes. Being rich in bioactive compounds – such as phenolic compounds, saponins, organic sulphides, and polysaccharides – garlic is not only a food, but it is also part of the traditional medicine in the Mediterranean region, India, and China [7].
Having a repertoire of beneficial bioactive compounds (like polyphenols and flavonoids) conferring it anti-inflammatory, immunomodulatory, cardioprotective, anticancer, antidiabetic, anti-obesity, antihypertensive, antibiotic, and antioxidant properties, garlic is considered as one of the most important vegetables and spices in the world (Tab. I) [8]. Consequently, garlic consumption has been reported to decrease non-communicable diseases such as hypertension, cardiovascular problems, cancer, obesity, and diabetes [9,10].
In addition to the bioactive compounds mentioned above, garlic contains 28% (w/w) carbohydrates (such as starch, sucrose, glucose, and fructose) and fatty acids (such as palmitic acid, linoleic acid, oleic acid, and linolenic acid) [17-20]. Since ancient times, garlic has been used as an anti-microbial and anti-inflammatory agent. In addition, it reduces the risk of chronic cardiovascular problems, suppresses and cures cancer, promotes immunological function, lowers cholesterol, detoxifies harmful compounds, restores physical strength, enhances resistance against stress and pathogens, and mediates antiaging, anti-cancer, hepatoprotective and renoprotective effects [14]. Garlic can be consumed raw, dried, or cooked, as a whole or in an extract form. Its consumption as an extract has been observed to reduce the risk of initiation, development, and proliferation of several types of cancers, such as breast, skin, uterine, colon, cervix, and gastric cancer [21, 22]. The presence of organosulfur compounds in garlic makes it a potent inhibitor of cancer cell proliferation and an inducer of apoptosis

Tab. I. Some important phytochemicals and bioactive constituents of garlic.

Types	Percent (g/ 100 gW)	Bioactive Compounds	Bioactivities	Therapeutic effects	References
Sulphur-containing compounds	2.3%	Thiosulphinates like allicin, allylmethyl-, methylallyl- and trans-1-propenyl-thiosulfinate	Allicin inhibits the growth of *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli*, *Bacillus cereus*, *Helicobacter pylori*, and *Streptococcus thermophilus*	Antimicrobial, urease inhibition	[11, 12]
		OrganoSulphur volatiles, including Diallyl disulfides (DADS), Diallyl sulfides (DAS), Diallyly trisulfides (DATS), sulfur dioxide, E/Z-ajoene, S-allyl-cysteine (SAC), and S-allyl-cysteine sulfoxide (alliin), S-allyl mercapto cysteine (SAMC)	SAC AND SAMC have strong radical scavenging activities, DAS and DADS enhance the activity of glutathione reductase	Antioxidants, prevent damage caused by free radicals, anti-cancer	[13, 14]
		Vinyldithiins including 2-vinyl-4H-1,3 dithiin	Lowers platelet aggregation	Antioxidants, cardioprotective, prevent myocardial infarction and ischemic stroke, reduce the risk of gastric and colon cancer	[15,16]
Phenols	1.5%	β-resorcylic acid, pyrogallol, protocatechuic acid, gallic acid, rutin, and quercetin	Scavenge free radicals, relax coronary arteries, prevent myocardial	Antioxidants, cardioprotective effects	[17, 18]
Non-sulphur containing saponins		β-cholorogenin, diosgenin, desgalactotigonin-rhamnose, proto-desgalactotigonin-rhamnose, voghieroside D1, sativoside B1-rhamnose, and sativoside R1 gitogenin and proto-desgalactotigonin	Inhibit fungal pathogens, protect against reactive oxygen species, prevent DNA damage	Antifungal, antitumor, antithrombotic, and cholesterol-lowering effects	[14, 19]
Amino acids	1.2 %	Arginine, leucine, glutamic acid, and aspartic acid	Arginine is a precursor of neurotransmitter nitric oxide, they smooth muscle relaxation and lower blood pressure	Neurotransmission, antihypertensive	[20]

in many types of cancers [14, 22, 23]. Although studies proving the therapeutic potentials of garlic are based on in vitro and in vivo animal models, some small-scale clinician trials in humans have provided an insight into the nutraceutical potential of garlic in both raw and commercial forms (Tab. II).

Legumes

Legumes such as alfalfa, green beans, clover, peanuts, lupines, peas, soybeans, broad beans, dry beans, chickpeas, dry peas, and lentils are important source of vegetable-based proteins and are important ingredients of many world-famous diet plans. Peas, beans, chickpeas and lentils are considered cornerstones of many ancient diet patterns, including the Mediterranean diet. These legumes are not only low in fat with no cholesterol, but are also heavily loaded with proteins, starch, minerals, vitamin, and fibre, making them as important as cereals in our daily diet. In the Med Diet legumes are taken daily in moderate proportions in cooked, baked, and raw form, as sprouts or salads. Being more proteinaceous than cereals, these legumes can be used as a primary source of

Tab. II. The biological activities of garlic and its active components as shown by clinical trials.

Bioactivities	Subjects/ patients	Study design	Interventions and duration	Results	References
Antioxidant properties	92 obese patients	Placebo-controlled randomized double-blind trial	400 mg of garlic extract per day for 3 months	Enhanced production of antioxidant	[24]
	46 untrained boys	Randomized controlled trial	250 mg garlic capsule per day for 8 weeks	Lowered oxidative stress and enhanced resistance and endurance during training	[25]
	44 pregnant women	Placebo-controlled randomized double-blind trial	1 mg of allicin plus 400 mg garlic per day for 9 weeks	Reduced oxidative stress	[26]
	42 menopausal women	Randomized double-blind controlled trial	1200 µg allicin per day for one year	Reduced oxidative stress	[27]
Anti-inflammatory properties	120 healthy individuals	Placebo-controlled randomized double-blind parallel intervention study	2.56 g aged garlic extract (AGE) per day for 90 days	Enhanced immune system functions	[28]
	120 healthy subjects	Randomized double-blind placebo-controlled nutrition intervention	2.56 g aged garlic extract per day for 90 days	Improved immune system function, less cold and flu symptoms	[29]
	60 healthy volunteers	Randomized controlled trial	1 g to 3 g of garlic powder 6.0 and 24.0 h respectively	Immunostimulatory effect	[30]
	51 healthy but obese adults	Placebo-controlled double-blind randomized trial	3.6 g aged garlic extract per day for 6 weeks	Reduced inflammation	[31]
Lipid lowering effects	160 type 2 diabetic patients	Randomized control trial	500 mg of garlic powder and 1.1 mL of olive oil for 3 months	Prevented dyslipidaemia	[32]
	150 hyperlipidaemic patients	Single-blind placebo-controlled study	1 mg allicin and 400 mg garlic in tablet, twice daily for 6 weeks	Lowered lipid levels	[33]
	75 healthy adults	Placebo-controlled randomized double-blind trial	10.8 mg allicin (3 garlic cloves) per day for 12 weeks	Lipid-lowering effects	[34]
	70 diabetic patients with dyslipidaemia	Placebo controlled randomized single-blind study	Garlic tablet 300 mg, 2 times daily for 12 weeks	Improvements in dyslipidaemia	[35]
Antidiabetic effects	210 type 2 diabetes mellitus patients	Placebo-controlled single-blind study	Garlic tablet 300 - 1500 mg per day plus Metformin 500 mg twice a day for 24 weeks	Reduced HbA_{1c} and fasting blood glucose levels	[36]

▶

Tab. II. *Continues.*

Bioactivities	Subjects/ patients	Study design	Interventions and duration	Results	References
Antidiabetic effects	Two 38-subject groups having diabetes	Double blind trial	750 mg capsule containing onion and garlic 20% (w/w), nettle leaf 20% (w/w), berry leaf 10% (w/w), walnut leaf 20% (w/w), fenugreek seed 20% (w/w), and cinnamon bark 10% (w/w), thrice daily for 12 weeks	Decreased HbA_{1c} and fasting blood sugar level.	[37]
Bone diseases	80 overweight or obese postmenopausal women with knee osteoarthritis (OA)	Placebo-controlled parallel-design randomized double-blind trial	500 mg garlic tablet twice daily for 12 weeks	Improved OA symptoms	[38]
	76 overweight or obese postmenopausal women	Placebo-controlled randomized double-blind parallel design trial	1000 mg garlic tablet per day for 12 weeks	Reduced pain severity	[39]
	44 postmenopausal osteoporotic women	Double-blind randomized controlled clinical trial	2 garlic tablets per day for 8 months	Immunomodulatory effects	[27]
Antimicrobial effects	45 children	Randomized double-blind controlled clinical trial	2 mL garlic or garlic with lime formulation, used as mouth rinse, once a day for 2 weeks	Economic and effective alternative to sodium fluoride mouth rinse	[40]
Antiviral effects on respiratory viral infections	796 children	Double-blind placebo-controlled randomized trial	First stage Allicor 600 mg and second stage Allicor 300 mg tablets per day for 5 months	Effective in the prevention of nonspecific acute respiratory infections, without any side effects	[41]
Anticancer effects	57,560 men and women	Comparison-based study	One bulb of garlic per day for 9 years	Reduced risk of colorectal adenoma	[42]
	1,424 lung cancer cases and 4,543 healthy controls	Population-based case control study	Weekly administration of 8.4 g raw garlic or 33.4 g garlic components for 7 years	Dose-dependent protective association between raw garlic and lung cancer	[43]
	5,033 patients with gastric cancer (aged 35-74 years)	Double-blind intervention study	Synthetic allitridum 200 mg daily and 100 µg selenium every other day for 1 month per year, for a total of 3 years	Protection from gastric cancer	[44]
	3,365 *H. pylori* positive volunteers, with participants and risk for gastric cancer	Placebo-controlled blinded randomized trial	200 mg aged garlic extract and 1 mg steam distilled garlic oil 2, twice daily for 7.3 years	Decreased incidence of gastric cancer and mortality	[45]
Cardioprotective effects	157 postmenopausal asymptomatic women	Placebo-controlled double-blind clinical trial	Garlic herbal preparation containing 500 mg isoflavonoid for 12 months	Suppression and prevention of atherosclerosis	[46]

▶

Tab. II. *Continues.*

Bioactivities	Subjects/ patients	Study design	Interventions and duration	Results	References
Cardioprotective effects	92 obese patients	Placebo-controlled randomized double-blind nutritional intervention	Daily intake of 400 mg of garlic extract for 3 months	Suppressed inflammation and improved endothelial biomarkers of cardiovascular problems	[24]
	60 patients with mild hypercholesterolemia	Randomized controlled trial	6 g of aged black garlic twice a day for 12 weeks	Enhanced cardioprotective effects, beyond gold standard medication	[47]
	55 patients with metabolic syndrome	Randomized double-blind study	AGE 2400 mg daily for 52 weeks	Decreased low attenuation plaque (LAP) formation in coronary arteries	[48]
	51 coronary heart disease patients	Placebo-controlled randomized double-blinded study	150 mg Allicor garlic tablet 2 times per day for 12 months	Significant reduction in cardiovascular risk, by 1.5-fold in men ($p < 0.05$) and 1.3-fold in women	[49]
Antihypertensive effects	100 hyperlipidemic patients	Randomized study	Mixture of garlic and coriander, 2 g daily for 60 days	Improved lipid parameters and reduced blood pressure	[50]
	79 patients with uncontrolled systolic blood pressure	Dose-response trial	AGE 240/480/960 mg containing 0.6/1.2/2.4 mg of S-allylcysteine daily for 12 weeks	Marked reduction in systolic blood pressure	[51]
	41 moderately hypercholesterolemic patients	Double-blind crossover study	AGE 7.2 g daily for 10 months	Reduced systolic and diastolic blood pressure	[52]

amino acids. Protein extracts from soybeans are used as an alternative to meat [53]. In addition, legumes are rich in phosphorus, potassium, chromium, copper, selenium, zinc, magnesium, and folic acid, which have numerous health benefits, like cell growth, energy production, nerve and muscle function [54, 55].

Consuming legumes in moderate proportions and as part of a balanced diet has been observed to reduce the risk of hypertension, type 2 diabetes, obesity, cardiovascular diseases, stroke, and dislipidemia [56-58]. The low glycemic index of legumes and the presence of many non-nutrient phytochemicals (such as saponins, phytosterols, lectins, phytoestrogens, phytates, and amylase and trypsin inhibitors) confer several health benefits to legume consumers, such as an enhanced protection against cancer, free radicals-induced damage, cardiovascular diseases, and hypercholesteremia [59].

Moreover, legumes reduce oxidative stress, promote gut microbial diversity, colon health, and suppress inflammatory conditions and cancer [57-60]. The non-nutrient content of legumes, previously considered as hazardous to health, has now been proven to be important from a nutraceutical point of view. The health benefits of some non-nutrient compounds found in legumes is presented in Table III.

Clinical studies on legume consumption

Clinical studies have shown legumes to be useful in lowering blood sugar levels. For instance, in a randomised study 121 subjects having type 2 diabetes were given low-GI diet, containing one cup/day or ~ 190 g of cooked legumes or wheat fibre foods for 3 months: the results indicated that the patients that were given legumes had considerably decreased triglyceride levels, systolic and diastolic blood pressure, A1C, and blood glucose levels [70].

Similarly, regular consumption of legumes was shown to reduce total and low-density lipoprotein (LDL) cholesterol levels. A meta-analysis study reviewing 10 randomized, controlled trials based on non-soy legumes consumption for a minimum of 3 weeks resulted in lowered cholesterol levels in the participants. In another trial, 31 subjects having type 2 diabetes were given a legume-free therapeutic diet for heart disease. Alternatively, they were given the same diet but replacing red meat with legumes thrice a week. The results showed promising decreases in triglycerides, LDL cholesterol, fasting blood glucose, and insulin levels [71].

Being rich in minerals, potassium, magnesium, and fibre, legumes play a positive role in managing high blood pressure [72]. For instance, marked reductions in triglyceride levels, blood pressure, waist circumference

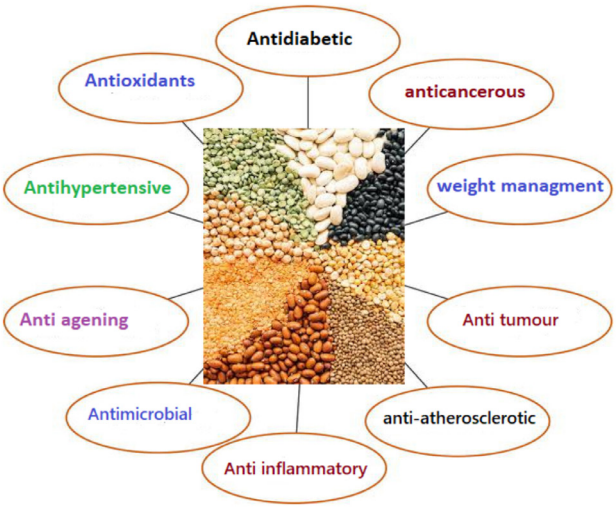
Tab. III. Bioactive compounds of legumes and their bioactivity.

Classification	Bioactive compounds	Sources	Bioactivity	References
Total phenolics	Gallic acid, protocatechuic acid, syringic acid, p-Hydroxybenzoic acid, vanillic acid, trans-p Coumaric acid, phydroxybenzoic acid, ferulic acid, sinapic acid	Adzuki bean, mung bean, kidney bean, white lupine, soybean, chickpea, red lentils	Antioxidant, anti-inflammatory, antihypertensive, anti-atherosclerotic, antitumor, ACE inhibitor, and antidiabetic and antiaging activities	[61- 64]
Saponins	Azukisaponin IV, VI, V, II, I, and azukisaponin III, soyasaponin and saponin B	Adzuki bean, mung bean, peas	Capture free radicals and stimulate antioxidant enzymes	[65]
Proanthocyani-dins	Procyanidins, prodelphinidins, rhamnosides	Mung bean	Antioxidant, tyrosinase inhibitor	[66]
Anthocyanins	Delphinidin-glucoside, cyanidin-galactoside, cyanidin-glucoside, pelargonidin-glucoside	Black soybean, red kidney bean	Antioxidant, antimicrobial anti-inflammatory, and antidiabetic	[63, 64]
Tocopherols	δ-Tocopherol, β,γ tocopherols	Soybean, black soybean, white kidney bean, cowpea, whole bean, kidney bean, black-eyed and pinto bean	Antioxidant and anticancer	[63, 64]
Carotenoids	Lutein and zeaxanthin isomers	Lentil, red kidney bean, cowpea	Antioxidant properties	[64]
Flavonoids	Catechin, epicatechin, quercetin-3-oglucoside, myricetin, kaempferol-3-orutinoside and kaempferol-3-o-glucoside, quercetin	Soybean, chickpea, mung bean, red lentils, kidney bean, black soybean, black turtle bean	Antioxidant properties	[67, 68]
Condensed tannins	Catechins	Lentil, black soybean, black turtle bean, adzuki bean, mung bean	Antioxidant, antimicrobial, anti-HIV, and anti-tumour activities	[69]

and weight were observed in 113 obese people consuming 1:2 ratio of legume servings to whole grains for 18 months [73]. Meta-analysis of the results of eight trials with more than 500 participants, 50% of which were obese or overweight, concluded significant reductions in systolic and mean arterial blood pressure in subjects who consumed a cup of legumes daily for 10 weeks [74]. In addition, people who consume legumes regularly tend to have lower body mass indices (BMI > 30 kg/m2) as compared to non-consumers [75]. This provides substantial evidence that a Mediterranean-style eating plan, characterised by daily consumption of legumes, is effective for weight loss [76].

Despite their numerous health benefits (Fig. 1), legumes are still not consumed at an optimum level in various areas of the world. This adds to the fact that, despite being the healthiest dietary pattern, the Med Diet is still restricted mainly to its region of origin. Developing healthy dietary programs and creating awareness would help people reap the full benefits of the typical spices, herbs, and other constituents of the Med Diet.

Fig. 1. Health benefits of legumes.



Conclusion

Traditional diets, such as the Med Diet, are not only budget friendly but also rich in healthy nutrients that provide an overall healthy lifestyle, with reduced risk of chronic and life-threatening diseases such as CVDs and cancer. Being rich in beneficial phytochemicals, garlic and legumes should be included in daily meal plans: these cardioprotective, anti-cancerous, antidiabetic and antihypertensive ingredients in our food will not only satisfy our culinary demands, but also promote a healthy life.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; ZN, GB, MCM, BA, VV, GM, AI: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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Review

Foods of the Mediterranean diet: citrus, cucumber and grape

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Keywords

Mediterranean diet • Citrus • Cucumber • Grape • Phytochemicals

Summary

Fruit and vegetables are excellent sources of health-promoting bioactive compounds and nutraceuticals. Regular consumption of fruit and vegetables helps prevent the onset and progression of many non-communicable diseases. The Mediterranean diet envisages consumption of healthy vegetables and fruit on a daily basis for maximum health benefits. Traditional use envisages vegetable-based and fruit-based diets, and many studies scientifically proved the beneficial effects of Mediterranean vegetables and fruits. Rich in bioactive phytochemicals, citrus, cucumbers and grapes have antioxidant, anti-inflammatory, antimicrobial, cardioprotective, anti-ageing and anti-cancer properties. Studies indicate that intake of citrus, cucumbers and grapes reduces hypertension, hyperlipidemia, skin problems and infections and improves the health of the cardiovascular and nervous systems. These beneficial effects are mediated by several bioactive molecules present in Mediterranean diet vegetables and fruits, such as citrus, cucumbers and grapes. Indeed, they contains flavones, isoflavones, tannins, polyphenols and many beneficial natural molecules. This review focuses on the bioactive ingredients in citrus fruit, cucumbers and grapes, all components of the Mediterranean diet, and their health effects. A deep understanding of Mediterranean diet's components, as well as clinical trials to test natural molecules beneficial effects, will permit to further explore the therapeutic potential of the Mediterranean diet in several pathological conditions.

Introduction

The Mediterranean diet is widely accepted to be a very healthy diet. Whole grains, legumes, fruit, vegetables, white meat, fish, nuts and olive oil are all essential components. This diet is beneficial for human health and wellbeing because of its abundance of antioxidants, fibre, vitamins, minerals, phytosterols, probiotics, omega 3 and omega 6 fatty acids and phytosterols.

Citrus fruit is popular in the Mediterranean region. Though not native to the region, it has become an important commodity because of its place in the Mediterranean diet. Various citrus fruits have been used traditionally to treat illnesses in many Asian countries. Due to their medicinal qualities, citrus secondary metabolites such flavonoids, alkaloids, limonoids, coumarins, carotenoids, phenolic acids and essential oils are precious for maintaining human health. Their qualities include cardiovascular and neuroprotective properties and antioxidant, anti-inflammatory and anti-cancer effects [1-15].

The cucumber *Cucumis sativus* L. (family Cucurbitaceae) is a vegetable widely consumed throughout the world. It has a high water content and relatively few calories, as well as strong antioxidant, lipid-lowering and anti-diabetic effects. By eliminating pockets of toxins and old debris, cucumber has a purifying effect on the body. The skin is nourished by application fresh cucumber juice, which has a calming effect on irritation and reduces edema. Cucumber can also calm the body and reduce the pain of sunburn. The fruit is cooling, hemostatic, tonic and effective against excessive thirst and heat stroke. The seeds are used to treat constipation and have a cooling effect on the body. Cucumber is a source of cucurbitacins, cucumerin A and B, cucumegastigmanes I and II, vitexin, orientin, isoscoparin 2′′-O-(6′′′–(E)-p-coumaroyl) glucoside and apigenin 7-O-(6′′-O-p-coumaroyl glucoside) [16-32].

Grapes are rich in primary and secondary metabolites such as polyphenols. Polyphenols and phenolic acids have strong anti-inflammatory and anti-cancer effects and immunomodulatory properties. Although their distribution varies greatly in the different tissues, the pericarp and seeds of grapes are rich in polyphenols and phenolic acids. The leaves and seeds of the grapevine are tasty and healthy ingredients of Mediterranean cooking.

Citrus phytochemicals and their health-promoting effects

Citrus fruit is a distinctive component of the Mediterranean diet and adds richness and variety and to its health-promoting effects. It has many nutritional and healthy properties. It has been used for centuries to treat illnesses, such as bronchitis, tuberculosis, coughs, colds and menstrual disorders [1]. Citrus fruit is rich in vitamins, minerals and fibre and without sodium, fat and cholesterol. It also contains secondary phytochemicals, such as carotenoids and

polyphenols, that prevent non-communicable diseases such as cardiovascular disease and cancer. Citrus fruit is rich in vitamin C which plays a key role in the absorption of inorganic iron, thus helping to alleviate anemia if administered with appropriate medicines [2, 3]. Vitamin C also promotes formation of collagen. Although citrus is not an excellent source of iron or zinc, its vitamin C content mediates release of iron and zinc from other foods, thereby maintaining iron status [2]. Citrus contains cellulose, hemicellulose and pectin which make up 60 to 70% of its total fibre content. Together with lignin, this form of fibre may decrease absorption of glucose after consumption of carbohydrates and reduce reabsorption of bile acids, lowering plasma levels of cholesterol [1-3].

Citrus fruit contains many important secondary metabolites in its peel, pulp, seeds, pressed oil and juice (Tab. I). Their concentrations vary between citrus species and in the various parts of the fruit [1-3]. Hesperidin and flavonoids have been linked to the anti-inflammatory and antioxidant activities of phenolic acids, such as caffeic, chlorogenic and ferulic acids [2]. The compounds responsible for these benefits include coumarins, essential oils (limonene) and flavonoids such naringin, naringenin, hesperidin and nobiletin (auraptene, imperatorin) [1]. Citrus also contains triterpene compounds like limonoids that have been remarked for their anti-cancer properties in animal models (stomach, skin, colon and lung) and in human colon adenocarcinoma cells and human breast cancer cells. Flavonoids extracted from citrus can also lower blood sugar, triglycerides and cholesterol levels. The flavanones hesperidin, naringenin, naringin and neohesperidin, as well as polyethoxylated flavonoids (nobiletin, tangeretin) are responsible for these qualities [1, 2].

Cucumber phytochemicals and their health-promoting effects

Cucumbers (*Cucumis sativus* L.) belong to the Cucurbitaceae family and are widely cultivated all over the world. They are eaten raw in salads, fermented (pickled) or cooked [17]. *C. sativus*, one of the 30 species of cucumbers, has the highest commercial value [18, 19]. The therapeutic potential of *C. sativus* leaves, fruits and seeds is extensively mentioned in ayurvedic medicine for management of ageing [20, 21]. The cucumber is of-

Tab. I. Phytochemicals in the citrus family and their bioactivity.

Components	Bioactivity	References
Folate (Folic acid / folacin)	Promotes cell proliferation and red blood cell synthesis, prevents neural tube defects, stabilizes genetic material and may protect against cancer and heart disease.	[4,5]
Potassium	Minerals and sodium can retain biological fluids. Low sodium and high potassium levels may lower blood pressure.	[4,5]
Dietary fibre	Includes resistant starch, soluble and insoluble polysaccharides, pectin and certain other components. Boosts intestinal flora and shortens the time food spends in the gut. Some types of fibre help lower blood fat levels. May lower risk of certain malignancies, heart disease and digestive issues including constipation.	[5]
Non-nutrient phytochemicals		
Coumarins Polymethoxyflavones	Anti-obesity effects. Citrus peels are rich in coumarins, a class of polyphenols. Auraptene, the most prevalent coumarin in citrus, is found in grapefruit, trifoliate oranges, sour oranges and particularly *Citrus natsudaidai*. Products made from citrus fruits like marmalade and grapefruit juice maintain auraptene activity. Prevent formation of lipid droplets	[6]
Polyphenols	Anti-microbial, anti-viral, anti-allergic, anti-inflammatory, anti-proliferative and anti-carcinogenic activity; astringent flavors. Anthocyanins are responsible for many of the colors of fruit and vegetables.	[5, 7-10]
Flavonoids		
Nobelitin Citromitin Tangeretin	Hepatoprotective, anti-obesity and anti-hyperglycemic effects. Prevent D-galactosamine-induced liver damage	[11]
Kaempferol Quercetin Apigenin Naringenin	Anti-microbial effects Cell-cell signaling antagonists, biofilm formation suppressors	[12]
Hesperidin	Anti-allergic effects Reduces granulocytes degranulation and allergic symptoms	[13]
Limonin Nomilin	Hepatoprotective, anti-obesity and anti-hyperglycemic effects Improves indicators of liver injury and inflammation	[14]
Essential oils	Anti-microbial effects Inhibit pathogens and microorganisms of decomposition	[15]
Chimpi	Anti-anxiety effects Strong anxiolytic effect equivalent to fluoxetine.	[16]

ten used to treat skin problems such as sunburn and eye inflammation. It is thought to revive, cool, heal, soothe and soften irritated skin and to prevent itching. Bioactive substances in several chemical classes have been isolated from this plant. A distinguishing feature of this species are cucurbitacins. Cucumber fruits are 96.4% water, 0.4% protein, 0.1% fat, 2.8% carbohydrate, 0.3% minerals, 0.01% calcium, 0.03% phosphorus, 1.5 mg/100 g iron and 30 IU/100 g vitamin B. They also contain enzymes such as proteases, ascorbate oxidases, succinic dehydrogenase and malic dehydrogenase [19]. They have a significant amount of ascorbic acid [20] while pulp and peel extracts contain lactic acid (7-8% w/w), which has demonstrated antioxidant properties [17]. The tetracyclic cucurbitane nuclear skeleton of cucurbitacins is arbitrarily split into twelve groups that form many cucurbitacins with antiproliferative properties [22].

Phytochemicals of cucumber seeds

Cucumber seeds contain compounds such flavonoids, tannins, saponins and steroids [23]. Indeed, ethanol extract of cucumber seeds contained flavonoids, terpenoids, tannins, cardiac glycosides, phenols and carbohydrates [24]. Moreover, methanol extract of cucumber pulp contained tannins, alkaloids, saponins, glycosides, terpenes, phenolics and glycosides [25, 26]. The cotyledons of various varieties of *C. sativus* seedlings contained cucurbitacins A B, C, D, E and I [27] that have cytotoxic, antitumor, hepatoprotective, anti-inflammatory, antibacterial, anti-helminthic, cardiovascular and anti-diabetic properties [28]. Finally, cucumber contains two newly discovered primary C-glycosyl flavonoid compounds: cucumerin A and cucumerin B. Cucumber leaves and fruits contain many flavonoids, such as vitexin, isovitexin, and orientin, and carotenoids [29-33]. Table II summarized the main phytochemicals present in cucumber plant.

Pharmacological activity of cucumber phytochemicals

Antioxidant activity

Cucumber (*Cucumis sativus* L.) contains several free radical scavenging compounds that reduce oxidative stress by reducing superoxide mutase, guaiacol peroxidase, glutathione reductases and ascorbic acid peroxidases [20, 34]. Other antioxidants include butylated hydroxyl anisole (BHA) which is a more effective free radical scavenger than ascorbic acid. Tannins, polyphenols and flavonoids in cucumber also show radical-scavenging effects [34].

Anti-cancer activity

Cucumber contains many compounds with anti-cancer properties [35]. For instance, methanol and acetone extracts of cucumber has shown cytotoxicity against cancer cell lines HeLa and Michigan Cancer Foundation-7. Ethanol extract of cucumber leaves is rich in glycosides, alkaloids, tannins, proteins, amino acids, phytosterols, steroids, terpenoids and saponins; it inhibited the growth of HeLa and HepaG2 cancer cell lines in the MTT assay. Significant anticancer activity was seen at doses of 62.5, 125, 250 and 500 mg. Ethanol extract was more effective against HepG2 than against HeLa. According to the authors, the triterpenoids in the extract were responsible for the anti-cancer activity [35].

Antibacterial and antifungal properties

Ethyl acetate and methanol extracts of cucumber fruit have been reported to have antimicrobial activities, while methanol extract of cucumber seeds is reported to inhibit entero-pathogens *S. aureus*, *P. aeruginosa* and *E. coli*. Volatile cucumber oil has shown antibacterial and antifungal properties against gram-negative and gram-positive bacterial strains [36]. The research demonstrated an effect of *C.s.* against two major fungi. The authors concluded that *C.s.* seeds may have antifungal potential [37]. The study showed that ethanol extract of *Cucumis sativus* Linn. screened positively for antifungal activity against six fungi compared to reference griseofulvin [37].

Cytotoxicity

Ethanol extracts of Cucumis sativus showed cytotoxicity against brine shrimp nauplii in a brine shrimp toxicity assay. Toxicity rates were concentration-dependent. The LC50 and LC90 of the extract against brine shrimp nauplii were 75 µg/ml and 120 µg/ml, respectively [37].

Hepatoprotective activity

Cucumis sativus has been demonstrated to protect against oxidative stress caused by cumene hydroperoxide. Antioxidant and radical-scavenging compounds of *Cucumis sativus* fruit extract readily cross the cell membrane and protect against intracellular generation of reactive oxygen species. Aqueous extract of *Cucumis sativus* fruit acts as a hepatoprotective and antioxidant against cumene hydroperoxide-induced hepatotoxicity [38].

Hypoglycemic and hypolipidemic activity

Animal models such as alloxan-induced diabetic rats (AIDRs) have been used to test the hypoglycemic and hypolipidemic effects of cucumber. For instance, ethanol extracts of fruits of the Cucurbitaceae family, in-

Tab. II. Phytochemicals in cucumber plant.

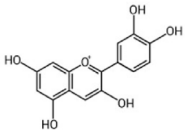
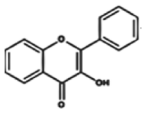
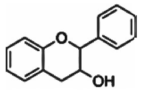
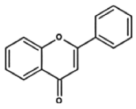
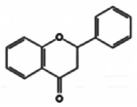
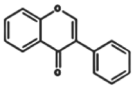
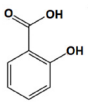
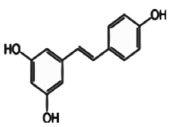
Plant	Part of plant	Class of compounds	References
Cucumber	Seed	Tannins, saponins, terpenoids and steroids	[23, 24]
	Pulp	Tannins, alkaloids, saponins, glycosides, terpenes, phenolics and glycosides	[25, 26]
	Cotyledons	Cucurbitacins A B, C, D, E and I	[27]
	Leaves and fruits	Flavonoids, carotenoids	[29-33]

cluding cucumber, white pumpkin and ridge gourd, significantly reduced blood sugar levels in AIDRs. They also lowered the high lipid profiles of these rats. Ridge gourd was shown to significantly improve liver glycogen levels in AIDRs. The study suggested that cucumber fruit extracts may prove beneficial as supplements in the treatment of hyperglycemia and hyperlipidemia, which frequently coexist in diabetic patients. However, additional research is required to screen individual chemical compounds, identify antidiabetic lead compounds and determine their mechanisms of action [39].

Grape phytochemicals and their health-promoting effects

Grapes are grown in many parts of the world but are perhaps best known in California in the United States and in the Mediterranean from Portugal to Lebanon and Syria.

Tab. III. Health benefits of phytochemicals present in grapevine and other sources.

Grapevine phytochemicals	Other sources	Health benefits	References
Anthocyanin: natural non-toxic water-soluble flavonoid pigments 	Mulberries, black currants, red currants, sweet cherries, blue berries, strawberries, plums, red onion, red raspberries, black chokeberries	• Antimicrobial effects • UV protection • Weight loss • Protection against type 2 diabetes • Decreased risk of cancer • Improved cognitive function • Reduce inflammation	[54-57]
Flavanols 	Onions, kale, red wine, tea, peaches, berries, tomatoes, lettuce, scallions, broccoli	• Reduce blood pressure • Improve blood flow to the brain and heart • Prevent blood clots fight cell damage	[57-59]
Flavan-3-ols 	White, green, oolong and black tea, apples, purple and red apples, blue berries, strawberries, chocolate	• Antioxidant • Anticarcinogen • Antimicrobial • Cardiovascular preventive • Antiviral • Neuroprotective	[57, 58, 60]
Flavones 	Parsley, red peppers, celery, chamomile, peppermint	• Antioxidant • Anti-inflammatory • Antimicrobial • Anticancer	[57, 58, 61]
Flavanones 	Limes, lemons, oranges	• Inhibit apoptosis • Induce peripheral and cerebral vascular blood flow • Improve cognitive performance	[57, 58, 62]
Isoflavones 	Soy, red clover, green tea, split peas, pigeon peas, peanuts, chickpeas, lima beans	• Prevent cardiovascular disease, osteoporosis, hormone-dependent malignancy and cognitive decline	[57, 58, 63]
Phenolic acids 	Berries, herbs, spices, cocoa, flax seeds, vegetables, olives, coffee, tea	• Antioxidant preventing cell damage • Anti-inflammatory	[64]
Resveratrol 	Peanuts, berries, cocoa, blueberries, bilberries, cranberries	• Antibacterial • Antifungal • Antioxidant • Anti-inflammatory for arthritis and skin • Prevents cancer, diabetes, Alzheimer's disease	[65-69]

Many Mediterranean sites produce magnificent wines, famous for their flavor and bouquet [40, 41]. Grapes contain high concentrations of phenolic acids, flavanols, flavon-3-ols [42], myricetin, peonidin, flavonoids, resveratrol, quercetin, tannins, anthocyanins, cyanidin, ellagic acid and proanthocyanins [41-49]. Besides the phytochemicals in the fruit, pulp, seeds and leaves of the grapevine, the wine derived from grapes also contains polyphenols and antioxidants [49]. The primary polyphenols in wine are resveratrol, anthocyanins, catechins and tannins (proanthocyanidins and ellagitannins) [50, 53]. Red wine and white wine are an integral part of the Mediterranean diet; they have alcohol concentrations of 14% and 11%, respectively, significantly lower than 35%, the alcohol content of spirits [51-53]. The polyphenol component of wine can have great health-protective effects [50-52], whereas white wine has fewer bioactive compounds than red wine; distilled drinks like liquor and spirits have virtually no bioactive compounds [46]. Different health effects are anticipated for alcoholic beverages due to their different chemical compositions.

Like wine, other bioactive elements of the Mediterranean diet, including nuts, fruit and vegetables (which mostly contain flavonoids) and olive oil (mainly hydroxytyrosol, tyrosol and oleocanthal), can also have cardioprotective effects through various synergistic pathways [53]. Besides containing many potential health-promoting phytochemicals (Tab. II), grapes are also rich in potassium, a mineral that maintains the body's fluid balance and can help lower high blood pressure and reduce the probability of developing heart disease and stroke. Grape seeds are rich in vitamin E, which helps keep the skin supple and moisturized. Grapes contain other components that may help prevent acne and improve the hair by boosting blood flow to the scalp. Resveratrol in grapes can strengthen the immune system, promote wound healing and prevent bacterial infections. Grape resveratrol slows the normal aging process by inhibiting cell death and reducing age-dependent deterioration of cells. Antioxidants such as flavan-3-ols and resveratrol in grapes have been observed to be effective against cancer of the mouth, throat, pancreas, prostate and colon, among others [68, 69]. Being rich in water and fibre, grapes help bowel movements, thus maintaining a healthy digestive system. Grape seed extract has been shown to play an important role in combating irritable bowel syndrome [70], while grape skin is rich in melatonin that plays a role in circadian rhythms [71]. The consumption of certain varieties of grapes, rich in polyphenols and fibre, has been shown to reduce the onset and incidence of cardiovascular disorders [72]. For instance, a randomized, parallel-group, controlled trial with 34 non-smoking adults (13 hypercholesterolemic and 21 normocholesterolemic) who were given supplements of 7.5 g/day grape antioxidant fibre containing 1400 mg polyphenols and 5.25 g dietary fibre for 16 weeks showed a significant reduction in blood pressure and normalisation of lipid profile [73]. Other studies have indicated that grapes and their phytochemical constituents have cardioprotective effects by lowering blood pressure, lipids, platelet function and thrombosis [74].

Conclusions

Vegetable- and fruit-based nutraceuticals have become popular for many pathophysiological conditions and cosmetic uses at traditional and commercial scale. Although the bioactive phytochemicals of these essential ingredients of the Mediterranean diet are backed by much evidence of traditional use, carefully designed clinical trials are required to further explore their therapeutic potential in different pathophysiological conditions.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; ZN, Kristjana D, Kevin D, BA, VV, GM, AI: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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REVIEW

Foods of the Mediterranean diet: lacto-fermented food, the food pyramid and food combinations

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Keywords

Mediterranean diet • Lactic acid bacteria • Fermented foods • Functional foods • Bioactive compounds

Summary

The Mediterranean diet proved to be one of the healthiest diets in the word. It has several beneficial effects and it prevents many non-communicable diseases, such as cancer, cardiovascular diseases, and obesity. Before being a culinary regime, the Mediterranean diet is characterized by specific cultural heritages and traditions, also influencing the lifestyle of the populations. The Mediterranean diet follows the so-called food pyramid, comprising several food combinations. Indeed, it is mainly composed by vegetables, fish and dairy products, while red meat and sweets are poorly consumed. Processed foods are mainly avoided, apart from lacto-fermented ones, the first processed foods consumed by humans. Food fermentation by microorganisms not only improves the functionality of bioactive metabolites, but also increases the shelf life and organoleptic properties of the food. Lactic acid bacteria play a vital role in transforming the food constituents, thereby enhancing their nutritional and functional properties. In addition, these foods introduce beneficial bacteria into gut microbiota, thus maintaining a healthy gut microbiome and corresponding gut-brain axis, thus providing an overall improvement in health and a reduced risk of non-communicable diseases and metabolic disorders.
This review will focus on the Mediterranean diet, on its characterising food pyramid and food combinations, and on lacto-fermented foods, one of the components of the Mediterranean diet with the most beneficial effects.

Introduction

The Mediterranean diet was awarded Intangible Cultural Heritage of Humanity status by UNESCO in 2010 [1, 2]: it is not only a diet, but also a tradition and symbology of the Mediterranean region, its cuisines, and its people, and also a healthy way of living [1]. Mediterranean people have traditionally based their cuisine on the interactively woven ideas, habits, and values of the Mediterranean community, as well as the individual groups and subgroups of each Mediterranean country. The Mediterranean cuisine is a blend of their traditions and tastes, and it symbolizes the richness of the Mediterranean heritage [2].
The Mediterranean cuisine is mainly comprised of cereals, fruits, and vegetables (which can be consumed raw, cooked, or pickled), white meat, plenty of olive oil, fermented foods, and dairy products. Fermented foods have been conventionally used in the Mediterranean countries since ancient times, owing to their organoleptic properties and long shelf-life. Currently, lacto-fermented foods (that is, foods that have been fermented by lactic acid bacteria or LAB, usually belonging to genera such as *Lactobacillus*, *Leuconostoc*, and *Streptococcus*) have gained much focus because of their health-promoting properties [3]. These types of foods are naturally rich in lactic acid bacteria, which depend upon a fermentable sugar for their metabolism and growth [4]. The product of fermentation is either lactic acid alone (homo-fermentation) or a combination of acetic acid, lactic acid, carbon dioxide and ethanol (heterofermentation) [5]. The lactic acid fermentation of sugars in raw foods not only coins a peculiar flavour and texture to the food, but also releases an array of signalling molecules and health-promoting compounds that interact with the gut microbiota [6], thus bringing many health benefits to it. Thanks to the properties of the lactic acid bacteria and their metabolites, many of these foods are classified as "functional foods" [7]: owing to their myriad of positive effects on human health, these foods are considered as reliable and inexpensive means of improving human health.

LAB-fermented foods and their health benefiting effects

Fermented probiotics and dairy products

Milk is a good source of lacto-fermented foods, such as yogurt and cheese. The lactose in milk is fermented to lactic acid by the LAB *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* [8]; this process decreases the milk pH, thus preventing the development of pathogenic microbes. In addition, lactic acid fermentation generates many useful metabolites – such as

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essential amino acids, bioactive compounds, vitamins, minerals, and exopolysaccharides – that enhance the nutritive value of the product [9].

Today, a wide variety of fermented dairy products is available, ranging from plain yogurt to flavoured yogurt and finely textured Greek yogurt, which contain absorbable forms of vitamins and minerals [10]; branched-chain amino acids (BCAA), which enhance muscle growth and maintain the body bioactive peptides, with health promoting effects [8, 9]; unsaturated fatty acids, which help to absorb fat-soluble vitamins A, D, E, and K, and conjugated linoleic acid. The conjugated linoleic acid exhibits anti-cancerous properties, by inducing apoptosis specifically in breast cancer cells [11, 12]. The bacteria in yogurt contribute to the transient microbiota and thus improve the gut environment. Several studies have also suggested beneficial effects of yogurt in managing type 2 diabetes. For instance, a study based on meta-analysis of randomised controlled trials regarding use of yogurt for managing type 2 diabetes mentioned that its regular consumption results in a reduction in complications [13]. This might be due to the alteration and modulation of gut microbiota, which prevent dysbiosis and improve the overall digestion. In addition, the gut microbiome plays an important role in improving liver and cognitive functions by participating in the gut-brain axis [14]. For instance, the consumption of yogurt along with fruits helps in improving the non-alcoholic fatty liver disease (NAFL) [15]. Similarly, yogurt supplementation decreases the deposition of the myeloid-beta plaques in the brain cortex and hippocampus in the early stages of life [16, 17]. This indicates that yogurt is beneficial for improving gut microbiota and the corresponding gut-brain axis, which modulates the overall health of an individual and prevents non-communicable diseases and metabolic disorder [16-18].

Kefir

Kefir is a natural complex probiotic, derived from kefir grains by the combined action of LAB, Acetic Acid Bacteria (AAB), and yeasts, enveloped in a slimy matrix composed of EPS and proteins [19]. Traditionally, Kefir has been used as a health-promoting and life-prolonging fermented milk [20], as it is said to possess anticancer, antidiabetic, antidepressive, antiallergic, antiasthma, and immunomodulatory effects [21, 22]. Kefir plays an important role in improving the gut microbiota by suppressing the bacteroids and increasing LAB and Bifidobacterial species, thereby improving hypertension, inflammation, and fasting sugar levels in patients suffering from metabolic disorders [23, 24]. In addition, it has been implicated in managing NAFL and high-fat diet induced obesity. A study on mouse model reported that 0.2 mL supplementation of Kefir decreased the incidence of obesity by 60% in test mice, as compared to controls, by reducing systemic inflammation and blood cholesterol levels. This could be due to the fact that kefir LAB colonize the gut epithelium, thereby exerting an influence on overall gut microbiota by niche exclusion and lactic acid production, which reduces the pH by creating an acidic environment that suppress the growth of bacteroides and induces the expression of genes encoding beneficial digestive enzymes [24]. In addition, the probiotics in kefir upregulate the expression of peroxisome proliferator-activate receptor, thereby playing a vital role in beta oxidation of fatty acids in the liver and thus in the NAFL disease management [25]. Moreover, kefir bioactive metabolites, EPS, and sphingolipids exhibit anticancer properties, possibly by mediating the signalling pathways involved in proliferation and apoptosis of cancer cells [26]. Kefir has also been indicated in improving sleep quality and reducing the incidence of mental disorders in postmenopausal women. Furthermore, it helps in bowel movements, modulates immunity, improves both physical and mental health (reducing anxiety, depression, and stress) and, thus, the individual's overall quality of life [27]. In addition, kefir releases short-chain fatty acids (SCFAs) in the gut, which improves bone density and bone formation, which also prevents fractures in the elderly [28]. This repertoire of health benefits, of course, can be retrieved when used in moderate amounts. In fact, the Mediterranean diet regime includes kefir consumption in moderate quantities, along with other dairy products like Greek yogurt and feta cheese.

Cheese

Another popular fermented dairy product of the Mediterranean region is cheese (such as pecorino, halloumi, brie, chevre, manchego, feta, Parmigiano Reggiano, ricotta...), whose consumption in low to moderate amounts is recommended in this diet [29]. Cheese is good for lactose intolerant people, as the LAB completely consume this sugar during the prolonged ripening of the cheese. During the initial fermentation stages, the LAB use milk carbohydrates, leaving behind indigestible oligosaccharides, whose consumption exerts prebiotic effects and enhances the beneficial gut microbiota [30]. Not only cheese is an excellent source of prebiotics, but it also has strong anti-inflammatory properties. For instance, in a recent study it was observed that cheese produced using *L. delbrueckii* subsp. *lactis* CNRZ327 and *P. freudenreichii* ITG protected against epithelial cell damage and colitis in mice, as compared to controls [31]. In addition, studies have reported the cholesterol-lowering effects of cheddar and Turkish cheese [32]. Similarly, *Lactobacillus*- and *Lactococcus*-fermented cheese products contain bioactive peptides that have satiety regulation, antimicrobial, antithrombotic, anticarcinogenic, hypotensive, stress-relieving, mineral absorptive and anti-inflammatory properties [33].

Vegetable fermented products

The long history of traditional pickles and fermented vegetables indicates both their culinary and health-promoting potential. A recent shift to use of fermented food has brought attention to traditionally fermented vegetables that were being used throughout the world since ancient times, such as kimchi (fermented vegetables with spices and seafood), sauerkrauts (fermented cabbage), gundruk (fermented leaf), and various kinds of pickles.

Fermentation and pickling not only enhance the bioactivity of these vegetables, but also increase their shelf life [34].

Sauerkrauts

Sauerkrauts are produced by spontaneous or selective fermentation of cabbage by specific bacteria: for instance, *Lactobacillus sakei*-fermented vegetables and sauerkrauts show three times more bioactivity as compared to those fermented by any other strain [35]. Fermentation enhances the cabbage's amount of vitamin C, fibre, ethanol, organic acids (e.g. lactic acid, maleic acid, acetic acid, and succinic acid), short chain fatty acids (e.g. propionic acid), and acetaldehyde. It also enhances the bioactivity of its glucosinolates, which are normally not bioavailable in the fresh product. Sauerkraut exerts many beneficial effects on health, such as improvement of gut microbiota in patients suffering from IBS, as indicated by the changes in fecal microbial composition [36]. Sauerkrauts show antitumor activity by inducing apoptosis of cancer cells, exerting inflammation-modulating effects, and inhibiting tumour invasion in different tissues [35]. Besides that, owing to its vitamins and organic acids, it has a strong antioxidant potential [37]. It also exhibits antidepressive effects by inhibiting Mono Ammino Oxidase (MAOs), thus helping in anxiety, depression, and even in the onset of Parkinson's disease [38].

Fermented table olives

Table olive are the most common traditional fermented vegetables in the Mediterranean region, with an increasing demand worldwide and with Italy, Spain, Egypt, Turkey, Greece, Algeria, and Portugal as the main producers. Their production exceeded 2.9 million tons in 2017-2018, which was still less than the overall demand [39]. The fermentation of oleuropein not only brings added value to the end product in terms of bioactive compounds, dietary fibres, fatty acids, and antioxidants, but, owing to the bitter taste it gives to the olives, it increases their culinary value as well [40]. The process of olive fermentation is quite complex, as it involves a wide array of microorganisms and LAB (e.g. *Lactobacillus plantarum* or *Lactobacillus pentosus*) and yeasts (e.g. *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Candida boidinii*, etc.) [41, 42]. The fermentation of olives by these microbes adds flavor, improves texture, and ensures consumer safety [40].

The probiotic trait of olives makes them an excellent source of gut friendly bacteria that stick to the fruit and are ingested along with it. The probiotics contained in fermented olives bring several health benefits, such as protection against different kinds of cancer, bowel irregularities, intestinal infections, allergic reactions, constipation, and indigestion [43]; apart from having immunomodulatory, antioxidant, and antiatherogenic properties. Fermented olives are also rich in oleic acid, which has a protective effect against breast, prostate, and colon cancer [44, 45]. Moreover, table olives contain hydroxytyrosol, a polyphenol with anti-inflammatory and anti-microbial activities, proposed for the treatments of several diseases, among which lymphedema and viral infections [46-53]. Table olives also contain high levels of oleocanthal, a natural COX-inhibitor that also protects against certain cancers and neurodegenerative disorders [54]. Furthermore, fermentation of olives inhibits the growth of many pathogenic and spoilage microorganisms [55]. This implicates that fermented olives are a potential source of undiscovered healthy microbial strains and bioactive compounds, which might be the secret behind the health promoting effects of Mediterranean diet.

Fermented meat and meat products

Meat fermentation is the most ancient and commonly used form of fermentation. Traditional fermented meat products are valuable and popular for a variety of reasons, and are used in many popular cuisines. Fermented meat products are one of the most economically important commodities in Europe, specifically in the Mediterranean countries, where they are representative of a rich cultural heritage [56]. Meat fermentation comprises many biochemical, microbiological, and chemical changes, which bring added value to fermented meat products in terms of taste, colour, aroma, and odour. Lactic acid bacteria play a core role in the fermentation of meat by reducing its pH levels and facilitating the production of bacteriocins, which prevent the growth of pathogenic and spoilage microorganisms and ultimately improve the safety, stability, and shelf life of these products [57]. Preserved fish and fermented meat sausages has also been an important tradition of the Mediterranean culture [58]. Preserved fish was the main ingredient of *garum*, an extremely popular fish sauce in ancient times that was used as a condiment in the ancient Greek, Roman, and Byzantine cuisines [59], which closely resembles the fermented anchovy sauce called *colatura di alici* that is still produced in Campania, Italy. Dry fermented sausages are also an important fermented meat product of the Mediterranean region [60]. Table I reports a list of lacto-fermented foods and of the microorganisms involved in their fermentation.

The Mediterranean food pyramid

The Mediterranean food pyramid is representative of the Med Diet and was developed based on the eating habits of long-living adults in the Mediterranean Region [68]. The pyramid follows general guidelines on choice of food items and not their quantities. Nutritional experts, sociologists, anthropologists, and agriculturists have collectively contributed to the development of what we currently call the Mediterranean diet by adapting the pyramid to the new way of life of the Mediterranean region [69]. The new pyramid follows the pattern of the old one, with plant-based staple foods that should be consumed in larger quantities (such as cereals, fruits, and vegetables) at its base, the ones to be consumed in moderate amounts in the upper levels, and the ones to be consumed in low amounts at the top.

Tab. I. Lacto-fermeted foods and the microorganisms that induce their fermentation.

Source	Microorganism(s)	Fermented Food Product	References
Milk	*S. thermophilus, L. delbrueckii ssp. bulgaricus*	Cheese, Yogurt	[61]
Meat	*Lactobacillus sake, L. plantarum*	Sausages (e.g. Salami)	[62]
Grains	*Sacchromyces cerevisiae, L. brevis*	Yeast bread, beer, sake, Chinese rice wine and rice vinegar	[63]
Plants	*L. plantarum, L. brevis*	Kimchi, sauerkraut, olives, Szechuan pickled vegetables	[64]
Legumes	*A. soyae, Z. rouxii, T. halophilus*	Fermented bean curd, bean paste, miso, soya sauce	[65]
Fruits	*S. cerevisae*	Wine, vinegar	[66]
Fish and shellfish	*Lactobacillus brevis*	Fermented fish, fish sauce, shrimp paste	[67]

The Med Diet, however, is not just the choice of certain foods rather than others, but is also the way the inhabitants of this region select, prepare, and enjoy said food together. The new Med Diet food pyramid includes ingredients from all the food groups in appropriate proportions and frequencies, and is addressed generally to the healthy adult population; however, it is flexible enough also to cater pregnant women, children, and people with different pathophysiological conditions [70]. Putting together foods from various levels of this food pyramid makes a balanced healthy diet and promotes a healthy lifestyle. The guidelines established by the pyramid for daily, weekly, and occasional foods are represented in Table II [69, 70].

The Mediterranean food combinations

Perhaps the most important thing in adapting to this culinary regime is to understand its cultural heritage and the traditions behind various food combinations. It is not simply a diet, but a lifestyle: not only it involves healthy eating, but also certain ways of cooking, seasonality of products, and socialization and community interactions [71]. Accordingly, to retrieve all the health-promoting benefits of this dietary regime, one must understand the Mediterranean food combinations.

Addressing the issue of food consumption in the Mediterranean region means perceiving it as a practice that is so well-knitted to the rest of the activities making up the

Tab. II. The Med Diet food pyramid: food choices, servings, and frequencies.

Foods	Quantities/serving	Frequency	Benefits
Wholegrain cereals	One or two servings per meal in the form of bread, pasta, couscous, rice, millet, etc.	Daily	Source of iron, magnesium phosphorous, micronutrients
Vegetables	Two servings per meal at lunch and dinner (at least one serving should be raw)	Daily	Source of antioxidant, anticancer, and antidiabetic compounds
Fruits	One or two servings per meal	Daily	Satisfy sweet cravings and source of antioxidants
Dairy products	One or two servings of low-fat yogurt, Greek yogurt, and different types of cheese	Daily	Source of saturated fats and calcium maintenance of bones
Olive oil	One tablespoon per person for salad dressing, moderate consumption in cooking	Daily	Has a central position in the pyramid as a principal source of dietary lipids, maintains the blood lipid profile, has cardioprotective properties, lowers the risk of developing atherosclerosis
Spices, herbs, garlic, onions, olives, nuts, and seeds	Handful of olives, nuts, and seeds as snacks. Garlic and onion in salads, cooking main course dishes, soups, etc.	Daily	Improve flavour and palatability of the food and are rich in healthy lipids, proteins, vitamins, minerals, and fibre
Fermented beverages	One glass per day (women) or two glasses per day (men), recommended during meals	Daily	Source of probiotics and maintain gut microbiota
Animal (fish, red meat, eggs) and plant proteins	Both animal and plant proteins can be consumed, with animal proteins only for taste (fish: two or more servings, red meat: two servings, eggs: two to four servings)	Weekly	Source of healthy amino acids and fats
Sugary and unhealthy fat rich foods	Very low amounts	Occasional	Med Diet regime supports a very low and occasional consumptions of sweets and confectionaries, to avoid negative health effects

food event that it cannot be considered alone. For instance, consumption of the Mediterranean diet cannot be separated from its production, cultural traditions, social practices, and methods that have evolved with history and are built around food and nutrition [72]. For this very reason the Med Diet is not just a dietary regime, but a concept that encompasses cultural heritages, values and traditions, biodiversity sustainability, and, ultimately, health and wellbeing.

The Culinary Distinctions of the Mediterranean Diet

Mediterranean cuisine includes foods from the Mediterranean basin, which includes countries like Syria, Turkey, Egypt, Spain, Italy, and Greece. This region is famous for its rich history, which results in a wide diversity in cultures, social structures, ethnic groups, occupations, and religions [73]. This cultural diversity is strongly reflected in the combinations of condiments and procedures, collectively constituting a culinary system that encompasses a common historical and regional framework. The distinguishing criterion for the Mediterranean cuisine within the different countries is the choice of the staple food that is eaten by all socioeconomic classes. The second distinguishing feature is the cereal or groups of cereals, along with the accompanying elements, condiments, and methods [72].
The third element of distinction is the significant regional and cultural difference among various countries of the Mediterranean, which enables us to divide the Mediterranean into three culinary regions: Southern Europe, Eastern Mediterranean, and North Africa [74].

Southern European cuisine

Southern European cuisine is typical of Southern France, Italy, and Spain, and is characterised by specific distinctive ingredients. Unlike other Mediterranean cuisines, wine is an important element of this cuisine. In addition, the main source of meat is pork, preferred over lamb/mutton or goat. The staple foods are pastas, leavened breads, and rice; and a wide variety of grains are used. Food flavoring is done by using garlic, mustard, anise, tomatoes, capers, anchovies, and pine nuts [75].

Eastern Mediterranean cuisine

This culinary region comprises Middle Eastern cuisine and the culinary traditions of Turkey, Greece, Egypt, Syria, Lebanon, Palestine, Israel. The prominent foods of this region include yogurt, which is widely consumed and also used in sauces and condiments, and fresh cheeses like halloumi and feta, that can be either cooked or consumed raw in a wide variety of culinary assortments. The flavouring is carried out by mint, parsley, sumac, and lemon juice, with pomegranates and nuts as common ingredient in spreads and sauces. Cereals and grains are consumed, also in the form of pitas and lavash. Bulgur wheat is also added to salads, like tabbouleh. The main sources of animal proteins are mutton, lamb, goat and poultry, for example in the form of grilled kebabs, kibbeh, and stir-fried gyros. Chickpeas are used as a meat substitute and are either cooked whole, fried, or in the form of a paste, known as hummus [76].

North African cuisine

This cuisine is distinguished from others by its plentiful use of spices: cumin, saffron, coriander, cinnamon, chilies, cloves, and paprika are regularly used in the cooking traditions of Morocco, Algeria, Tunisia, and Libya. Harissa and ras el hanout are two intense spice mixtures, mostly used in Moroccan cuisine, that give heat to stews and sauces. Preserved dried lemons are used to add a peculiar, brined taste to the North African cuisine. Commonly used grains and cereals are couscous and granular semolina, often consumed with lamb, mutton, and goat meat-based dishes and stews [77]. Chicken and beef are also regularly used in this cuisine. Dried fruits like apricots, dates, and raisins are frequently used, both raw and in cooked dishes. One of the most famous dishes of this region is the Moroccan tagine, a blend of slow-cooked stew of vegetables, meat, and sauce, named after the cone-shaped earthenware pot in which it is cooked [78]. Hibiscus tea and juices containing hibiscus extract are popular drinks of this region [79].

Conclusions

The Mediterranean cuisine is a blend of the values, practices, and exchange of ideas in the Mediterranean basin, which shaped and developed the culinary habits and appetites of the diverse Mediterranean cultures. It is a complex and interactive food system: not just a mere selection of foods, but a network of cultural practices that has come up with a healthy dietary regime. This wonderful cultural heritage needs to be preserved not only for the culinary satisfaction of the people of the world, but also for providing mankind with a diet that sustains health and wellbeing.
The Mediterranean diet, being the healthiest diet regime of the world, is a repertoire of healthy food choices that, when consumed in the recommended manner and followed in a holistic way, as do the Mediterranean people, can sustain a healthy and long life. An important role among the beneficial functional foods of this dietary regime are the fermented foods, which not only improve the gut microbiota, but also create an improved immune system, better bowel movements, reduce harmful gut bacteria, and maintain the gut-brain axis, which is the key to human health. In addition to the aforementioned health benefits, fermented foods have antioxidant and anti-cancerous properties, making them ideal as low-cost nutraceuticals. Adapting to this diet regime and adhering to its recommendations can support a healthy lifestyle, both in healthy individuals and in people suffering from underlying pathophysiological conditions.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; ZN, GB, MCM, BA, VV, GM, AI: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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REVIEW

Modern vision of the Mediterranean diet

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Keywords

Mediterranean diet • Anticancer effects • Antidiabetic effects • Antimicrobial effects • Modification of hormone release

Summary

The Mediterranean diet is the most well-known and researched dietary pattern worldwide. It is characterized by the consumption of a wide variety of foods, such as extra-virgin olive oil (EVOO), legumes, cereals, nuts, fruits, vegetables, dairy products, fish, and wine. Many of these foods provide several phytonutrients, among which polyphenols and vitamins play an important role. Data from several studies have strongly established that nutrition is a key factor in promoting a healthy lifestyle and preventing many chronic diseases. In particular, a large number of studies have established the protective effects of the Mediterranean diet against several chronic diseases, among which are diabetes, cardiovascular diseases, cancer, aging disorders, and against overall mortality. Animal and human translational studies have revealed the biological mechanisms regulating the beneficial effects of the traditional Mediterranean diet. Indeed, several studies demonstrated that this nutritional pattern has lipid-lowering, anticancer, antimicrobial, and anti-oxidative effects. Moreover, the Mediterranean diet is considered environmentally sustainable. In this review, we describe the composition of the Mediterranean diet, assess its beneficial effects, and analyze their epigenomic, genomic, metagenomic, and transcriptomic aspects. In the future it will be important to continue exploring the molecular mechanisms through which the Mediterranean diet exerts its protective effects and to standardize its components and serving sizes to understand more precisely its effects on human health.

Introduction

The Mediterranean diet (MD), defined by Ancel Keys in the 1960s, is one of the most well-known and well-researched dietary patterns worldwide [1]. The MD is the traditional dietary pattern followed by the inhabitants of the Mediterranean region. Historically, in the countries close to the Mediterranean Sea, the main diet has included an abundance of different non-starchy vegetables, seeds, nuts, marginally refined whole-grain cereals, and legumes [2].
Since the 1960s, MD has been extensively studied to understand its role in the prevention of chronic and/or degenerative diseases, cardiovascular diseases, metabolic syndrome, cognitive decline, and cancer [3].
Furthermore, MD is considered an environmentally sustainable dietary pattern [4]. Specifically, an epidemiological study revealed the association of MD with the decreased incidence of cardiovascular diseases [5]. Similarly, other observational and epidemiological studies reported an inverse relationship of MD with disease risk and mortality in various types of cancers [6-8]. The interventional trial PREDIMED (Prevención con Dieta Mediterránea) compared MD with the control diet and documented a significant reduction in the incidence of diabetes and cardiovascular diseases in the MD group [8].
A recent meta-analysis based on observational studies and clinical trials highlighted the beneficial effects of MD on various chronic diseases, such as cardiovascular diseases, diabetes, myocardial infarction, coronary heart disease, neurodegenerative diseases, cancer incidence, and overall mortality [9, 10]. Furthermore, studies have analyzed the effects of MD on cognitive function, aging parameters, and improvement in quality of life [11]. MD has been linked to the healthy aging concept, which is defined as the lack of major chronic diseases, good mental and physical condition, absence of depression, absence of function-limiting pain, good social functioning, and independent performance of daily activities [8].

Mediterranean diet components and their characteristics

A significant feature of MD is the daily consumption of several phytonutrients, such as plant phenols and vitamins as follows.

Extra-virgin olive oil (EVOO)

One of the main characteristics of MD is the regular consumption of EVOO, which contains a mixture of essen-

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tial dietary fatty acids. The consumption of olive oil is considered the main reason for a long life span amongst Mediterranean populations [1]. EVOO is the major source of unsaturated fatty acids and other components, such as fat-soluble vitamins, polyphenols, chlorophylls, and phytosterols [12, 13]. The polyphenols present in olive oil possess anti-inflammatory, antioxidant, neuroprotective, cardioprotective, anticancer, anti-obesity, anti-diabetic, antimicrobial, and antisteatotic effects. These effects are mainly caused by the presence of secoiridoid (anti-feeding deterrents of the *Oleaceae* family, such as iridoid glycoside) derivatives, among which oleuropein, oleacein, and oleocanthal, and simple phenols, such as tyrosol and hydroxytyrosol [3, 14-20].

Legumes, cereals, and nuts

Humans have been cultivating legumes for centuries and consuming them in the form of porridge and pulses. Pulses are highly nutritious and can be easily prepared and stored for long periods. Undoubtedly, these features of legumes are the cause for their success and their incorporation in the traditional diets of various countries. The most common legumes of MD are beans, lentils, and chickpeas. Legumes are usually mixed with different cereals, fish, meat, and vegetables. Similarly, for thousands of years, seeds and nuts (hazelnuts, almonds, tree nuts, pistachios, etc.) have been considered a staple food and consumed daily. Nuts and legumes have been routinely consumed all over the Mediterranean region, Asia, and America [1]. The main components of pulses and beans are flavanols, a type of polyphenols with a ketone group in their chemical formula, which reduce endothelial dysfunction, decrease cholesterol and blood pressure, and regulate energy metabolism [21]. Moreover, people living in the Mediterranean countries regularly consume cereals, such as rice and wheat, in the form of pasta, bread, couscous, etc. These cereals, along with potatoes, constitute the main sources of energy and carbohydrates [1].

Fruits and vegetables

The Mediterranean climate favors the production of several vegetables and fruits that constitute a major part of MD. Original Mediterranean vegetables include turnips, artichokes, lettuce, and radishes. Interactions with outside regions led to the introduction of new varieties of fruits and vegetables. For example, citrus fruits and eggplant were introduced from North Asia and India, whereas zucchini, tomatoes, potatoes, peppers, corn, and green beans entered the Mediterranean region from the Americas [1].

Dairy products

Traditionally, the consumption of milk and other dairy products has been low in the Mediterranean countries. However, plenty of land is devoted for raising goats and sheep for their meat, milk, and wool, thus facilitating the manufacture of yogurt, cheese, and other fermented dairy products [1].

Fish

The Mediterranean region possesses a rich tradition of fishing, which has led to high fish consumption. However, environmental contaminants have compromised the contributions of omega-3 fatty acids [1].

Wine

In the European Mediterranean countries, MD has been significantly associated with moderate wine consumption during meals. Wine is known to have originated during the Neolithic period, while the Greeks and Egyptians popularized the beverage by developing the techniques related to its refinement and preservation. Moreover, Romans extended grapevine cultivation across Italy and other countries, hence making wine an essential part of MD [1].

Mechanisms involved in Mediterranean diet effects

Latest advancements in all omics fields and bioinformatics have allowed their use in nutritional studies for enhancing the understanding of molecular mechanisms and changing paradigms [8, 22].

Mediterranean diet and transcriptomics

The use of transcriptomics makes it possible to analyze the specific effect of a diet or food on gene expression, thereby leading to a better understanding of specific mechanisms. It is possible to unravel which gene expression is upregulated or downregulated by the influence of certain foods. In humans, several researchers have analyzed the effects of MD and its components on the transcriptome using selected candidate genes as well as the whole transcriptome. The PREDIMED study has examined alterations in canonical pathways of the cardiovascular system. Nine of these pathways were altered by MD + virgin olive oil, whereas four pathways were modified by MD + nuts. Overall, the results showed that MD modulates crucial pathways associated with cardiovascular risk, such as renin–angiotensin, atherosclerosis, hypoxia, angiopoietin and nitric oxide signaling, and endothelial nitric oxide synthase signaling pathways. This finding supports the idea that MD could exert beneficial effects by altering the expression of genes associated with cardiovascular diseases. Interestingly, the study noted that the atherosclerosis signaling pathway was significantly downregulated after the MD + EVOO intervention (Tab. I) [23, 24].

Mediterranean diet and epigenomics

The term epigenomics refers to a wide range of genomic modifications without involving changes in the DNA sequence, which lead to alterations in gene expression. The epigenomic profile may be linked to increased car-

Tab. I. Genes of the atherosclerosis signaling pathway that were downregulated after the MD + EVOO intervention.

Gene symbol	Gene name
IL1RN	Interleukin 1 receptor antagonist
IL1β	Interleukin 1-beta
ICAM1	Intercellular adhesion molecule 1
TNF-α	Tumor necrosis factor, alpha

Tab. II. Immune and inflammatory-linked genes for which methylation pattern was modified by 5 years of MD in peripheral blood cells.

Gene symbol	Gene name
EEF2	Eukaryotic translation elongation factor 2
IL4I1	Interleukin 4-induced gene 1
COL18A1	Collagen, type xviii, alpha-1
PLAGL1	Plag1-like zinc finger 1
LEPR	Leptin receptor
PPARGC1B	Peroxisome proliferator-activated receptor-gamma, coactivator 1, beta
IFRD1	Interferon-related developmental regulator 1
MAPKAPK2	Mitogen-activated protein kinase-activated protein kinase 2

diovascular risk and aging [25]. Three types of epigenetic biomarkers are often observed based on epigenetic regulators: DNA methylation, noncoding RNA synthesis, and histone modification. A study involving 36 participants investigated the alterations induced in the methylome of peripheral blood cells after 5 years of MD (Tab. II) [26]. Similar results were obtained by studies that evaluated the effects of MD on inflammation at the epigenetic level [8, 27, 28].

Mediterranean diet and genomics

The first omics approach was focused on the study of single nucleotide polymorphisms that influence diseases associated with the metabolic status. With technological advancements, genome-wide association studies and, subsequently, next-generation sequencing technologies were applied to explore multiple polymorphisms in a single experiment [29-31] Currently, studies focusing on gene–diet interactions are involved in examining the heterogenic responses of identical dietary patterns, which means that different individuals exhibit different responses to the same MD components. PREDIMED revealed that polymorphisms in specific genes associated with cardiovascular disease risk display significant gene–diet interactions with MD (Tab. III) [8]. The influence of MD on microRNA-binding site polymorphisms was observed via the analysis of the gain-of-function mutation polymorphism (rs13702) of microRNA-410 in the *LPL* 3′-untranslated region. The findings revealed a gene–diet interaction and demonstrated that MD enhanced reduced triglyceride concentrations and stroke risk, whereas in the control diet, these beneficial effects were lost [8, 32].

Mediterranean diet and metagenomics

The gut microbiota plays an important role in the relationship between dietary habits and health. Several studies analyzed the effect of MD components on microbiota, both at the species level and at the metagenomic level. Some of the studies reported beneficial effects of MD on the microbiota, and other studies examined the favorable effects of MD on health by simulating the profiles of beneficial microbiota. Moreover, the presence of metabolomic markers in urine or plasma indirectly reflect the microbiota activity. The incorporation of metabolomics and metagenomics, along with exposomics (the study of all the exposures of an individual in a lifetime and how those exposure relate to health) and genomics, will certainly provide informative results on the mechanisms of action of MD in the years to come [8, 33, 34].

Mediterranean diet and bioinformatics

Computational and bioinformatic methods play a vital role in investigating the effects of MD. The latest bioinformatic tools and highly efficient data-generation methods have enabled the collection of huge amounts of information and rapid analyses of data. Currently, various bioinformatic tools and techniques, such as networking and pathway analyses, are being applied to understand the complexity of MD effects at the systems biology level. Significant advancements are expected in the near future, which are likely to enable us to better understand the molecular basis of the multidimensional effects of MD [8, 35, 36].

Effects of the Mediterranean diet on disease pathways

MD exerts many beneficial effects on human health and prevents chronic diseases via various mechanisms.

Lipid-lowering effects

The initial mechanistic studies explaining the inverse relationship of MD with cardiovascular risk focused on high monounsaturated fatty acid and low saturated fatty acid contents of MD. These studies also examined other conventional risk factors, such as plasma lipid concentration, glucose metabolism, and blood pressure [8, 37, 38]. The results of the PREDIMED study showed that MD is able to improve the protective role of

Tab. III. Genes for which polymorphisms are associated with cardiovascular disease risk and display significant gene-diet interaction with MD.

Gene symbol	Gene name
MLXIPL	Mlx-interacting protein-like
TCF7L2	Transcription factor 7-like 2
CLOCK	Circadian locomotor output cycles kaput
LPL	Lipoprotein lipase

HDL by reversing cholesterol transport and enhancing cholesterol efflux capacity via reduction of the activity of cholesteryl ester transfer protein, thereby increasing HDL's ability of cholesterol esterification and its vasodilation capacity [39, 40].

Protection against oxidative stress and inflammation

Traditional MD is rich in antioxidant components such as vitamin E, β-carotene, vitamin C, and flavonoids, minerals such as selenium, and natural folate [2]. The results of a large case–control study called INTERHEART revealed the beneficial effect of dietary antioxidants on coronary heart disease [41]. Insufficient intake of dietary antioxidants might escalate the risk of atherosclerotic plaque formation owing to alterations in lipoprotein oxidation. Recently, the participants of a randomized clinical trial who adhered to an MD + EVOO dietary pattern demonstrated a significant decrease in inflammatory markers and oxidized circulating LDL [42]. The PREDIMED study showed that MD exerts anti-inflammatory effects on the cardiovascular system and is able to reduce both systolic and diastolic blood pressure [43]. Specifically, PREDIMED reported that serum levels of several genes decreased after 3-5 years of MD intervention (Tab. IV) [44].

Oxidative, inflammatory, and nitrosative stresses are the most common causes for neurodegeneration, whereas antioxidant molecules, such as polyphenols from olive oil, restore neuronal function by improving the redox status. Additional beneficial effects of MD include hypoglycemic, antioxidant, antiviral, antimicrobial, cardioprotective, antitumor, anti-inflammatory, neuroprotective, and antiaging effects [45].

In transgenic mouse models of Alzheimer's disease, hydroxytyrosol was found to alleviate oxidative stress in the brain and mitochondria as well as neuroinflammation by inducing the expression of the Nrf2-dependent gene. Similarly, the administration of oleuropein for 8 weeks at a dose of 60 mg/kg/day was able to decrease oxidative stress and increase mitochondrial function via activation of the Nrf2 pathway in spontaneously hypertensive rats. Moreover, tyrosol, at a dose of 240 mg/kg, was able to offer protection against lipopolysaccharide-induced acute lung injury via inhibition of NF-kB and activation of AP-1 and Nrf-2 pathways [3, 46].

Tab. IV. Genes for which serum levels decreased after 3–5 years of MD intervention.

Gene	Gene name
IL-6	Interleukin 6
IL-8	Interleukin 8
IFN- γ	Interferon, alpha-1
IL-5	Interleukin 5
IL-7	Interleukin 7
IL-1β	Interleukin 1-beta
TNF-α	Tumor necrosis factor, alpha
IL-12p70	Interleukin 12

In an animal model of rheumatoid arthritis, the phenolic extracts of EVOO protected the joints and decreased proinflammatory mediators via inhibition of MAPK and NF-κB signaling in activated synovial fibroblasts. In the same model, the polyphenolic extracts of EVOO inhibited IL-6, TNF-α, IL-1β-induced matrix metalloproteinases, microsomal PGE synthase-1, and IL-1β-induced cyclo-oxygenase-2 [3, 47].

Anticancer effects

Since the last decade, several *in vivo* and *in vitro* studies have revealed anticancer effects of hydroxytyrosol from olive oil against numerous malignant cell types, which could be attributed to different mechanisms of action. Most of the studies have been focused on colon cancer, which is the third most prevalent cancer worldwide and is associated with a high death rate in developing countries. Because of its autooxidation properties, the accumulation of H_2O_2 is considered one of the most significant anticancer mechanisms of hydroxytyrosol. However, several studies have highlighted the proapoptotic and antiproliferative mechanisms of hydroxytyrosol based on the type of cancer cells studied [48, 49]. The analysis of androgen-dependent prostate cancer cells showed that hydroxytyrosol inhibits the expression of the androgen receptor and androgen receptor-responsive prostate-specific antigen secretion [50].

Furthermore, in hepatocellular carcinoma cells, hydroxytyrosol exerts anticancer effects by inhibiting proliferation and inducing apoptosis and G2/M cell cycle arrest. Moreover, hydroxytyrosol could lead to angiogenesis and tumor growth inhibition *in vivo* via the inhibition of NF-κB and PKB/Akt pathways. The proapoptotic and antiproliferative effects of hydroxytyrosol are also linked to inhibition of the lipogenic enzymes farnesyl diphosphate synthase and fatty acid synthase in human hepatoma cells, which are related to aggressive tumor behavior [51].

Antidiabetic effects

Several *in vivo* animal studies on diabetes have established the beneficial effect of oleuropein or olive leaf extracts rich in oleuropein against type 2 diabetes. Clinical trials that enrolled people with type 2 diabetes mellitus have reported significant reductions in fasting plasma glucose levels and glycated hemoglobin levels after treatment with 500 mg/day of olive leaf extracts for 14 weeks. Another clinical trial on overweight middle-aged men reported significant improvement in the responsiveness of pancreatic β-cells and insulin sensitivity after supplementation with olive leaf extracts, 51 mg oleuropein, and 9.7 mg hydroxytyrosol on a daily basis [14, 52].

In animal model of diabetes, significant reductions in serum glucose, oxidative stress, and cholesterol levels were observed after oleuropein treatment. Moreover, oleuropein promoted glucose-stimulated secretion of insulin in pancreatic β-cells via the stimulation of the ERK/MAPK signaling pathway and inhibition of amylin

amyloid cytotoxicity, which is the most prominent characteristic of type 2 diabetes [53, 54].

Antiatherogenic effects

Oleuropein and hydroxytyrosol present in MD inhibit monocytoid cell adhesion and endothelial activation. These effects are attributed to the antioxidant and anti-inflammatory activities of oleuropein and hydroxytyrosol [14, 55, 56].

Effects on autophagy

Autophagy is essential for the efficient development and functioning of cardiomyocytes. Moreover, the process plays a vital role in regulating the inflammatory response produced by macrophages, most likely via restriction of the activity of inflammasomes and generation of macrophage foam cells by lipid turnover modulation. Autophagy also modulates neurodegenerative diseases and metabolism dysregulation. Therefore, the beneficial effects of MD might influence the regulation of autophagy [57, 58].

Researchers have observed that polyphenols from MD exert a direct effect on autophagy. Resveratrol, a polyphenol present in nuts, wine, and grapes, is an autophagy inducer [59]. The effects of resveratrol on autophagy might be explained by its enhancing effect on the activity of deacetylase sirtuin 1, which in turn regulates the activity of several autophagy-related proteins. Likewise, polyphenols present in virgin olive oil, such as oleocanthal and oleuropein, have been reported to enhance autophagy [8, 60].

Modification of hormones and growth factors

Short-chain fatty acids that are produced by the metabolism of oligosaccharides and resistant starch present in MD by the gut microbiota can induce satiety by obstructing gastric emptying, thereby increasing the production of gut hormones, such as glucagon-like peptide-1 and peptide-YY. Importantly, in addition to weight loss, MD causes a substantial decrease in fasting glucose and C-peptide levels as well as free and total testosterone levels [61].

In women, MD causes a significant increase in plasma levels of sex hormone binding globulin and insulin-like growth factor binding protein 1 and 2, which reduce the biological activity of estradiol, insulin-like growth factor 1, and testosterone [62]. Additionally, lower glycemic index, lower branched-chain amino acid intake, and higher monounsaturated and n-3 fatty acid intake might exert beneficial effects in decreasing insulin resistance along with compensatory hyperinsulinemia [63, 64]. Furthermore, the high fiber contents of MD could increase fecal mass and estrogen excretion, which results in decreased plasma levels of estradiol and estrone [65]. The vegetables present in MD are rich in chemical compounds that offer potential benefits against different types of cancer, such as lycopene in tomato; organosulfur compounds in onion and garlic; capsaicin in hot pepper; indol-3-carbinol, isothiocyanates, and sulforophane in cruciferous vegetables; monoterpenes in oranges and lemons; polyacetylenes in pumpkin and carrots; spermidine and ferulic acid in whole grains; and ginkgetin in capers. Moreover, estrogenic molecules with low potency, such as biochanin A, formononetin, daidzein, coumestans, and genistein found in beans, can compete with the endogenous estrogens for binding to estrogen receptors, hence blocking their mitogenic effects [2, 66].

Antimicrobial and antiviral effects

Studies have reported that hydroxytyrosol exhibits *in vitro* antimicrobial properties against various gastrointestinal tract and respiratory infectious agents, such as *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Haemophilus influenzae*, *Salmonella typhi*, *Moraxella catarrhalis,* and *Staphylococcus aureus*, at reduced inhibitory concentrations as well as foodborne pathogens, such as *Listeria monocytogenes*, *Yersinia enterocolitica,* and *Salmonella enterica*. Furthermore, the antimicrobial activities of hydroxytyrosol oleate and hydroxytyrosol acetate against *Staphylococcus epidermidis* and *Staphylococcus aureus* were evaluated [67, 68]. The results from such studies established that hydroxytyrosol inhibits the hemolytic activity of streptolysin O released by *Streptococcus pyogenes*. Additionally, hydroxytyrosol demonstrates antibacterial activity against *Propionibacterium acnes* and mycoplasmas, such as *Mycoplasma pneumoniae* [69]. Hydroxytyrosol also appears to display inhibitory properties against human immunodeficiency virus (HIV)-1, preventing it from entering the host cell and binding to its catalytic site, thus inhibiting viral entry and integration. Studies have also reported the inactivation of influenza A viruses by hydroxytyrosol, thus suggesting that the antiviral mechanism of hydroxytyrosol might require the presence of the viral envelope [14, 70]. Finally, hydroxytyrosol exerts a similar antiviral mechanism against SARS-Cov-2 virus, resulting in a potential treatment benefit against COVID-19 infection [71-75].

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; AKK, MCM, GB, BA, VV, GM, AI, LS, STC, KLH: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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REVIEW

Implication of the Mediterranean diet on the human epigenome

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Keywords

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Summary

Epigenetics, defined as "hereditary changes in gene expression that occur without any change in the DNA sequence", consists of various epigenetic marks, including DNA methylation, histone modifications, and non-coding RNAs. The epigenome, which has a dynamic structure in response to intracellular and extracellular stimuli, has a key role in the control of gene activity, since it is located at the intersection of cellular information encoded in the genome and molecular/chemical information of extracellular origin. The focus shift of studies to epigenetic reprogramming has led to the formation and progressive importance of a concept called "nutriepigenetics", whose aim is to prevent diseases by intervening on nutrition style. Among the diet types adopted in the world, the renowned Mediterranean Diet (MD), being rich in unsaturated fatty acids and containing high levels of whole grain foods and large quantities of fruits, vegetables, and legumes, has shown numerous advantages in excluding chronic diseases. Additionally, the fact that this diet is rich in polyphenols with high antioxidant and anti-inflammatory properties has an undeniable effect in turning some cellular pathways against the disease. It is also apparent that the effects of polyphenols on the epigenome cause changes in mechanisms such as DNA methylation and histone acetylation/deacetylation, which have a regulatory effect on gene regulation. This review presents the effects of long-term consumption of nutrients from the MD on the epigenome and discusses the benefits of this diet in the treatment and even prevention of chronic diseases.

Introduction

The concept of epigenetics, which was first defined by Conrad Waddington in 1942, was discussed as the gene-environment interaction established between genotype and phenotype in the developmental process [1]. The currently accepted definition of epigenetics, which etymologically means "above gene", is "hereditary changes in gene expression that occur without any change in the DNA sequence". Although the 21st century is defined as the "century of epigenetics", it carries out studies in this field by targeting various epigenetic marks, such as DNA methylation, histone modifications, and non-coding RNAs, in the control of cellular processes. In the genome, which is constituted of all of the genetic information contained in cell DNA, these epigenetic marks have extremely stable and dynamic structures, but they can also lead to changes in the epigenetic state when influenced by environmental factors [2].

On the other hand, the complex modifiers associated with genomic DNA that enable the transfer of unique cellular and developmental identity constitute the epigenome. In other words, the epigenome, which includes any gene expression modifier that is independent from the DNA sequence in said gene, is the chain of chemical tags that can modify the DNA and the structures associated with it. Therefore, the epigenome is at the intersection of the cellular information encoded in the genome and the molecular/chemical information of extracellular origin.

Over the last two decades, once genetic information has become understandable, the focus of studies in this field has shifted to epigenetic reprogramming. In this context, studies about nutrition, which is an indispensable part of human life, have drawn attention to the importance of epigenetic studies, especially those focusing on the mission of preventing diseases. Among the many types of diet, it is frequently discussed within the framework of precision and preventive medicine that the Mediterranean Diet (MD) plays an effective role in reducing the prevalence of many chronic diseases. This review will discuss current approaches to the role of the MD in epigenome control, as personalized medicine and disease prevention strategies are gaining importance today.

An overview of the elements that make up human epigenome

Together with the genome, the epigenome establishes a unique gene expression program to define the functional identity that is unique to each cell type, plus the

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developmental or disease processes. At the same time, the epigenome plays a role in the development of the organism's ability to respond to some environmental stimuli. Therefore, unlike the stable genome, the epigenome is characterized by a dynamic and variable behavior in response to intra- and extracellular stimuli. Maintaining this dynamic structure and activating gene regulation mechanisms from the developmental process essentially require the formation of epigenetic memory. In the formation of this epigenetic memory, the interactions of the epigenome with the environment affect the activation process of genes. Therefore, it is worthy of consideration that the epigenome of an individual is not only hereditary, but it can also be shaped by exposure to certain environmental factors during the prenatal period [3]we are still far from understanding the molecular events underlying phenotypic variation. Epigenetic modifications to the DNA sequence and associated chromatin are known to regulate gene expression and, as such, are a significant contributor to phenotype. Studies of inbred mice and monozygotic twins show that variation in the epigenotype can be seen even between genetically identical individuals and that this, in some cases at least, is associated with phenotypic differences. Moreover, recent evidence suggests that the epigenome can be influenced by the environment and these changes can last a lifetime. However, we also know that epigenetic states in real-time are in continual flux and, as a result, the epigenome exhibits instability both within and across generations. We still do not understand the rules governing the establishment and maintenance of the epigenotype at any particular locus. The underlying DNA sequence itself and the sequence at unlinked loci (modifier loci.

Epigenetic modifications enable chromatin organization and maintain cellular function by creating inherited transcription conditions. This type of regulation occurs through DNA and histone and chromatin modifications, which occur by packaging histone-binding proteins. The epigenome is shaped differently depending on the cell type, usually involving reversible mechanisms via its epigenetic marks. The mechanisms responsible for this regulation are DNA methylation – the most important element of DNA modifications that have a direct effect on the epigenome – and chromatin modifications, including histone acetylation, histone methylation, histone deimination, glycosylation, ADP ribosylation, histone ubiquitination, histone sumoylation, histone phosphorylation, nitrosylation, biotinylation. Apart from these mechanisms, non-coding RNA-mediated RNAi mechanisms, which have an important role in targeted gene silencing, have been studied frequently in recent years, thus opening a new horizon in studies in the field of epigenetics.

DNA MODIFICATIONS THAT ACT DIRECTLY ON THE EPIGENOME: DNA METHYLATION

Epigenetic marks are an important component of the epigenome because they cause diverse effects on gene expression. Since the transformation into a specific cell type from the very early stages of embryogenesis is considered as a "software" encoded in the epigenome, it can be concluded that the epigenome plays a leading role in defining cell functions [4]. Notwithstanding, within the scope of the measures taken to prevent metabolic syndromes (such as diabetes) in the early stages of life, the fact that dietary styles cause permanent changes in DNA methylation is among the issues that have been frequently emphasized in the recent years. In fact, there is growing evidence that nutrition has an impact on DNA methylation, which is one of the largest and most studied epigenetic mechanisms that play a key role in gene expression maintaining [5].

The core of this mechanism is the conversion of cytosines in regions where CpG islands (sometimes also called "CpG clusters") are dense to 5-methyl cytosines (5mC), undertaking the task of suppressing gene expression by blocking transcription factors [6]. DNA methylation, which plays an important role in gene expression and chromatin organization and is a part of the normal developmental process, largely occurs in the early postnatal period and during development. Recent studies demonstrated a strong link between DNA methylation and human diseases, directing post-genome era studies in this field. In this respect, its effect on nutrigenomics is a separate research topic in itself.

The mechanism of DNA methylation in mammalians is based on DNA methyltransferases (DNMTs), which are responsible for establishing methylation models throughout the genome, and methyl-CpG binding proteins (MBDs), which are responsible for reading the relevant marks. Among the DNA methyltransferases, those classified as DNMT3A and DNMT3B are the two main enzymes responsible for *de novo* methylation, having functions such as methylation remodeling and suppression during embryogenesis. DNMT1, another critically important, plays a role in establishing the methylation pattern and suppressing transcription by specifically targeting hemi-methlyated CpG sequences. Enzymes such as DNMT2 and DNMT3L, which play ancillary roles in the formation of the complex, have also been identified [7, 8]. Deregulations in DNA methylation, being a dynamic mechanism and playing a pivotal role in controlling the timing of cellular processes, have been associated with many diseases such as cancer, cardiovascular diseases, and predisposition to obesity [9].

Chromatin modifications that act directly on the epigenome: Histone modifications

Chromatin is an entire highly condensed DNA-protein complex, whose structural and functional unit is the so-called "nucleosome", that forms the backbone of essential nuclear processes – such as replication, transcription, and DNA repair – in all eukaryotes. It exists in two functional forms, euchromatin and heterochromatin, which are conceptually different from each other. Euchromatin is a highly loosely condensed form that is transcriptionally active and provides the environment for DNA-regulatory processes, while heterochromatin is often defined as its tightly condensed transcriptionally inactive form, devoid of DNA-regulatory activity [10]. Histones are evolutionarily well-conserved proteins.

There are two copies of each of the H2A, H2B, H3, and H4 histones in the octamer nucleosome, and their organization is completed when 147 bp DNA wraps around this octamer structure twice [11].
Histone modifications that develop after the translational process in the transfer and after the processing of epigenetic information include more than 200 modifications, which occur at the amino-terminal ends of the histone proteins. These modifications predominantly include cellular events such as acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation, and have been extensively linked to gene activity, gene silencing, or isolation between active and inactive gene regions– similar to DNA methylation [6]. All these modifications cause a change in the structure of chromatin, neutralizing the electrostatic charge of histone tails, as well as leading to the formation of different histone codes and activating or inactivating transcriptions [6, 12].
It should be noted that histone modifications have always been more striking than DNA methylation, due to the fact that the latter's properties can be reversed by applying certain nutrition styles throughout the life span from the periconceptional period [13]which cause heritable changes in gene expression without changes in DNA sequence. Nutrient-dependent epigenetic variations can significantly affect genome stability, mRNA and protein expression, and metabolic changes, which in turn influence food absorption and the activity of its constituents. Dietary bioactive compounds can affect epigenetic alterations, which are accumulated over time and are shown to be involved in the pathogenesis of age-related diseases such as diabetes, cancer, and cardiovascular disease. Histone acetylation is an epigenetic modification mediated by histone acetyl transferases (HATs. Therefore, the protection of chromatin structure by regional relaxations and rearrangements has a key role in processes like DNA replication, DNA repair, and gene transcription; it also underlies dynamic changes within the epigenome. The fact that the enzymes involved in the process of providing this dynamic structure work in opposition to each other show that such modifications have reversible properties. Although there is coordination in all types of histone modifications in the control of chromatin structure, the process is complicated by the interactions between DNA methylation and ATP-dependent chromatin remodeling elements [14].
Among the most interesting studies conducted to understand the role of nutrition in the regulation of these modifications are histone acetylation and deacetylation, which have been studied for decades in different aspects of omics sciences. In this context, it would be beneficial to briefly give the basic outlines of these mechanisms that play a key role in modulating the epigenome. Histone acetylation is a reversible covalent modification that is observed at specific lysine residues of histone tails, which causes the positive electrical charge of the target lysine to become neutral, thereby weakening the electrical attraction between histone-DNA or nucleosome-nucleosome [14]. The enzymes involved in the process are the Histone Acetyltransferases (HATs), which are able to catalyze the transfer of the acetyl ($O = C\text{-}CH_3$) group to the ε-amino group of the lysine chains at the N-terminus of histone tails, using acetyl CoA as a cofactor. Notably, these enzymes neutralize lysine's positive electrostatic charge, thereby causing relaxation of the attraction between histones and DNA. In contrast to this process, Histone deacetylases (HDACs) are involved in the deacetylation process (that is the recycling process of acetylation); these enzymes remove the $O{=}C\text{-}CH_3$ group in the ε-N-acetyl-lysine amino acid in histone and give the lysine a positive charge again. As a result of this reaction, the histone DNA connection is tightened and the local chromatin structure is closed to transcription in a stable state [15].
The elucidation of the molecular mechanisms underlying the reversibility of histone modifications, apart from showing new ways in the treatment scheme of many diseases, has led to the idea that nutrition styles will have an undeniable effect on shaping these histone modifications, and has also recently increased the interest in studies in this direction.

Non-coding RNA-mediated interactions: RNAi

The concept of RNAi was first introduced in 1998, when Andrew Fire and Craig Mello demonstrated the role of double-stranded RNA (dsRNA) in post-transcriptional gene silencing (PTGS) in *C. elegans*, demonstrating that non-coding RNAs play a central role in the gene expression of multicellular organisms [16]. RNAi has been an important part of research in the development of many treatment strategies in addition to the histone modifications because of its convenience in investigating gene function comprehensively, which also makes it a useful tool in the intervention and modification process in the mammalian genome [17]. This technique was developed as an anti-sense strategy and, since it targets the mRNA inside the cell, it is essential to transfer into the cells a DNA or RNA nucleic acid chain that is reverse complementary to this mRNA. Thus, the mRNA is degraded by catalytically active oligonucleotides, preventing the translation of mRNA. In fact, this mechanism is part of an advanced cellular defense system, developed to protect its genome against foreign pathogens. However, including small molecules – such as small interfering RNAs (siRNAs) and microRNAs (miRNAs) – in these pathways was proven to have important functions in gene regulation, which led to its use in molecular biology studies to suppress endogenous genes [18].
siRNAs are of fundamental importance in the mechanism of RNAi by downregulating target mRNAs through endonucleolytic cleavage. In the setup of the RNAi pathway, dsRNAs are reduced to fragments of 20-22 nucleotides, thereby binding to the complementary part of target mRNAs and degrading them [19]. Although the microRNAs have a similar pathway to siRNAs, they are formed from precursor RNA molecules with a stem-loop secondary structure and play a key role in biological processes such as development, differentiation, cell proliferation, and apoptosis. Dysregulations in miRNAs are associated with many chronic diseases (in particular

cancer) and other epigenetic changes that occur together, changing the chromatin structure and thus also the interaction of proteins that is necessary for transcription with DNA. Although it is not possible to go into details of the molecular mechanism of the RNAi process in this review, whose aim is to discuss the MD, nutrigenomic studies conducted in recent years focus on the effects of diet contents on the epigenome by targeting molecules in this regulatory pathway.

The influence of diet on shaping epigenetic mechanisms

Nutrition, being one of the indispensable elements for survival and healthy life, has become an interesting topic because of its evolution into many different patterns of social behavior created by different populations. The plant- and animal-derived nutrients bestowed on them by nature in the land each people inhabited not only shaped many different cultural cuisines, but also paved the way for the utilization of these nutrients as alternative medical treatments. Although there are differences in cultural bases, the global effect of the age we live in has led to the convergence of nutritional needs by coming together on a common denominator. As the most obvious example of this, the adoption of the Western Diet (WD) by almost all societies has made it the most accessible form among people's daily routine activities. However, it is obvious that this diet, being rich in saturated fatty acids, on the long run can cause disorders in glucose and insulin regulation, thus paving the way for the accumulation of fat in the body and an increased risk of many chronic diseases, such as cancer and metabolic syndromes [20, 21].

The emergence of numerous different diets in modern dietary patterns has led to a selective competition in establishing a dynamic balance between cellular metabolisms depending on the food source. In this context, observational studies and randomized controlled trials have revealed that the Mediterranean Diet (MD), which is rich in unsaturated fatty acids, has many advantages in reducing the risk to develop diseases such as Type-2 Diabetes Mellitus (T2DM), obesity, cardiovascular diseases (CVD), and cancer [22]. The MD is a sustainable diet type that is substantially rich in polyphenols, contains high levels of whole-grain foods, and supports the consumption of plenty of vegetables, fruits, and legumes [23, 24]. Additionally, it supports the intake at moderate levels of basic foods such as poultry, fish, and eggs, with the omega-3 fatty acids it contains; also, the consumption of red wine at meals is accepted as a part of this diet. Unlike the Western Diet, consumption of red meat and processed meat is allowed at a lower level, while confectionery products with low or scarcely any nutritional value are minimal [25].

Prior to associating nutrition with epigenetics, it is noteworthy to emphasize the importance of DNA repair mechanisms that are responsible for the protection of genomic stability against any DNA damage. These mechanisms, which preserve the stability of the genome, primarily detect lesions in DNA and enable mechanisms such as base/nucleotide excision repair mechanisms or non-homologous end-joining (NHEJ) and homologous recombination (HR), based on single-strand breaks (SSBs) or double-strand breaks (DSBs) [25, 26]. These repair mechanisms, which act as a shield against DNA damage, are conspicuously present in neoplastic diseases. Although germline mutations are effective in the formation of genomic instability in caretaker genes, particularly in hereditary cancers, this inactivation in sporadic cancers is characterized by the accumulation of DSBs as a result of collapsed DNA replication forks [27]. In this process, which is called oncogene-induced DNA replication stress model, the fact that the MD, known for its protective effect against cancer, provides the necessary metabolic substrates and strengthens genomic maintenance demonstrates that nutrition plays a dominant role in epigenome [25].

As mentioned above, many in vivo studies have shown that dietary habits not only affect the genomic stability of the organism, but they also have a shaping effect on the epigenome. For this purpose, the differences between high-fat and/or high-sugar diets were examined, revealing that the effects of dietary components on histone acetylation and deacetylation were more favorable to living a healthy life when the subjects followed calorie-restricted diets [13]. To illustrate this phenomenon with a few examples, the offspring of Japanese macaques that followed a high-fat diet during pregnancy had higher histone H3 acetylation levels, while the opposite was observed in the offspring of mothers fed a lower-fat diet [28]. Similarly, rats on a high-fat diet showed a significant decrease in liver regeneration abilities, suggesting that this may be consistent with a significant decrease in HDAC activities [29]. All these findings together show that high-fat and/or high-sugar diets can lead to metabolic syndromes by negatively affecting liver functions.

The tight link between nutrition and epigenetics from past to present: The Dutch Famine

Epidemiological studies carried out so far have underlined that the dietary style adopted in childhood and adolescence causes several alterations in gene expression levels; those epigenetic factors play an important role in this regard [30]. In fact, this knowledge became even more significant with the evaluation of metabolic parameters, strikingly revealing that the individual is not only dependent on his/her own diet, but also influenced by his/her parents' diet [5, 31]. It is undeniable that all the factors that the mother is exposed to during pregnancy might lead to epigenetic changes and, accordingly, the predisposition of the fetus to possible diseases that may develop throughout his/her life can be shaped at different levels [32-35].

For example, the famine suffered by the population of the Netherlands during World War II (September 1944-May 1945, known as the "Dutch Hunger Winter") that

Tab. I. Classification of polyphenol groups according to the dietary sources in the Mediterranean Diet and their effects on the cellular processes and epigenome they are involved in.

Subclasses of polyphenols	Phenolic compounds	Bioactive Compounds	Dietary sources in MD		Biological activity	Cellular processes involved	Epigenetic mechanism(s)	Reference(s)
Phenolic Acids	Hydroxybenzoic acids	p-hydroxybenzoic acid (pHBA), 3,4-dihydroxybenzoic acid (DHB; protocatechuic acid), 3,4,5-trihydroxybenzoic acid (Gallic acid), Vanillic acid	Red fruits, black radish, pomegranate, onions, berries, nuts		• Anti-oxidant and anti-inflammatory activities • Anti-tumorigenic and neuroprotective effects	• Cell cycle arrest, and induction of apoptosis • Promoting autophagy and inhibition of oxidative stress in neurons	Inactivation of HATs, activation of HDACs	[54, 78]
	Hydroxycinnamic acids	Cinnamic acid, p-Coumaric acid, Ferulic acid, Caffeic acid, Chlorgenic acid, Rosmarinic acid, Sinapic acid	Blueberries, kiwis, plums, cherries, coffee		Anti-oxidant and anti-inflammatory activities	Regulating autophagy and protective effect against DNA damage, cell cycle arrest, and apoptosis induction	Inhibiting HDACs and DNMTs	[60, 79]
Flavonoids	Flavonols	Quercetin, Kaempferol, Myricetin	Onions, broccoli, blueberries, apples, peppers, tomatoes		Free radical scavenging activity together with its anti-oxidant and anti-inflammatory properties	Inhibition of cell proliferation and tumor growth, induction of apoptosis	Influencing effects in miRNA expression patterns related to inflammation	[60, 80]
	Flavanones	Naringenin, Hesperetin, Eriodictyol	Citrus fruits (such as grapefruit, lemons, oranges, etc.)		Anti-oxidant and anti-inflammatory activities	Protection of pancreatic β cells in late stages of diabetes	Inhibitory effect on histone acetylation and decreased histone methyltransferase activity	[81]
	Isoflavones	Genistein, Daidzein, Glycitein	Legumes, soybeans, red clovers		Chemopreventive effects due to its anti-oxidant and anti-inflammatory effects	Inhibition of cell proliferation and tumor growth, induction of apoptosis	Dose-dependent epigenetic roles in DNMT inhibition and histone acetylation	[60, 82]
	Flavones	Apigenin, Luteolin	Cereals, parsley, artichokes, cabbages		Chemopreventive effects due to its anti-oxidant and anti-inflammatory effects	Inhibition of DNA replication, stimulation of apoptosis	Inhibition of DNMTs and HDACs activity	[60, 82]
	Flavanols	Catechins, Epigallocatechin-3-gallate (EGCG)	Green tea, cocoa, berries, nuts		Anti-oxidant and anti-angiogenic activity	Inhibition of cell proliferation and tumor growth, induction of apoptosis, protection against oxidative stress	Inactivation of DNMTs and HATs, activation of HDACs	[13, 48, 54]
	Anthocyanins	Cyanidin, Malvidin, Delphinidin, Pelargonidin, Peonidin, Petunidin	Berries, pears, apples, red cabbage, black soybean, grapes, blackcurrant, peaches		Anti-oxidant, anti-inflammatory, anti-mutagenic and anti-adipogenic effects	• Cell growth inhibition, cell cycle arrest and induced apoptosis • Decrease in inflammatory cytokines such as CRP and TNF-α	Alterations in H3K4me3 levels and DNMTs activities	[60, 83-85]
Other polyphenols and their bioactive compounds		**Dietary sources in MD**	**Biological activity**		**Cellular processes involved**	**Epigenetic mechanism(s)**	**Reference(s)**	
Stilbenes	Resveratrol, Pterostilbene, Pallidol	Red wine, grapes, blackberry, blueberry, mulberry, peanuts, etc.	Cardioprotective, neuroprotective, and chemopreventive effects		• Promoting apoptosis, inhibition of cell proliferation. • Regulating signaling pathways involved in meiosis, inhibitory effect on angiogenesis, inhibition of NF-κB activation	Inhibition of DNMTs and HDACs, leading to activation of HATs	[48, 51, 60]	
Lignans	Secoisolariciresinol, Matairesinol, Arctigenin, Nordihydroguaiaretic acid (NDGA), Pinoresinol	Extra-virgin olive oil (EVOO), tea, coffee, whole grains	Chemopreventive effects due to their anti-oxidant and anti-inflammatory effects		Cell cycle arrest and apoptotic activity	Induction of DNA demethylation and upregulation of H3K9me3	[86, 87]	
Curcuminoids	Curcumin, Demethoxycurcumin, Bisdemethoxycurcumin	Turmeric, ginger, curry powder	• Anti-oxidant, anti-inflammatory, anti-microbial effects. • Cardioprotective effects		• Inhibition of cell proliferation and tumor growth, induction of apoptosis • Ameliorates the dysregulations in the related pathway of neurodegenerative diseases	• Induced histone hypoacetylation and decreased DNMTs activity. • Decrease in HDAC1 expression levels by inhibiting matrix metalloproteinase-2 (MMP-2)	[48, 53, 60, 88, 89]	
Organosulfur compounds	Sulforaphane, Isothiocyanates	Cruciferous vegetables (broccoli, cabbage, kale, cauliflower, Brussel sprouts, etc.)	• Chemopreventive effects due to its anti-tumoral properties • Increased antioxidant and anti-inflammatory capacity in neurodegenerative diseases		• Cell cycle arrest and induction of apoptosis • Promoting autophagy and inhibition of oxidative stress in neurons	• Inhibition of HDAC activity, increased histone acetylation levels • Decreased DNA methylation levels in nuclear factor erythroid 2-related factor 2 (Nrf2) promoter	[90-95]	
Tyrosols	Hydroxytyrosol, Oleuropein, Ligstroside	Olive leaf, extra-virgin olive oil (EVOO)	• Antioxidant, anti-inflammatory, and antiatherogenic effects • Anti-viral properties		• Inhibition of proliferation in cancer cell lines by preventing DNA damage • Inhibition of viral replication by causing down-regulation of endocytosis-related genes	• Modulation of distinct miRNA expression levels • Inhibition of several HDACs	[24, 76, 77, 96, 97]	

caused the death of approximately 20,000 people, led to an understanding of the importance of fetus' nutrition during pregnancy and even in the period before conception [35-37]. According to available reports, children born to women who became pregnant during the famine were found to be more prone to diabetes, cardiovascular diseases, various kinds of cancer, and a number of mental problems later in life [36, 38, 39].

Considering the pregnancy periods separately, it was observed that the birth weight of babies born to women who suffered from the famine during the first trimester was not affected, but there was an increased risk of obesity and CVDs as compared to those who were exposed to starvation in adulthood [40]. As the strongest evidence of this situation on DNA methylation, it can be acknowledged that changing methylation levels in Insulin Receptor (*INSR*) and Carnitine Palmitoyltransferase 1A (*CPT1A*) genes shapes the susceptibility to related diseases, which play important roles in prenatal growth and fatty acid oxidation, correspondingly [41]. However, it has been observed that the children of women who were exposed to famine in the third trimester, which is among the later stages of pregnancy, presented a lower birth weight, while the incidence of hypertension increased, together with a significant increase in insulin resistance [42]. Although changes in the DNA methylation process are observed to play an important role in determining disease predispositions, insulin-like growth factor II (*IGF2*), which is known to have important functions in the developmental process and is one of the best characterized epigenetically-regulated loci, draws attention. Maternally imprinted *IGF2* is provided by the differentially methylated region (DMR), and *IGF2* DMR methylation is determined by various genetic factors, providing the preservation of the methylation mark until middle age [43, 44]. In fact, a study conducted by Heijman et al. has led to the observation that the methylation level of *IGF2* DMR is lower in babies born years later, when mothers are deprived of essential amino acids such as methionine (the methyl donor) during the periconceptional process. However, considering the results of the same study, it was found that the children of women who were exposed to famine later in pregnancy were born with lower birth weight, but there was no change in *IGF2* DMR methylation levels [35].

Mechanisms of the Mediterranean diet and its components in achieving epigenetic control on some diseases

Along with extreme factors (such as famine) that people may be exposed to throughout their lifetime, adopting unhealthy lifestyle habits such as a physically sedentary lifestyle, smoking, excessive alcohol consumption, and consuming too much fast foods show that individuals are at higher risk in terms of metabolic diseases, which are the gateway to many chronic diseases. Based on this information, researchers are focusing on the effects that the MD and its contents have on the human epigenome, which might be described as an unopened magic box. It is extremely interesting the fact that diet plays a decisive role in the epigenome maintenance and that the polyphenols contained in the main typical foods of the MD can shape DNA methylation and histone modifications. Although these studies are still scarce, it is possible to discuss in detail how these mechanisms are reflected in literature.

Polyphenols are bioactive molecules that are naturally contained in large quantities in fruit and vegetables and that are responsible for different biological functions: they are thus classified into different groups, such as phenolic acids, flavonoids, and anthocyanins [45]. Phenolic acids are then divided in two subgroups, hydroxybenzoic acids and hydroxycinnamic acids, while flavonoids are divided in many different subgroups, including flavonols, flavanones, flavones, isoflavones, flavanols, and anthocyanins [46]. In addition to flavonoids and phenolic acids, there are also separate groups of polyphenols defined as "secondary plant metabolites", including stilbenes, lignans, and sulforaphanes, which have strong anti-inflammatory and antioxidant effects in the cell and act as a shield against DNA damage [47]. In recent years, researchers have been focusing on studying the effects of these polyphenols, which are largely contained in the foods that make up the MD (being a fruit- and vegetable-based diet) on the epigenome. The biological properties of the polyphenol groups and their components mentioned here, as well as the effects they have on the cellular processes and epigenome they are involved in, are summarized in Table I.

The fact that polyphenols, thanks to their high antioxidant and anti-inflammatory properties, affect in different ways the regulation of epigenetic processes and metabolic activities shows that the MD is more than a diet [48]. Given the fact that diet and lifestyle habits (regular sports activity, healthy sleep, etc.) usually match, many studies reported that a diet rich in monounsaturated fatty acids (MUFAs) positively improves cardiovascular health [49, 50]. For example, cocoa flavonoids have an antihypertensive effect, lowering blood pressure by down-regulating functional genes such as *DNMTs* and *MTHFR*, which are involved in the epigenetic process of peripheral blood mononuclear cells [13, 51, 52]. Animal experimental studies on hypertension have shown that curcumin, which is included in curcuminoids, causes a significant decrease in HDAC1 expression levels by inhibiting the inflammatory marker matrix metalloproteinase-2 (MMP-2) and also causes an evident increase in Tissue Inhibitor of Metalloproteinases 1 (TIMP1) via Histone H3 acetylation [53]. Likewise, the flavanol Epigallocatechin gallate (EGCG), found in green tea, acts as an interesting HAT inhibitor [13, 48], which in among the treatment targets of cardiovascular diseases: inhibiting HAT activation leads to suppressing the activity of Nuclear Factor-kappa B (NF-κB), which is the main member of the inflammation pathway. Keeping all these mechanisms and their interactions into consideration, it is clear to see that the nutrition style of the MD has a

unique role in managing the epigenetic signature on preventing CVDs.

In addition to their cardioprotective effect, polyphenols also play an important role in protecting cognitive activity [54]. Recently, the neuroprotective effects of fish, hazelnut, and olive oil – all ingredients vastly used in the MD – and of their micronutrients, like omega-3 polyunsaturated fatty acids (ω-3 PUFAs), are resonating in the field of nutrigenomics. The ω-3 PUFAs found in fish oil help prevent age-related cognitive loss by affecting DNA methylation in different cells [55]. Resveratrol, which is found in red wine (moderately consumed with meals in traditional MD) and in fruits such as grapes, strawberries, and apples, plays a role in decreasing the risk of Alzheimer's Disease by inhibiting DNMTs and HDACs activities, which lead to the activation of HATs, via chromosome segregation [55-57]. In addition, animal experiments have shown that supplementation of S-adenosyl methionine (SAM), a methyl donor, can alleviate disease-related symptoms by recovering the methylation potential of the *PSEN1* gene [58, 59]. This structure of resveratrol, which significantly affects histone modifications, suggested that it may also have an active role in cell cycle and tumorigenesis processes [60]. In this regard, there are studies demonstrating that resveratrol, which is thought to be a key molecule in maintaining the epigenomic profile, is highly effective in inhibiting proliferation in breast cancer cells by suppressing the Enhancer of Zeste 2 Polycomb Repressive Complex 2 (EZH2), a lysine methyltransferase. The suppression of ERK1/2 phosphorylation is also the basis of the antiproliferative effect created by resveratrol, which has a synergistic effect with the suppression of EZH2 [61]. These effects were not only limited to histone modifications, but also demonstrated a potential therapeutic effect in neuroblastoma by causing the suppression of EZH2 by upregulation of various tumor suppressor microRNAs, like miR-137 [62].

In addition to all its anti-tumorigenic effects, resveratrol also has effects on obesity, which is one of the metabolic diseases caused by the Western Diet. Given the fact that nutritional styles the fetus/baby is exposed to throughout pregnancy and lactation are vigorously linked to the onset of adult diseases, and it has been hypothesized that obesity can be modulated by epigenetic memory in the offspring [63]. In this context, numerous studies have shown that polyphenols in the MD indirectly cause suppression of genes that regulate adipocyte differentiation and triglyceride accumulation through chromatin remodeling and various histone modifications. Furthermore, mice that were fed a high-fat diet showed increased DNA methylation in the *Leptin* and *Pparg2* gene promoters and proinflammatory mechanisms were entirely affected [64, 65]. To reinforce the effect of polyphenols on adipokinesis with an example, quercetin, a flavonol found in onions and kale, and resveratrol, a prominent stilbene, are observed to cause a significant decrease in the levels of CCAAT Enhancer Binding Protein (C/EBPα) and Peroxisome Proliferator-Activated Receptor (PPARγ), which are pro-adipogenic genes, and to negatively regulate adipokinesis [66-68]. Epigallocatechin gallate (EGCG), another type of polyphenol included in the flavanol subclass, which is prominently found in green tea, has long been shown to pave the way for lipolysis by suppressing lipogenesis with pre-adipocyte differentiation and providing beta-oxidation to fatty acids [69, 70]. In cancer, where EGCG is also a potential epigenetic modifying agent, it has gained more attention, as it has been shown to inhibit cell proliferation by binding to enzymatic substrates of DNMT3b and HDAC1 [71, 72]. Moreover, among chromosomal abnormalities, EGCG has been reported to also have a modulating effect at the epigenome level on imbalances in DNA methylation that occur due to the overdose of genes on the 21st chromosome in Down Syndrome [73].

Extra-virgin olive oil (EVOO), one of the most intriguing components of the MD, has a protective effect against many chronic diseases as it is rich in various flavonoids and phenolic acids, as well as other polyphenols such as Oleuropein (OL) and Hydroxytyrosol (HT). HT is among the important phenolic fractions that mainly compose olive oil, and it has many pleiotropic effects that enable it to influence biological functions thanks to its antioxidant, anti-inflammatory, and antiatherogenic effects [24]. In addition, recent studies carried out to investigate its protective effects against SARS-CoV-2 infection have shown that HT extracted from olive leaves effectively reduces viral replication by causing down-regulation of endocytosis-related genes [74, 75]. It was demonstrated that in cancer HT and other secoiridoid glycosides inhibit proliferation in certain cell lines by preventing DNA damage, supporting the idea to employ HT dose-dependent supplementation together with conventional treatments [76, 77]. However, it should be noted that when these olive oil-derived bioactive components are evaluated holistically around omics data, their synergetic effect on disease models might be taken more comprehensively in the near future.

Conclusion and future perspectives

The MD, as the name suggests, has been traditionally adopted by the populations living in the countries with a coast to the Mediterranean Sea, and it is proven that the polyphenolic compounds it contains (thanks to the many fruits and vegetables that make it up) play important roles in preventing many chronic diseases, thanks to their antioxidant and anti-inflammatory properties. Polyphenols are diet-derived natural compounds and, given their numerous beneficial effects on chronic disorders, they can be used as adjuvants in personalized and preventive medicine thanks to their effects on the individual's epigenome. Epigenetics, which is defined as heritable changes in gene expression without any change in the DNA sequence, leads to the shaping of the epigenome with chemical modifications that are caused by environmental factors. Nutrition styles and their effects on human health have attracted the attention of researchers working in the field of nutrition for many years, and

combining the findings obtained from observations with genetic research has led to the formation of the multidisciplinary science known as nutrigenomics. Numerous studies show that epigenome formation is related not only to the later stages of life, but also to the prenatal period, for example to the diet followed by the mother during pregnancy and even to the environment she was exposed before conception, thus leading to disease predispositions that the fetus may develop throughout his/her entire life. The fact that children born to women who were pregnant during the Dutch Hunger Winter, one of the most dramatic events engraved in human history, are more prone to many chronic diseases is the strongest reflection of the transfer of changes created on the epigenome to future generations.

Recently, there is a growing interest in addressing the effects of nutrition on the epigenome in the pathways of inflammation and metabolism. Within this framework, PUFAs such as ω-3 and ω-6, which together with polyphenols are among the main components of the MD, act as transcription factor ligands and play an active role in inflammation-related pathways; also, they are among the strongest evidence that they are a major metabolic regulator in related processes. Similarly, many studies have emphasized the effect of nutrition supplemented with folate- and B-vitamins-rich foods, like broccoli and Brussels sprouts, on the modulation of metabolic processes such as nucleotide synthesis and DNA repair, as well as its shaping effect on DNA methylation and histone modifications.

Considering the data presented in the literature so far, it is revealed that long-term consumption of the nutritional elements of the MD, rich in fruits and vegetables and in polyphenols, is of undeniable importance in the treatment and even in the prevention of many diseases by shaping the epigenome in different directions. It is foreseeable that in the future nutriepigenetics – already attracting a great amount of attention thanks to its role in maintaining a long and healthy life – will be supported by more in vitro and in vivo studies, thus providing new data to the literature, further clarifying its mechanisms, and emphasizing the importance of personalized medicine.

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Conflicts of interest statement

The authors declare no conflict of interest.

Author's contributions

MD, MB, and TB developed the study design and conceptualization of the research methodology. SK and NG contributed to the manuscript's writing. MD, HA, and MCE contributed to manuscript reviewing and editing processes. All authors have read and approved the final manuscript.

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Review

Effects of the Mediterranean diet on the components of metabolic syndrome

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Keywords

Mediterranean diet • Metabolic syndrome • Effect mechanisms

Summary

Metabolic syndrome, also as known as Syndrome X or Insulin Resistance Syndrome, is a complex health problem featuring visceral obesity (the main diagnostic criterion), insulin resistance, dyslipidemia and high blood pressure. Currently, this health condition has gained a momentum globally while raising concerns among health-related communities. The World Health Organization, American Heart Association and International Diabetes Federation have formulated diagnostic criteria for metabolic syndrome. Diet and nutrition can influence this syndrome: for example, the Western diet is associated with increased risk of metabolic syndrome, whereas the Nordic and Mediterranean diets and the Dietary Approach to Stop Hypertension are potentially beneficial. The Mediterranean diet can affect the components of metabolic syndrome due to its high dietary fiber, omega 3 and 9 fatty acids, complex carbohydrates, antioxidants, minerals, vitamins and bioactive substances, such as polyphenols. These nutrients and bioactive substances can combat obesity, dyslipidemia, hypertension and diabetes mellitus. The mechanisms by which they do so are generally related to oxidative stress, inflammation (the most common risk factors for metabolic syndrome) and gastrointestinal function. The literature also shows examples of positive effects of the Mediterranean diet on the metabolic syndrome. In this review of the literature, we shed light on the effects, mechanisms and dynamic relationship between the Mediterranean diet and metabolic syndrome.

Definition of metabolic syndrome

Metabolic syndrome, also known as Syndrome X or Insulin Resistance Syndrome is a complex abnormality associated with coronary artery disease [1]. Visceral obesity (abdominal obesity/android type obesity), insulin resistance (IR), hypertension and dyslipidemia are components of this cluster of metabolic problems [2]. There are many diagnostic criteria that have been developed by different organizations (Tab. I). Visceral obesity is the basic diagnostic criterion of metabolic syndrome and is associated with generalized low-level inflammation, accompanied by elevated serum concentrations of pro-inflammatory cytokines, such as tumor necrosis factor alfa (TNF-α), which can decrease insulin sensitivity in human tissues. Pro-inflammatory cytokines are known to affect negatively blood pressure hemostasis and lipid metabolism. Besides low-grade inflammation, visceral obesity is commonly related to overnutrition. A positive energy balance has some long-term comorbidities such as IR and coronary artery disorders. This explains why visceral obesity is the main diagnostic criterion of metabolic syndrome [2-4]. Other common complications include stroke, myocardial infarction and diabetes mellitus [5].

The prevalence of metabolic syndrome differs between countries. Aguilar et al. [6] reported a 33% overall prevalence of metabolic syndrome in the United States of America (USA) in 2012. Liang et al. [7] reported a 38.3% prevalence in 2018. In 2017, prevalence was 48.8% in Qatar [8] and 42.87% in Iran [9]. Metabolic syndrome, a preventable health problem, is therefore very common all over the world. This condition is definitely related to unbalanced and Western style nutritional habits and also sedentary lifestyle behaviors which are common even in childhood. Practices such as a healthy diet and physical activity may protect against metabolic syndrome. The literature shows benefits of the Mediterranean diet in decreasing risk of metabolic syndrome [10-12].

Diagnostic criterAia of metabolic syndrome

As already mentioned, obesity is the main diagnostic criterion for metabolic syndrome. Defined as undesirable weight gain, it is a common chronic non-communicable disease of our time [13].

Obesity, especially visceral/abdominal obesity, is associated with comorbidities such as IR, high blood pressure and dyslipidemia (e.g. low high-density lipoprotein, HDL and high triacylglycerol, TAG) [14]. Insulin resistance is defined as poor tissue response to the hormone insulin and it is definitely related to visceral obesity with its associated inflammation and oxidative stress. IR may increase dyslipidemia, atherosclerosis and other coronary artery disease risk factors. It is regarded as the first stage of type II diabetes mellitus [15]. Another modifiable cause of mortality is high blood pressure, which is a risk factor for renal dysfunction, myocardial infarcts and stroke. Angiotensin-converting enzyme (ACE) system impairment and high sodium intake are related to high

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blood pressure [16]. Dyslipidemia means low plasma concentrations of HDL, high TAG and/or low-density lipoprotein (LDL). There are two types of dyslipidemia: primary and secondary. Secondary dyslipidemia is related to issues such as obesity and IR which are related in turn to overnutrition [17]. Figure 1 shows these mechanisms as a summary.

Risk factors for metabolic syndrome

There are two types of risk factors for metabolic syndrome: those that can and cannot be altered. Diet, physical activity and smoking are alterable risk factors that people can change to reduce their risk of metabolic syndrome [23]. A Western diet is more likely to induce metabolic syndrome than certain other diet models [24], because it includes a large proportion of red meat, processed red meat products, refined grains, high-fat dairy products and few fruits, vegetables, nuts or legumes [25]. Saturated, trans and omega 6 (n-6) fatty acids, simple carbohydrates, sucrose and salt are major elements of the Western diet, which is poor in complex carbohydrates, dietary fiber and n-3 fatty acids [26]. The above elements can be associated with oxidative stress, inflammation, dyslipidemia and non-communicable disorders such as obesity and its comorbidities [27]. Another risk factor for metabolic syndrome is sedentary lifestyle [28]. Regular physical activity can increase energy expenditure, lipolysis and insulin sensitivity, while decreasing blood pressure. It can improve blood lipid parameters [29]. Thus, there is a relation between sedentary lifestyle and metabolic syndrome risk [28, 29]. Smoking, one of the worst habits, is linked to accumulation of abdominal fat and visceral obesity, IR, dyslipidemia, hypertension and other abnormalities which are all linked to oxidative stress and inflammation [30]. High alcohol consumption can also increase health risks, such as visceral obesity, poor insulin sensitivity, high blood pressure and abnormal lipid profile [31].

The Mediterranean diet is the most effective nutrition model which is a potential agent to decrease noncommunicable chronic disorders via its contents. It includes many beneficial nutrients that can influence metabolic pathways adversely affected due to chronic diseases, of course, has a potential impact on metabolic syndrome (see section 2.2). Accordingly, we review the recent literature on the links between the Mediterranean diet and metabolic syndrome.

Tab. I. Diagnostic criteria for metabolic syndrome according to various health organizations.

Organization	Visceral obesity	TAG	HDL	Blood pressure (BP)	Fasting plasma glucose	Other	Reference
• **World Health Organization (WHO)** • Diabetes, IR or impaired glucose tolerance PLUS two or more other criteria.	**Body mass index** > 30 kg/m^2 **Waist hip ratio** *For males* > 0.9 *For females* > 0.85	≥ 150 mg/dL	*For males* < 35 mg/dL *For females* < 39 mg/dL	≥ 140/90 mmHg	Impaired glucose tolerance or diabetes and/or IR	**Microalbuminuria** *Urinary albumin excretion rate* ≥ 20 µg/min *Albumin creatine ratio* ≥ 30 µg/mg	WHO [18]
– **International Diabetes Federation (IDF)** – Visceral obesity PLUS two or more other criteria.	**Waist circumference** Defined with ethnic-specific values	≥ 150 mg/dL (1.7 mmol/L) or specific treatment for this abnormality	*For males* < 40 mg/dL (1.03 mmol/L) *For females* < 50 mg/dL (1.29 mmol/L) or specific treatment for this abnormality	Systolic BP ≥ 130 mmHg or Diastolic BP ≥ 85 mmHg or treatment of previously diagnosed hypertension	≥ 100 mg/dL (5.6 mmol/L) or previously diagnosed with type II diabetes	-	IDF [19]
- **American Heart Association (AHA)** - At least three criteria.	**Waist circumference** *For males* ≥ 102 cm (≥40 inches) *For females* ≥ 88 cm (≥ 35 inches)	≥ 150 mg/dL (1.7 mmol/L) or specific treatment for this abnormality	*For males* < 40 mg/dL (1.03 mmol/L) *For females* < 50 mg/dL (1.29 mmol/L) or specific treatment for this abnormality	Systolic BP ≥ 130 mm Hg or Diastolic BP ≥ 85 mmHg or treatment of previously diagnosed hypertension	≥ 100 mg/dL or specific treatment for this abnormality	-	Grundy et al. [20] Alberti et al. [21] AHA [22]

The Mediterranean diet vs common diets

Various nutrition models, such as the Dietary Approach to Stop Hypertension (DASH), the Nordic diet and the Mediterranean diet have been designed as alternatives to Western style eating habits, which have spread all over the world [32]. DASH was developed to decrease the risk of hypertension and it is often prescribed for people diagnosed with this disorder. DASH may also be effective against obesity, coronary artery disease and related non-communicable disorders [33]. The Nordic diet is a nutritional model based on the nutritional habits of the Nordic peoples [34]. The Mediterranean diet is another region-specific nutritional model based on the healthy eating habits of Mediterranean peoples [35]. These three nutrition models (DASH, Nordic and Mediterranean diet) are rich in nutrients beneficial for health [33-35]. The Western diet contains many harmful elements which may increase the risk of non-communicable diseases [36]. The United Nations Educational, Scientific and Cultural Organization (UNESCO) has listed the Mediterranean diet as an Intangible Cultural Heritage [37].
From this point, it is possible to indicate, DASH is a therapeutic nutrition model and there is no food pyramid to make it easier for adherence to this nutrition model. Nordic diet is based on the Nordic region-related nutrition habits which is so difficult to adopt by other people elsewhere. And also, some recommendations are not compatible with the optimal nutrition principles such as canola oil consumption (due to omega 6 content). In this prospect, the Mediterranean diet has a general food pyramid with easy food consumption recommendations while each recommendation is objective and compatible with the principles of optimal nutrition.

Beneficial nutrients and bioactive substances in the Mediterranean diet

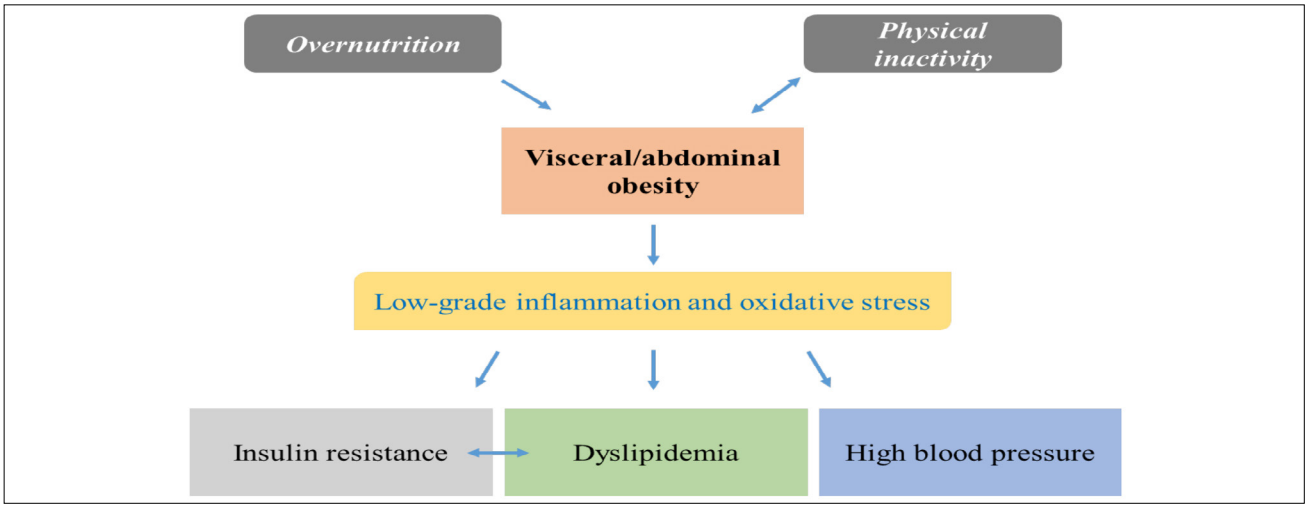
The Mediterranean diet includes items that are consumed with different frequencies, indicated in Table II as *often*, *moderate*ly and *rarely* [38, Tab. II].

Bach-Faig et al. [40] developed a Mediterranean diet pyramid with consumption frequencies and amounts. The pyramid also includes suggestions for physical and social activities. In 2020, Serra-Majem et al. [41] updated the pyramid with sustainability principles and Dayi et al. [42] replaced some items with traditional foods of Cyprus to facilitate users in that location and decrease human impact on the planet.
Certain items of the Mediterranean diet contain polyphenols such as naringenin, apigenin, kaempferol, hesperidin, ellagic acid, oleuropein, rosmarinic acid, resveratrol and quercetin, as well as dietary fiber, monounsaturated fatty acids such as omega 9 (n-9), polyunsaturated fatty acids such as omega 3 (n-3), complex carbohydrates and many vitamins (A, C and E) and minerals (calcium, potassium, magnesium etc.) which can reduce the risk of metabolic syndrome [43-46]. Figure 2 shows the nutrients and their food sources according to the basic Mediterranean diet pyramid.

Effects of dietary nutrients and bioactive substances on metabolic syndrome diagnostic criteria

Figure 3 shows the potential beneficial effects of the Mediterranean diet on the different components of metabolic syndrome. Olive oil is a typical item of the Mediterranean diet and its polyphenols can mitigate the risk of metabolic syndrome by reducing of visceral obesity, IR, blood pressure and lipid peroxidation. These polyphenols can also block signaling and expression of nuclear factor kappa B (NFKB), important risk factors for metabolic syndrome, thus decreasing secretion of proinflammatory cytokines [47, 48]. Another typical item of the Mediterranean diet is red wine. The main polyphenol in red wine, resveratrol, can exert anti-inflammatory and antioxidant effects [49, 50]. Resveratrol may also help regulate the human gut microbiota, an important component of metabolic syndrome, activate sirtuin 1, which is important for lipolysis, and activate adenosine mo-

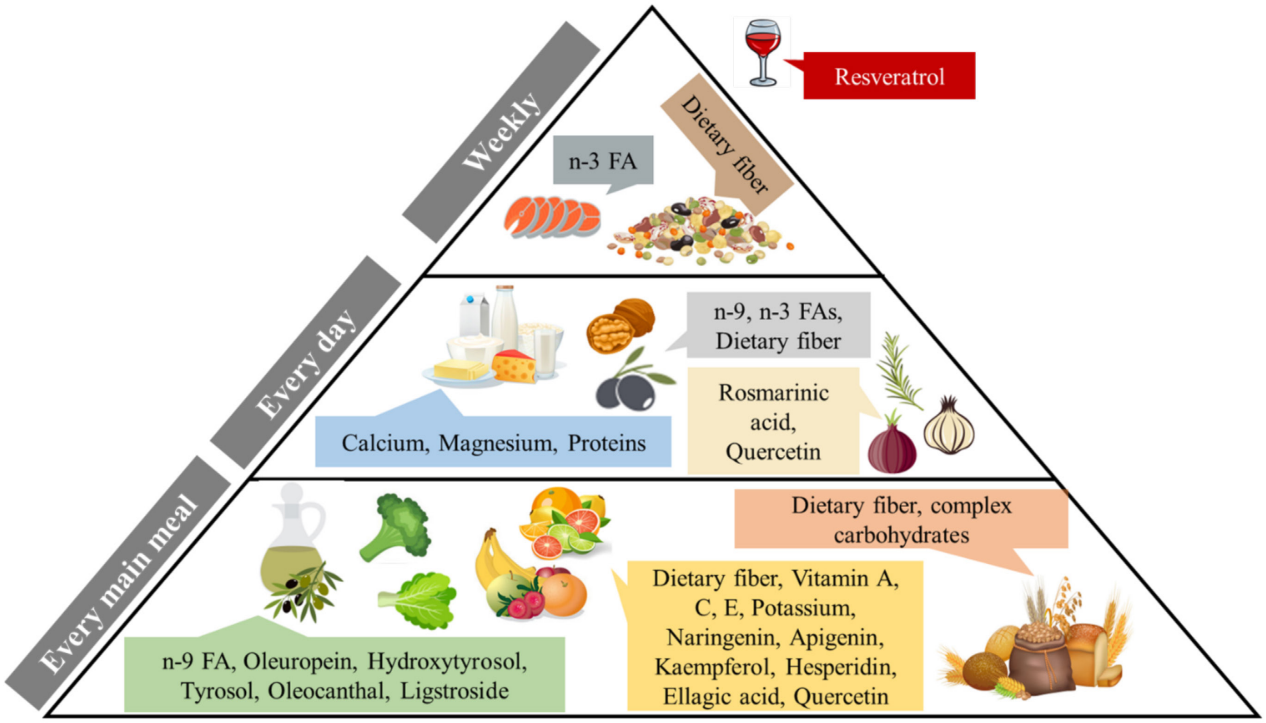
Fig. 1. Simple mechanism of development of metabolic syndrome.



Tab. II. Consumption frequencies of the various items of the Mediterranean diet [38, 39].

Consumption frequencies		
Often	**Moderately**	**Rarely**
Olive oil, vegetables, fruit, nuts, legumes, unprocessed cereals	Fish, red wine, dairy	Poultry, red meat, processed red meat products

Fig. 2. Some nutrients and polyphenols of the Mediterranean diet that may reduce the risk of developing metabolic syndrome [Prepared by authors, based on the references in section 2.1., 3rd paragraph].



nophosphate protein kinase, which can increase insulin sensitivity [49, 50].

Citrus production and consumption are common in the Mediterranean region. Citrus polyphenols can decrease advanced glycation end products and block NFKB expression, thus decreasing oxidative stress and inflammation in the human body. A decrease in oxidative stress and inflammation biomarkers may in turn increase insulin sensitivity, improve lipid metabolism and lower blood pressure. Polyphenols such as naringenin may improve energy metabolism thus reducing visceral obesity [51]. Mediterranean vegetables, fruits and spices are good sources of polyphenols, important bioactive substances which as we have said, can block oxidative stress- and inflammation-related pathways. They therefore increase plasma concentrations of HDL and decrease those of LDL, as well as improving IR, body mass index and blood pressure [52]. Dietary polyphenols can be an effective prebiotic, potentially decreasing pathogenic and increasing beneficial microorganisms of the gut microbiota. A balanced microbiota may be related to good glucose tolerance and insulin secretion, while decreasing lipogenesis and inflammation [53]. In summary, polyphenols typical of the Mediterranean diet can decrease inflammation, oxidative stress, IR, lipid oxidation, body weight, blood pressure and endothelial dysfunction, reducing the risk factors for metabolic syndrome [54].

The Mediterranean diet includes some beneficial fatty acids such as n-9 and n-3 (due to frequent consumption of olive oil and moderate consumption of fish), while containing few saturated and trans fatty acids [39]. Omega 9 fatty acids, especially oleic acid, have antioxidant and anti-inflammatory effects and may therefore improve pancreatic beta-cell functions, insulin sensitivity and endothelial function [55]. Oleic acid can affect hypothalamic function and decrease ghrelin secretion [55]. It may inhibit platelet aggregation. It can also decrease plasma concentrations of LDL and increase those of HDL [56]. The oleic acid and polyphenols of olive oil can inhibit the ACE pathway, thus regulating blood pressure [57]. Omega 3 fatty acids can also diminish metabolic syndrome criteria [58, 59].

There are two types of dietary fiber: soluble and insoluble [60]. Soluble and insoluble fiber both have potential beneficial effects on metabolic syndrome [61]. Soluble fiber increases gastric emptying time and macronutrient absorption by increasing intraluminal viscosity. It may therefore be effective for reduction of body weight and regulation of postprandial blood glucose levels. During

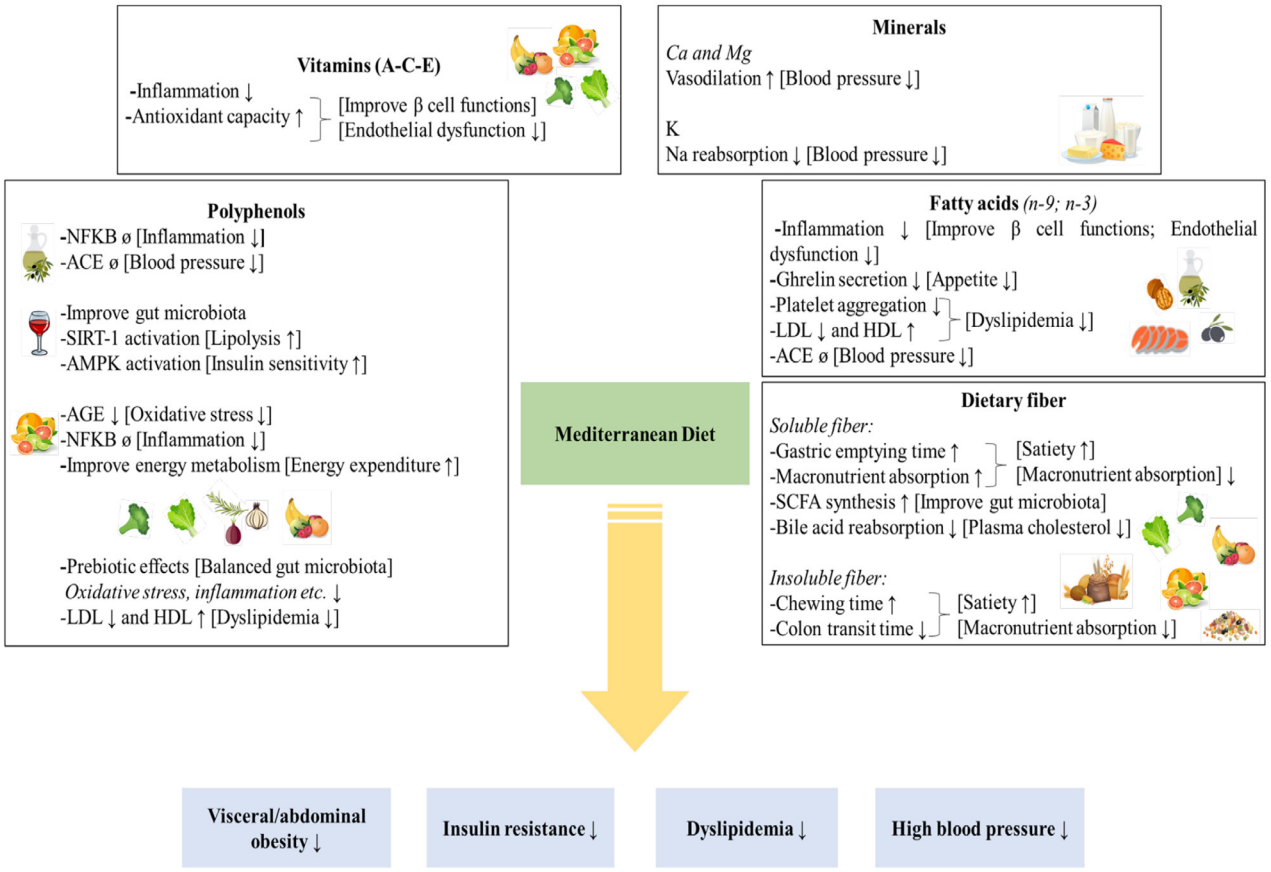


Fig. 3. Potential effects of the Mediterranean diet on different components of metabolic syndrome. Each box indicates major nutrients and nutritional substances with effects marked as up or down arrows, increased or decreased effect consequently. Furthermore, potential general effects were marked with brackets. [Prepared by authors, based on the references in section 2.2.1.

↓: Reduction; ↑: Enhancement; Ø: Inhibition []: Potential general effect

fermentation, soluble fiber produces short chain fatty acids, which can decrease glucose and fatty acid production by the liver, as well as absorption of macronutrients via inhibition of enterocyte contact of these [62]. In addition, soluble fiber reduces bile acid reabsorption so the liver has to produce more bile acid. Since cholesterol is a building block of bile acid, while our body produces new bile acid, plasma concentrations of cholesterol decrease [63]. Insoluble fiber increases chewing time and decreases colon transit time, which stimulates the vagus nerve and creates a sense of satiety. These mechanisms can lead to lower food intake and nutrient absorption which are important factors against obesity and IR [62]. According to the literature, complex carbohydrates can have similar effects [64].

Vitamin A, C and E are antioxidants that can protect against oxidative stress which plays a role in many non-communicable disorders such as IR, cardiovascular disease and cancer [65, 66]. Antioxidants can reduce stress on pancreatic beta-cells and tissues. These effects may increase insulin sensitivity and secretion which are important factors against IR and for promoting weight loss [67]. These vitamins also decrease proinflammatory cytokines and reactive oxygen species, improving endothelial function. They therefore have roles in blood pressure regulation, lipid metabolism and cardiovascular health [68, 69].

Minerals such as calcium, magnesium and potassium can have antihypertensive effects [70]. Low calcium intake can stimulate the renin-angiotensin pathway, increasing blood pressure through sodium reabsorption, while calcium deficiency stimulates parathyroid hormone secretion further increasing calcium uptake by cells, which can cause peripheral vascular resistance and an increase in blood pressure [71].

The calcium antagonist magnesium decreases calcium concentrations in cells and increases certain prostaglandin E series which in turn instigate vasodilation [72]. Likewise, potassium is a sodium antagonist which decreases reabsorption of sodium, a prohypertensive mineral, by the kidneys [73].

In addition to these nutrients and bioactive substances beneficial for metabolic syndrome, the Mediterranean diet features fewer harmful items, such as saturated and trans fatty acids, linoleic acid (n-6), cholesterol, simple carbohydrates, sodium, nitrites and nitrates, and contains lower total fats [38, 39, 74].

Based on these potential effects, the Mediterranean diet carries a chance to decrease the risk of metabolic syndrome and increase life expectancy. On the other hand,

the effects of the Mediterranean diet are not only limited to metabolic syndrome. So, it can be estimated that, governments/health authorities can decrease financial expenditure on health if they develop some programs to increase adherence to the Mediterranean diet.

Relation between other patterns of the Mediterranean diet and metabolic syndrome

Social and physical activities and fun are important components of the Mediterranean lifestyle. These factors are related to physiological and psychological wellbeing [40]. Regular physical activity has positive effects on health, such as decreasing fat mass, plasma levels of LDL and TAG, inflammation, oxidative stress and blood pressure, while increasing insulin sensitivity, glucose tolerance and plasma levels of HDL [75]. Due to these beneficial effects, regular physical activity has to be a complementary behavior to the Mediterranean diet to further decrease the risk of the metabolic syndrome. And also, WHO suggests at least 150 minutes/week of moderate-intensity physical activity for adults to be healthy. Thus, all the Mediterranean diet pyramids have regular physical activity suggestions at the base [40-42].

Even more, the adherence to the MD is also embedded into the attitude of individuals for encouraging their own food production. This brings up two positive features, firstly the activity levels are increased and secondly the environmental impact becomes self-rewarding. Therefore, this circle is a very proliferative one: the more the MD is favored, the more the self-productivity is achieved resulting in a stronger life style adaptation [42].

Social activities are important for mood: depression and anxiety are linked to many diseases, higher food intake and lower physical activity, which are risk factors of metabolic syndrome [76]. Furthermore, chronic melancholy may cause inflammation and oxidative stress and so be related to metabolic syndrome [77].

Table III list various studies on the subject. For the meta-analysis, 'number of studies' shows how many original studies are included and the sample size of studies has been shown as 'n'.

Results of some current studies support the potential effect mechanisms of the Mediterranean diet on metabolic syndrome which this review article shows. According to these results, the Mediterranean diet has been shown to be effective for weight loss, regulation of blood glucose and lipids, decreasing inflammation and blood pressure (Tab. III).

In conclusion, the Mediterranean diet is an effective nutritional model against non-communicable chronic disorders anywhere in the world. This review examined one such disease, metabolic syndrome, via a literature search for related mechanisms. Future research should address local Mediterranean food consumption by country, such as Cyprus, and its effects on the components of metabolic syndrome.

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None.

Conflicts of interest

There are no conflicts of interest.

Tab. III. Studies on the effects of Mediterranean diet on metabolic syndrome.

Authors	Type	Effects of Mediterranean diet
Kastoroni et al. [78]	Meta-analysis (Number of studies: 50; n: 534,906)	0.42 cm ↓ waist circumference 1.17 mg/dL ↑ HDL 6.14 mg/dL ↓ TAG 2.35 mm Hg ↓ systolic and 1.58 mm Hg ↓ diastolic BP
Huo et al. [79]	Meta-analysis (Number of studies: 9; n: 1178)	0.30% ↓ HbA1c 0.72 mmol/L ↓ FPG 0.55 μU/mL ↓ fasting insulin 0.14 mmol/L ↓ total cholesterol 0.29 mmol/L ↓ TAG 1.45 mm Hg ↓ systolic and 1.41 mm Hg ↓ diastolic BP
Richard et al. [80]	Original research (n: 26 males)	C-reactive protein (CRP) ↓ IL-6, IL-18 and TNF-α ↓ ≥8.5 cm waist circumference ↓ IL-6 and IL-18 ↓
Moosavian et al. [81]	Systematic review (Number of studies: 10; n: 856)	Improved body measurements, plasma lipid profile and glucose regulation
Mayneris-Perxachs et al. [82]	Original research (n: 424)	Incidence, reversion and prevalence of metabolic syndrome ↓
Pavić et al. [83]	Original research (n: 124)	HDL ↑ and systolic BP ↓ The Mediterranean diet was effective for the components of metabolic syndrome
Meslier et al. [84]	Original research (n: 82 healthy overweight and obese participants)	Plasma cholesterol and LDL ↓ Insulin sensitivity ↑ Systemic inflammation ↓

↓: Reduction; ↑: Enhancement

Authors' contributions

TD searched the literature and wrote the main outline of the article. MO contributed the concept of the article and revised the main outline of the manuscript.

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Review

Periconceptional Mediterranean diet during pregnancy on children's health

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Keywords

Congenital defects • Mediterranean diet • Periconceptional period • Pregnancy

Summary

During pregnancy, rapid and subtle physiological changes are observed from conception to birth. Nutrition and other lifestyle factors before and during pregnancy have been shown in the literature to influence the health of both mother and child. A healthy and varied diet during pregnancy can provide adequate energy and nutrients for both the mother and the growing fetus. Current research focuses on the periconceptional phase, which includes the early processes of gametogenesis, embryogenesis and placentation. A variety of abnormalities and pregnancy-related problems occur during this period, including congenital defects, fetal loss, miscarriage and preterm birth. A varied and balanced diet during periconception is important to maintain fetal development and growth. To date, numerous studies have been conducted to investigate the effects of consuming different nutrients, foods or food groups during pregnancy on the health of mother and child. For example, the Mediterranean diet is considered as a balanced, nutrient-rich diet due to the low consumption of meat products and fatty foods and the high consumption of vegetables, cheese, olive oil, fish, shellfish and little meat. While many studies have been conducted in the literature to investigate the effects of a Mediterranean diet during pregnancy on fetal health, the results have been inconclusive. The aim of this article is to review the current literature on the Mediterranean diet during pregnancy.

Introduction

Pregnancy has a complex nature involving growth, development and maturity processes. From fertilization to birth, a whole range of processes take place to prepare the fetus for life outside the womb [1]. Inherited diseases or chromosomal aberrations has been a central aspect of perinatal research and neonatal health. However, recent studies have been conducted to investigate the interaction between mother and fetus from different angles [2]. Pregnancy consists of two main periods including the embryonic period and the fetal period. The embryonic period lasts eight weeks, the foetal period lasts from the ninth week of pregnancy until birth [1, 3]. The first two weeks of the embryonic period are crucial because the embryo is extremely sensitive to an unfavourable environment (changed hormone concentrations, inadequate blood and nutrient supply), and in certain situations prenatal mortality may occur. From the second until the eigth week, the embryo is very sensitive to teratogenic drugs as embryonic development is initiated at this period. Finally, important physiological adaptations and less severe morphological changes occur during the fetal period. This period is critical due to changes that result in fetal programming that conditions the optimal or problematic organ systems affecting the whole organism [3, 4].

Some of the physiological changes that occur during pregnancy result in an increase in plasma volume and red blood cells, and a reduction in concentrations of nutrient-binding proteins and micronutrients that circulate in the body [5]. Dietary intake, limited exercise, smoking and alcohol use are critical to the health of women and their children during pregnancy and breastfeeding [6, 7]. Nutrition is considered one of the most critical environmental elements influencing embryo and foetal development and maternal health [8]. During pregnancy, the need for nutrients increases to support the growth and development of the fetus and to maintain maternal metabolism [9]. In recent years, several studies and comprehensive meta-analysis have shown that inadequate intake of important macronutrients and micronutrients can adversely affect pregnancy outcomes and neonatal health [9]. Deficiencies in micronutrients, in particular, have been linked to an increased risk of infertility, fetal structural abnormalities and long-term illnesses [8].

Since pregnancy is composed of different phases that continue, maternal nutrition and hormones have a significant influence on all phases of embryonic and foetal development, maternal nutrition and hormones have a significant impact on all stages of embryonic and fetal development [10]. Importantly, the periconceptional period is considered as a critical period for fetal development and health. During this period, a variety of

abnormalities and pregnancy-related problems occur, including congenital defects, fetal losses, miscarriages and preterm delivery [11]. According to the literature, periconceptional nutrition has an impact on fetal development and growth. It is important that the intake of certain nutrients during pregnancy, especially, the Mediterranean diet (MD) is considered a healthy and balanced diet. A number of studies have proven that it helps to prevent maternal and fetal diseases during pregnancy [11, 12].

Pregnancy and diet

Nutrition during pregnancy

Pregnancy is a 40-week period of life with different nutritional requirements for mother and child, and it is an important period of life for both. Pregnancy involves rapid and subtle physiological changes from conception to birth [7]. Diet and other lifestyle factors such as smoking and alcohol consumption before and during pregnancy and lactation have been shown to affect child health [13]. In addition, an unbalanced diet during pregnancy has been associated with serious pregnancy complications [14]. Although the role of nutrition in pregnancy has been investigated in several studies, the results have been inconclusive [15].

A healthy and varied diet during pregnancy can provide adequate energy and nutrients for both the mother and the growing fetus [5]. Several studies that investigated the effect of diet on pregnancy outcome focused on only one or a few nutrients [16]. High consumption of saturated fats, low consumption of polyunsaturated fats, carbohydrates and soft drinks, for example, have been associated with gestational diabetes mellitus (GDM) and hypertension [16]. In addition, calcium intake has been associated with a lower risk of preeclampsia and fewer preterm births [17]. Deficiencies in micronutrients such as folic acid, iron, and zinc and vitamins are common in pregnant women [18]. Low Fe consumption, for example, has been associated with anaemia, while low folate intake has been associated with neural tube abnormalities or preterm birth [7, 9]. Since vitamins and some minerals are important components of the immune system, deficiencies in these micronutrients can lead to harmful infections [8]. It is believed that micronutrient supplementation may be beneficial in preventing adverse pregnancy outcomes [19].

Extensive research has been conducted over the past decade to determine the relationship between intrauterine fetal development, postnatal phenotype programming and the risk of developing noncommunicable diseases including cardiovascular disease and neoplastic disease [20, 21]. An unfavourable nutritional status during pregnancy, namely reduced iodine, folic acid and protein intake, has been associated with intrauterine cerebellar development, altered cerebellar methylation patterns and higher lipoperoxidation in the children [22, 23]. In addition, consumption of fish and polyunsaturated fatty acids improved embryo morphology following *in vitro* fertilization (IVF) treatment [24]. Currently, research focuses on the periconceptional period which is 14 weeks before and up to 10 weeks after conception and includes the early processes of gametogenesis, embryogenesis, and placentation [25]. Researchers have reported that an unhealthy maternal lifestyle during periconception has negative effects on reproductive outcomes [7, 26]. Importantly, reproductive failures occur during the periconceptional period, i.e. implantation failure, early pregnancy loss, miscarriage, congenital malformations in newborns, and fetal growth failure [26]. Maternal periconceptional nutrition affects fetal programming, placental cognition and fetal and maternal competition for food [27]. In addition, diet can influence embryonic development through biochemical signals in the environment of uterus. It has been shown that optimal nutrition during periconception promotes fetal growth and development [15]. Studies in animals and humans have shown that epigenetic events expressed later in adulthood are related to early embryonic stages and the quality of maternal periconceptional nutrition [7].

Notably, maternal one-carbon (I-C) metabolism plays an important role in the DNA methylation patterns of the offspring that influence postnatal gene expression and disease progression [26]. One carbon metabolism is altered by environmental factors such as inadequate folate and folic acid intake, lifestyle and medication use as well as common polymorphisms such as the methylenetetrahydrofolate reductase (*MTHFR*) C677T polymorphism [26, 28]. Consequently, genetic and environmental varaibles influence I-C metabolism as well as intrauterine DNA methylation, gene expression and the transcriptome may explain the association between maternal environment and long-term outcomes [20, 26].

In summary, the health of every human being from conception to adulthood requires a varied and balanced diet. The developmental processes already take place during pregnancy and are influenced by the mother's diet. Therefore, nutrition should be planned in order to avoid unfavourable pregnancy consequences.

Mediterrranean diet in gestation

Fetal development depends on maternal nutritional and metabolic conditions. The baby's physiology and metabolism can be permanently altered and shaped by the intrauterine environment [29]. Several studies have investigated the association between the intake of various nutrients, foods or food groups during pregnancy and maternal and fetal diseases [30]. The Mediterranean diet, with its low intake of meat products and high-fat foods, is a balanced, nutritious diet that is considered a standard for diet quality because of its components such as vegetables, cheese, olive oil, fish, shellfish and little meat [31]. The health effects of this diet were described as early as the 1950s. The American scientist Ancel Keys was the first to note the effects of diet and lifestyle on cardiovascular disease [32].

The Mediterranean diet appears to provide adequate caloric and nutrient intake in appropriate amounts and proportions. It is distinguished by its high methyl donor content

for one-carbon metabolism, which is engaged in growth and programming activities, notably during the periconceptional period [33] Many studies have been undertaken to investigate the effect of Mediterranean diet on maternal health and offspring health. For instance, the Mediterranean diet is associated with a higher chance of clinical pregnancy and live birth after IVF, and a lower incidence of infertility [34, 35]. A significant study undertaken by Australian experts found that the Mediterranean diet protects against hypertensive problems during pregnancy (HDP) [36]. Similarly, other studies found that diets rich in vegetables and fruits were associated with pre-eclampsia and gestational hypertension [37, 38]. In contrast, a higher risk of preeclampsia was observed in women who adhered to a Western diet [38].

In another study, higher consumption of animal proteins before pregnancy was linked to an increased risk of gestational diabetes, whereas higher consumption of plant-based proteins was linked to a lower risk of metabolic disorders [39]. Furthermore, in a study carried out in Iran, where the consumption of fast food is very high, it was found that the consumption of chips in particular increased the risk of gestational diabetes [40]. Sibling studies have shown that those who were exposed to a diabetic environment in utero have a higher risk of developing diabetes, regardless of whether they have a genetic predisposition [41].

Moreover, other studies that have examined the effect of diet on neonatal and fetal development have shown that children of women who have a diet rich in vegetables and fruits have a lower risk of developing congenital limb defects [42]. Similarly, the risk of developing orofacial cleft is lower in offspring of women who have a periconceptional diet rich in fruits and vegetables [43]. In addition, a "one-carbon" diet high in fish and seafood has been associated with a lower incidence of congenital heart disease (CHD) and septal abnormalities [43, 44]. In a study, eating lean fish during periconceptional period has been associated with higher risk of low birth weight [45]. Finally, several studies have found that the Mediterranean diet reduces the risk of small gestational age (SGA) in newborns [46, 47].

Although the impact of the diet on the health of pregnant women and offspring have not been clearly established, the benefits of the Mediterranean diet for mothers, fetuses, and offspring are well documented. This diet should be promoted before, during, and after pregnancy.

Mediterranean-style diet and common health problems in pregnancy

Mediterranean diet and gestational diabetes and glucose intolerance

Gestational diabetes mellitus (GDM) is a glucose intolerance that occurs during pregnancy [48]. Depending on the diagnostic threshold and the population studied, 1-28% of all pregnancies are affected [49, 50]. GDM is associated with poorer pregnancy outcomes and a higher risk of maternal and child morbidity in the long term [51]. Age of the mother and a family history of type 2 diabetes and obesity are among the nonmodifiable risk factors associated with GDM [52]. GDM, in particular, type 2 diabetes (T2D) has a genetic component and aggregates in families. It has been demonstrated that women who have a diabetic sibling have an increased risk of developing GDM [53, 54]. Furthermore, identifying modifiable risk factors linked with GDM is critical for developing effective preventative approaches and avoiding negative health effects [55]. Several studies focused on dietary components during pregnancy as one of the modifiable factors contributing to GDM [56]. High sugar consumption, for example, is thought to contribute to an inflammatory process that may be associated with insulin resistance [57]. Although a number of studies have been undertaken to determine the link between dietary patterns and GDM, the results have been inconclusive [58, 59].

Two studies found that eating high amounts of dietary fibre has been linked to lower risk of GDM, but this finding is not confirmed by other studies [60]. The results of another study show that a high glycaemic load in the diet contributes to the development of GDM [61]. Interestingly, several studies have been conducted to investigate the effects of fat subtypes on GDM [48, 62]. It was found that saturated fats are sigificantly associated with the development of GDM, while polyunsaturated fats may be protective against GDM. On the other hand, information from observational studies suggests that a healthier diet, such as a Mediterranean diet, reduces the risk of GD [58, 63].

The MedDiet Project addresses the importance of the Mediterranean diet, highlights that eating fruitts, vegetables and whole grains may have preventive effects on GDM. A meta-analysis documented an important correlation between the risk of developing GDM and vitamin D deficiency [64]. A case-control study composed of 299 pregnant women diagnosed with GDM showed that adherence to the Mediterranean diet before pregnancy was associated with a lower risk of GDM [65]. Another study of 1076 pregnant women who followed a Mediterranean diet found a lower incidence of GDM and improved glucose tolerance [66].

Maternal Mediterranean diet and fetal/ neonatal insulin sensitivity

Insulin resistance impairs glucose metabolism, resulting in decreased glucose uptake in various tissues. It is well established that the intrauterine environment affects the growing offspring in a variety of metabolic ways, ranging from metabolic health to the development of metabolic diseases [67]. Intrauterine environment, glucose homeostasis and insulin homeostasis have been shownt to be affected by maternal diet. Pregnancy independent of maternal diet is characterized by increased development of insulin resistance, which may contribute to the development of GDM and some complex diseases in offspring later in life [68].

Importantly, animal studies have demonstrated changes in placental lipid transfer as a function of uterine lipid concentration and uterine circulation [69]. An increase in saturated fat in the womb has been shown to decrease insulin sensitivity and increase the glucose/insulin ratio in the child, resulting in inefficient glucose excretion. Insulin sensitivity has been found to increase with higher levels of omega-3 fatty acids. In addition, increased level of perinatal omega-6 fatty acids have been associated with the incidence of obesity in rodents [70]. Because the Mediterranean diet is high in monounsaturated fatty acids (MUFA), it has been shown to reduce the risk of cardiovascular disease by improving insulin sensitivity and blood lipids [71]. Another study highlights the importance of dietary habits in early pregnancy. Inadequate nutrition has a major impact on metabolic changes that cause prediabetic features at birth [72].

Maternal Mediterranean diet and neonatal lipoprotein profile and homocysteine

The studies mainly present findings on lipids and lipoproteins from infancy through adolescence and maturity. The presence of a proatherogenic environment, such as hypercholesterolemia during pregnancy, has previously been shown to increase oxidized lipids in the fetus and at birth. According to one study, lipid streaks grew relatively minimally during pregnancy, but their extent increased rapidly in later life compared to fetal development [73, 74].

Hyperhomocysteinemia is associated with the development of degenerative diseases such as kidney disease and neurological disorders. Therefore, in recent years, researchers have paid more attention to total homocysteine in serum [75]. For example, atherosclerosis is still a major cause of morbidity and mortality around the world [73]. There is evidence that the atherogenic process begins and is accelerated when levels of oxidised low-density lipoprotein (oxLDL) are elevated during pregnancy [2].

To date, the relationship between Mediterranean diet and many diseases has been studied. It has been suggested that inadeqauate consumption of Mediterranean diet is associated with lower risk of developing degenerative diseases However, the effects of the Mediterranean diet on coronary heart disease during pregnancy have not been adequately studied [73]. Mothers who follow a Mediterranean diet during pregnancy have been found to give birth to infants with low levels of insulinemia and insulin resistance [76]. Moreover, in recent years, research has focused on paraoxonase (PON-1) enzyme since it has antioxidant features and prevents low-density lipoproteins (LDL) from oxidation. Also, it has been suggested that this enzyme has pleiotropic antioxidant effects [77]. Furthermore, it has been observed that semisynthetic diets may contain compounds such as proteins, fiber, minerals, and vitamins that may affect lipids and lipoproteins. In one study, adherence to a Mediterranean diet during pregnancy was associated with lower homocysteine levels in Brazilian newborns [78]. In another study, following the Mediterranean diet was found to affect lipoprotein levels. A decrease in homocysteine levels was also observed, which was associated with improved glucose metabolism and higher maternal body weight [73].

Pregnancy and lipoprotein-associated coronary heart diseases

Undernutrition of fetuses in middle to late gestation results in disappropriate fetal growth, and leads to coronary heart disease [79, 80]. In particular, total fatty acids in plasma are significantly related to lipoprotein metabolism. In addition, several lipoprotein components such as cholesterol and saturated fat have been shown to be associated with cardiovascular health [81, 82].

Maternal hyperinsulinemia and increased tissue insulin sensitivity are some of the mechanisms leading to maternal fat deposition in the early stages of pregnancy [70, 73]. In later stages of pregnancy, low levels of lipoprotein lipase (LPL) and postheparin LPL activities are observed [70]. Changes in these metabolic processes reduce fat accumulation in maternal adipose tissue. Thus, increased lipolytic activity of adipose tissue leads to increased breakdown of fat deposits developed during the first trimester of pregnancy. In the liver, free fatty acids and glycerol are converted to acyl-CoA that leads to formation of glycerol-3-phosphate and triglycerides (TG), and packaged into very-low-density lipoprotein (VLDL). In addition, lipolysis increases and VLDL is produced and excreted in large amounts in later stages of pregnancy due to insulin resistance [70].

These metabolic processes in late pregnancy lead to an increase in VLDL levels and consequently, VLDL-TG and TG levels. In some pregnant women, transport of VLDL by cholesterol leads to an increase in total plasma cholesterol at the end of pregnancy. The increased level of VLDL in the late stages of pregnancy could therefore explain the increase in total cholesterol (TC) [70]. It is also suggested that the dietary mechanisms controlling low-density lipoproteins are less significant during pregnancy than in the pre-pregnancy period. Since TC seems to be the result of the temporary metabolic situation during pregnancy, TC should have a modest value in the diagnosis in pregnancy [73]. Detection of high levels of TC, could be used to determine neonatal risk for developing cardiovascular disease (CVD) [83]. In another study, CVD risk markers such as TC were shown to be detectable at 4 years of age at birth and in the respective parents [84].

The association between diet and lipoproteins in the fetus and at birth have not been extensively studied for several reasons. One of the possible reason was that the average levels of TC are similar in different populations of newborns [73]. According to the results of a study, Mediterranean diet during pregnancy may have an effect on systolic and diastolic blood pressure in children. In addition, the Mediterranean diet during pregnancy correlated with lower leptin levels, which may predict lower fat mass and TG in children [85].

Mediterranean diet and fertility

The influence of lifestyle factors on reproductive success has been extensively documented. To date, the influence of diet on fertility has been studied, focusing on individual foods and food categories [86]. In several studies, specific diet types and food groups have been widely studied, but the results have been incoclusive. According to some researchers, pregnancy rates after IVF have been increased by following a healthy diet [87]. Specifically, women who strictly adhered to the Mediterranean diet were found to have a lower risk of not becoming pregnant. According to the literature, male diet can have an impact on fertilization. Importantly, consumption of whole grains, cereals, vegetables, and fruits in large quantities is significantly associated with fertility and treatment outcomes [88]. According to some researchers, the high content of vitamin B6, folic acid and vegetable oils in the Mediterranean diet is one of the factors that can explain the positive effect of the Mediterranean diet on fertility [34]. Although the results of several studies are conclusive, further studies are needed to investigate the effects of the Mediterranean diet on IVF success rate [89].

Folate mechanism and congenital structural anomalies and fetal demise

The importance of the intrauterine environment for early embryonic development has been emphasized in recent years. In particular, folic acid has attracted the interest of researchers due to its importance in a number of clinical conditions [90]. Folate is necessary for cellular processes such as nucleotide synthesis and DNA repair. Furthermore, it acts as a cofactor for enzymes involved in one-carbon metabolism. Folate plays a central role in cell division and it is required during pregnancy and infancy. Folate plays a central role in cell division and is needed during pregnancy and infancy. During pregnancy, fetal, placental and maternal tissues need more folic acid [91, 92]. Folic acid deficiency, on the other hand, is associated with a number of diseases comprising birth defects and neural tube defects [93,94]. With an incidence of 0.2-10 per 1000 live births, neural tube defects (NTDs) are among the most common congenital anomalies of the central nervous system [95]. NTDs are serious birth defects caused by the failure of closure of the neural tube around 28th day after conception [96]. Genetic and environmental factors are involved in the pathogenesis of NTDs. Although the pathogenesis of NTDs is not yet clear, deficiencies in one-carbon metabolism are thought to be one of the causes the formation of NTDs [97].

Infants with anencephaly and spina bifida show several complications during their lifetime [98]. Randomized clinical trials have shown that folic acid consumption by mother during periconception is significantly associated with a reduced risk of developing NTDs [99, 100]. In addition, folic acid may also protect against other congenital abnormalities such as preeclampsia [101]. Folic acid intake and a folic acid-enriched diet are recommended by World Health Organization (WHO) and other government organizations in numerous countries for women of childbearing age. The incidence of NTDs was reduced by 50-75% with folic acid supplementation in the periconceptional period [102].

The synthetic version of the naturally occurring folates is folic acid (pteroylmonoglutamic acid) (pteroyl-L-poliglutamic acid). The basic difference between folic acid and folate is that the B9 vitamins differ in the amount of glutamate molecules they contain. Folates are found in many foods, primarily in green vegetables [103]. It has been shown that a maternal Mediterranean diet enriched with vitamin B12, niacin, iron and magnesium was significantly associated with spina bifida in the offspring [102]. In a study, western and prudent diets were compared. As a result, it has been shown that the that the children of women who followed the former dietary pattern were at higher risk for having cleft lip despite folic acid supplementation [102, 103]. Finally, a significant association was found between the risk of spina bifida (SB) and inadequate maternal consumption of a Mediterranean diet, possibly related to increased oxidative stress due to deficiencies of vitamins B6, B12, and folates [103].

Conclusion

In conclusion, pregnancy is a complicated process involving growth, development and maturation. Maternal nutrition's importance has undoubtedly been the subject of several studies, as it affects growth, maturation and fetal development. It has been shown that inadequate intake of essential macronutrients and micronutrients can negatively affect the outcome of pregnancy and neonatal health. Particularly, the periconceptional period is considered as a critical period for fetal development and health, as various malformations and pregnancy-related disorders occur during this time, including congenital anomalies, fetal losses, miscarriages, and preterm births. Importantly, a Mediterranean diet throughout pregnancy, especially in periconception, contributes to more appropriate growth from the first stage of pregnancy.

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Conflict of Interest

The authors have no conflict of interest to declare.

Author's Contributions

PT,HC conceptualized the content, and wrote the manuscript; MCE assisted with the revision of manuscript; PT

conceptualized the content and critically revised the manuscript. All authors read and approved the final manuscript.

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REVIEW

Effects of the Mediterranean diet polyphenols on cancer development

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Keywords

Mediterranean diet • Polyphenols • Cancer

Summary

Globally, the second most common mortality reason is cancer. There are two types of risk factors for cancer: intrinsic (unmodifiable) and non-intrinsic (modifiable). Bad lifestyle behaviors are among the exogenous non-intrinsic risk factors that can be related to 30-50% of cancer development risk, among which can be counted the Western lifestyle. On the contrary, a potentially good lifestyle model to prevent cancer is the Mediterranean diet (MD), which is a plant-based nutrition model. The Mediterranean diet includes many beneficial nutrients and nutritional substances, such as dietary fibers, fatty acids, anti-oxidant and anti-inflammatory substances, etc. Among these beneficial substances, an important group is the one composed by polyphenols, the most common plant-synthesized secondary metabolites. Being a plant-based nutrition model, the Mediterranean diet provides many polyphenols, such as resveratrol, quercetin, phenolic acids, catechins, anthocyanins, oleocanthal, oleuropein, rosmarinic acid, gallic acid, hesperidin, naringenin, ellagic acid, etc. These substances show anti-proliferative, pro-apoptotic, anti-inflammatory, anti-oxidant, anti-migration, anti-angiogenic, anti-metastatic, and autophagy stimulator effects, which can potentially reduce cancer development risk, as was shown by some in vivo and in vitro studies on this topic. In this review of the literature we shed light on the effects and potential interactions between the Mediterranean diet polyphenols and cancer development.

Introduction

Definition of Cancer

Cancer is a disease that is characterized by abnormal (uncontrolled) cell growth [1]. There are some hallmarks of cancer cells, such as "sustaining proliferative signaling", "evading growth suppressors", 'avoiding immune destruction, "enabling replicative immortality", "tumor-promoting inflammation", "activating invasion & metastasis", "inducing or accessing vasculature", "genome instability & mutation", "resisting cell death", and "deregulating cellular metabolism" [2]. Cancer is the second mortality reason in the world; nearly ten million people have died from cancer in 2020 [3].

Risk factors for cancer development can either be intrinsic (unmodifiable) and non-intrinsic (modifiable). Intrinsic risk factors are related to random errors in DNA, while non-intrinsic risk factors are divided in two groups: endogenous non-intrinsic risk factors (like biologic aging, genetic susceptibility, DNA repair machinery, hormones, growth factors, inflammation, etc.) and exogenous non-intrinsic risk factors (like radiation, chemical carcinogen substances, tumor-causing viruses, bad lifestyle behaviors, etc.) [4]. Especially, bad lifestyle behaviors – such as Western-style nutrition habits, sedentary life, smoking (both active and passive), high alcohol consumption, obesity, etc. – can increase cancer risk [5], being related to the development of cancer cells in 30-50% of cases [6].

Cancer Prevention via Nutrition: the Mediterranean Diet

The MD is an important nutrition model for the prevention of non-communicable diseases, such as diabetes mellitus, cardiovascular diseases, cancer, etc [7]. thanks to its many beneficial effects, among which are its anti-oxidant, anti-inflammatory, anti-proliferative, anti-angiogenesis, anti-metastatic activity, and so on. [8].

Yiannakou et al. [9] reported that in their cohort study (18 years median follow-up, n: 2966) a higher adherence to the Mediterranean diet resulted in a decreased cancer risk (≥ 25%), especially in women, but the MD was also effective in reducing cancer risk in non-smoker men. Barak and Fridman [10] reported that the MD reduced overall cancer risks, especially digestive tract cancers, as proven by a systematic review (28 trials, 570,262 participants). In another study (20.3 years follow-up, n: 120,852), researchers observed that the MD was significantly effective in reducing overall cancer risk in females, but not in males [11]. In their meta-analysis (117 studies, n: 3,202,496) Morze et al. observed that the highest adherence to the MD reduced cancer mortality in the general population [12]. Apart from prevention, adherence to the MD as a medical nutrition treatment showed beneficial effects also on the reduction of mortality risk in survivors of colorectal, head and neck, respiratory, gastric, liver, and bladder cancer. The reasons behind these effects of the MD are: a high consumption of olive oil, vegetables, fruits,

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Tab. I. Traditional Mediterranean diet principles [14, 16].

Higher consumption	Moderate consumption	Lower consumption
Olive oil, whole grains, legumes, seeds, vegetables, fruits	Fish, red wine, dairy	Poultries and white meat, red meat, processed red meat products.

and legumes; moderation in eating red wine, fish, and dairy products; and low consumption of red meat and processed red meat products, poultry and other white meats, desserts, etc. [8, 13-15]. Table I shows the main suggested consumption frequencies of these foods.

The foods that are suggested for higher or moderate consumption provide the organism with beneficial fatty acids, dietary fiber, anti-oxidant and anti-inflammatory nutrients, and many more nutritional substances [15]. Due to the positive relationship between their nutritional contents and human health, these are effective functional foods for the MD [17].

Among the many nutrient and beneficial substances that are contained in these products, a major role in the MD is played by polyphenols [18].

Polyphenols of the Mediterranean Diet

The name "Mediterranean diet" (MD) derives from the fact that this plant-based dietary model was created and developed in the Mediterranean region [16], but also people of different cultural origins can adapt to it with ease [19]. As previously stated, the MD includes a high number of polyphenols [18]. Kapolou et al. reported that moderate adherence to m the MD was related to an increase in the dietary intake of polyphenols [20]. To stay alive, plants synthase some metabolites, both primary and secondary. Among secondary metabolites, the most common are polyphenols [21], which are an important component of the MD, it being characterized by a high amount of plant-based food (such as olive oil, vegetables, fruits, legumes, red wine...) consumption [14]. Figure 1 shows the main polyphenols of the MD.

The MD is characterized by moderate red wine consumption, which is however influenced by social norms and religious beliefs [14]. The main polyphenol ingredient of red wine is resveratrol [22], but red wine also contains quercetin, myricetin, phenolic acids, catechins, anthocyanidins, etc. [23].

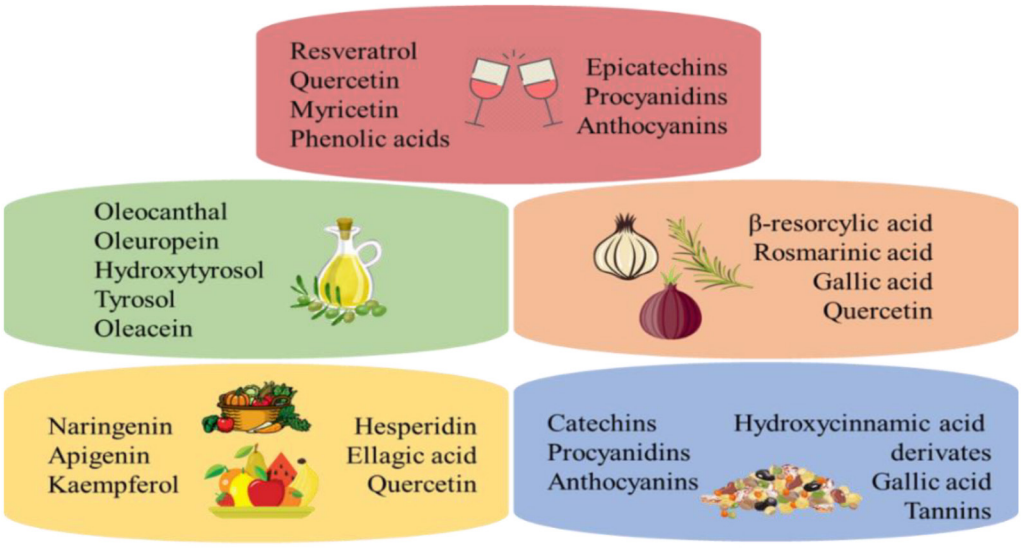
Another main component of the MD is olive oil [14], which contains the highest amount of oleocanthal, oleuropein, and other phenolics (Fig. 1) [24]. Fruits and vegetables are also good sources of polyphenols: some of the main ones contained in these foods are quercetin, kaempferol and ellagic acid [25]. Furthermore, citrus – a very common fruit in the Mediterranean region – contains naringenin, apigenin, hesperidin, and other polyphenols [26].

The Mediterranean diet is also characterized by the use of garlic, onion, and rosemary as food flavoring [27, 28]: these plants contain important polyphenolic compounds like β-resorcylic acid, rosmarinic acid, quercetin, gallic acid, etc. [29, 30]. In addition, the consumption of legumes, which are another important component of the MD [14], provides many beneficial polyphenols [31].

Effects of Some Polyphenols on Cancer Development

Resveratrol, one of the stilbene compounds, shows potentially anti-proliferative, pro-apoptotic, anti-inflammatory, anti-oxidant, anti-angiogenic, metastasis inhibitor effects, which are important to prevent cancer [32, 33]. Quercetin, a flavonol, may show anti-oxidant, anti-proliferative, pro-apoptotic, anti-inflammatory, anti-angiogenic, autophagy stimulator, metastasis inhibitor effects [34, 35] that are shown also by another

Fig. 1. Some of the main polyphenols of the Mediterranean diet. Each box shows a plant-based food group in the MD.



flavonol, called myricetin [36]. Catechin and epicatechin (two flavanols) are potential stimulators of apoptosis and cancer cell death, also showing anti-inflammatory and anti-oxidant effects [37].
In addition to these effects, catechins – especially green tea catechins – are potentially effective also in inhibiting migration, angiogenesis, and metastasis [38]. Anthocyanins can decrease cancer risk via anti-proliferative, anti-inflammatory, anti-oxidant, pro-apoptotic, anti-metastasis effects [39, 40].
Phenolic compounds from olive oil – such as oleocanthal, oleuropein, hydroxytyrosol, tyrosol, oleacein, etc. – potentially have anti-oxidant, anti-inflammatory, anti-proliferative, anti-angiogenic, pro-apoptotic effects [41, 42]. Another important polyphenolic compound against cancer is rosmarinic acid, a phenolic acid that potentially increases apoptosis, necrosis, and accumulation of Reactive Oxygen Species (ROS) and decreases cell proliferation, inflammation, and more [43]. Gallic acid and β-resorcylic acid, which are both phenolic acids, showed anti-cancer effects in some studies (Tab. II).
In addition, phenolic compounds from vegetables and fruits, especially those citrus-sourced, have potentially anti-inflammatory, anti-oxidant, anti-metastasis, anti-proliferative, and pro-apoptotic effects [25, 44], a characteristic shared also by tannins [45]. It is thus possible to say that these polyphenolic compounds, typical of the MD, have potentially anti-cancer effects due to their ability to affect some cancer-related pathways. Figure 2 shows a summary of their main effects.
Currently, some in vitro and in vivo studies showed the above-mentioned effects of a number of polyphenols; their results are listed in Table II.

Discussion

The above-mentioned effects of a number of polyphenols are testified by some in vitro and in vivo studies in the literature. According to these studies, some of the Mediterranean diet polyphenols are potentially effective in decreasing the development of the cancer types that are most common worldwide, such as lung, colon, brain,

Tab. II. In vivo and in vitro study samples about the effects of some polyphenolic compounds on cancer development. In the last column, a downward-facing arrow means that the compound is known to induce a reduction, while an upward-facing arrow means that the compound is known to induce an increase.

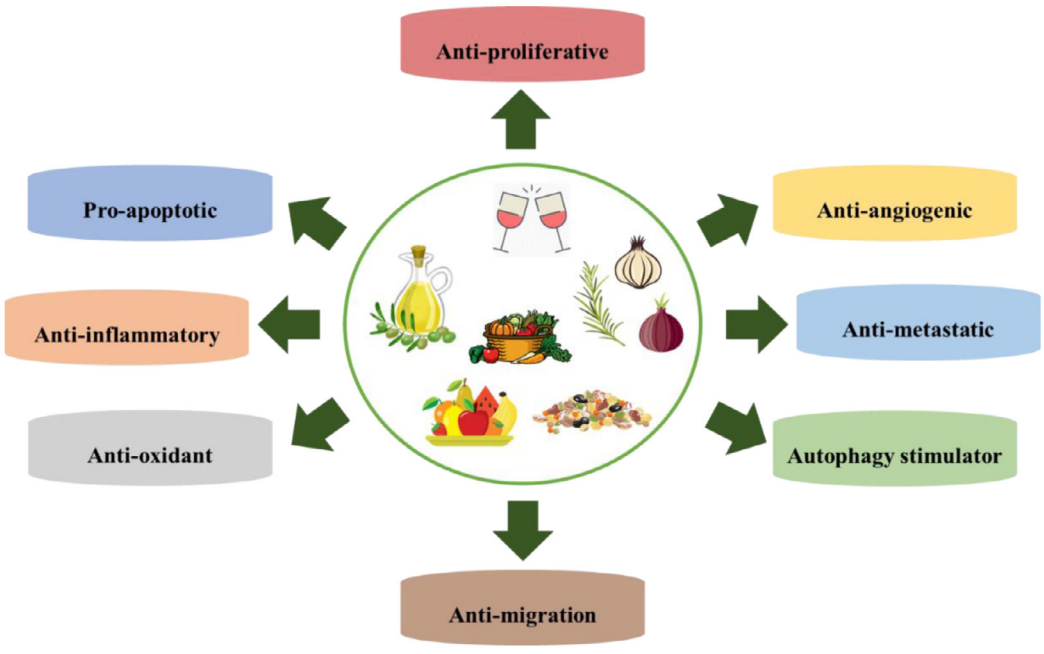
Reference	Polyphenolic compound	Results
[46]	Resveratrol (in vitro)	On colon cancer cells: -Apoptosis ↑ -Proliferation ↓ -Angiogenesis ↓
[47]	Resveratrol (in vitro)	On glioblastoma cells: -Growing ↓ -Migration ↓ -Proliferation ↓
[48]	Resveratrol (in vivo and in vitro)	In female rats: -Breast tumor incidence ↓ On breast epithelial cells: -Anti-oxidant activities ↑ -Apoptosis ↑ -Migration ↓
[49]	Resveratrol (in vivo and in vitro)	Both on mice and on follicular thyroid cancer cells: -Tumorigenesis ↓
[50]	Quercetin (in vitro)	On glioblastoma cells: -Cell death ↑
[51]	Quercetin (in vivo and in vitro)	In female rats: -Tumor volume ↓ (both colon carcinoma and breast cancer cells) On colon carcinoma, prostate adenocarcinoma, pheochromocytoma, breast cancer, acute lymphoblastic leukemia T, myeloma, lymphoid Raji, and ovarian cancer cells: -Apoptosis ↑ -Cell death ↑
[52]	Myricetin (in vitro)	On breast cancer cells: -Apoptosis ↑
[53]	Myricetin (in vitro)	On prostate cancer cells: -Metastasis ↓ -Cytotoxicity ↑ -Apoptosis ↑

▶

Tab. II. *Continues.*

Reference	Polyphenolic compound	Results
[54]	Anthocyanins from bilberry (in vitro)	On colon cancer cells: -Mitochondrial damage ↑ -Apoptosis ↑ -Proliferation ↓
[55]	Anthocyanins from blueberry (in vitro)	On melanoma cells: -Proliferation ↓ -Apoptosis ↑
[56]	Oleocanthal (in vitro)	On lung cancer cells: -Progression ↓ -Metastasis ↓
[57]	Olive oil phenols (in vitro)	On bladder cancer cells: -Apoptosis ↑ -Proliferation ↓
[58]	Rosmarinic acid (in vitro)	On melanoma cells: -Metastasis ↓ -Invasion ↓ -Proliferation ↓ -Apoptosis ↑ -Chemoprotective drug sensitivity ↑
[59]	Naringenin (in vitro)	On lung cancer cells: -Migration ↓ -Invasion ↓ -Proliferation ↑ -Apoptosis ↑
[60]	Tannins (in vivo)	In rats: -Antioxidant capacity ↑
[61]	Some phenolic acids (in vitro)	On breast cancer cells: -Apoptosis ↑ -Proliferation ↓
[62]	Gallic acid (in vitro)	On lung cancer cells, in combination with cisplatin (chemo drug): -Proliferation ↓ -Apoptosis ↑
[63]	β-resorcylic acid lactones (in vitro)	On lung adenocarcinoma and colorectal cancer cells: -Cytotoxicity ↑ -Proliferation ↓

Fig. 2. Potentially beneficial effects of MD phenolic compounds on cancer development.



breast, thyroid, and prostate cancers. The results of these studies are listed in Table II.

Conclusion

In conclusion, the main polyphenols of the MD have some solid potentially anti-cancer effects. Polyphenols are nutritional substances, not nutrients. Thus, for these substances there is no Dietary Recommended Intake (DRI), overdose, and such. On the other hand, most of the current studies are in vitro. From this point onward, there is a need for in vivo studies, which can show both the beneficial and the adverse effects of these substances on the human body. Consequently, a broader scope, including farm to fork concept as well as an epidemiological approach, can shed more light on this topic.

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Conflicts of interest

There are no conflicts of interest.

Authors' contributions

TD searched the literature and wrote the main outline of the article. AO contributed to the concept of the article and revised the main outline of the manuscript.

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Review

A review of the Mediterranean diet and nutritional genomics in relation to cancer in women

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Keywords

Women's cancer • Nutritional genomics • Nutrigenetics • Nutrigenomics • Mediterranean diet

Summary

Cancer is the leading cause of death among women all over the world. Female tissue-specific cancers are the most commonly diagnosed among women and account for most cancer-related deaths. The main risk factors for women's cancer are hereditary factors, specific exposure to dangerous chemicals, disorders such as hormone imbalance, and lifestyle. High body mass index, low physical activity, low intake of fruit and vegetables, smoking, excessive alcohol consumption, lack of cancer screening and treatment are the most common risk factors. Nutrigenetics and nutrigenomics are both part of nutritional genomics. Nutrigenetics is how a person's body reacts to nutrients based on his/her genotype. It can be used to create a personalized diet, maintain a person's health, avoid disease, and if necessary to sustain therapy. Nutrigenomics studies the impact of nutrition on gene expression and the epigenomic, proteomic, transcriptomic and metabolomic effects of dietary intake. There is evidence that diet matters for different women's cancers, and is related to cancer progression, survival and treatment. The optimum combination for cancer prevention is a diet rich in vitamins and fibre, with low meat consumption, low milk intake and moderate use of alcohol. The Mediterranean diet looks to be an optimal diet with a good nutrition pattern, qualifying it as a therapy to prescribe.

A review of the Mediterranean diet in women's cancer and nutritional genomics

Cancer is a major cause of death among women, with a an incidence of 9.2 million new cases and 4.4 million deaths per year according to the International Agency for Research on Cancer (2020) in high- and middle-income countries [1]. Cancer is increasing in all countries, irrespective of income, due to population growth and aging. Females constitute 49.5% of the global population, and a larger proportion of the population over 60 years of age, where cancer is more common, irrespective of income, due to a change in average lifespan and factors that contribute to death rates [2]. Female tissue-specific cancers such as breast, ovarian, uterine and endometrial account annually for over 3,000,000 cancer-related incidents [3]. Breast, colorectal, cervical and lung cancer are the types most frequently diagnosed in women and the cause of most cancer-related deaths [4].

Many risk factors are linked to the incidence of cancer and are mainly personal and environmental. Genetic factors, exposure to chemical substances, disorders such as hormone imbalance and unhealthy lifestyle are the main risk factors. The five most common are high body mass index, low physical activity, low intake of fruit and vegetables, smoking and excessive intake of alcohol. Absence of cancer screening and treatment also play a role, especially in undeveloped countries, as well as chronic infections (Hepatitis B and C, *Helicobacter pylori*, human papillomavirus and Epstein-Barr virus) [5, 6].

Smoking is considered to be number one risk factor, causing 22% of deaths due to cancer. Individual genetic and hereditary factors contribute significantly to the transformation of healthy cells and precancerous lesions into malignancies [7]. Number two risk factor is unhealthy lifestyle: poor diet, obesity, sedentary lifestyle and physical inactivity. A change in dietary habits is estimated to improve avoidance of cancer by 30 to 50% [8].According to the World Health Organization, the five leading behavioral and dietary risks are responsible for approximately one-third of cancer deaths: high body mass index, low consumption of fruit and vegetables, smoking, low physical activity and excessive consumption of alcohol [9].

Ovarian, endometrial, vulvar, vaginal and cervical cancer are all tumors of the female reproductive system. The incidence of ovarian, vulvar and vaginal cancers is low (1.7%, 0.3%, and 0.1%, respectively in 2018). Risk factors unrelated to diet are early menarche, nulliparity, menopause after age 55, smoking and heredity, while diet may be significantly linked to cervical and endometrial cancer [5]. Cervical cancer, the fourth most common cancer and the fourth cause of death worldwide is closely linked to diet [10]. Regular consumption of fruit and vegetables, and thus nutrients such as vitamins

E, C and A, carotenoids, folates and minerals, may help reduce the risk of cervical cancer due to the role played by these nutrients in protecting against and inhibiting the proliferation of cancer cells and preventing DNA damage [11].

Endometrial cancer is primarily caused by unbalanced and/or prolonged exposure of the endometrium to estrogens. Unless counterbalanced by progestogens, this increases endometrial cell mitotic activity, resulting in increased DNA replication and an increase in the probability of somatic mutations. Women who enter menopause late, are nulliparous, have polycystic ovary syndrome, use estrogen replacement therapy (without progestogens) or are obese are at risk of the above unbalanced or prolonged exposure [12]. Hormonal regulation of the menstrual cycle is linked to endometrial inflammation. Chronic endometrial inflammation is linked to being overweight or obese [13].

Breast cancer is the most common malignant cancer in women and the second most frequent cancer worldwide [14]. It is a heterogeneous disease. The gene-expression profile is classified into two major groups according to estrogen receptor (ER) expression: ER-expressing positive (ER+) is significantly related to hormonal factors, unlike ER-expressing negative (ER-) [15].

Many risk factors are linked to cancer onset: menopause lowers the risk of breast cancer, while hormonal dysregulation and prolonged exposure to endogenous hormones, like estrogen and estrogen receptor positivity, are associated with increased risk. Aging and genomic mutations impact cancer progression. Heritable mutations in DNA repair genes *BRCA1* and *BRCA2*, apart from being associated with breast cancer development, are also indicators of endometrial and ovarian cancer. Tumor suppressor protein 53 (TP53) mutations, angiogenic factors and signaling molecules are other remarkable factors [3].

There is growing interest in the links between susceptibility to cancer, prognosis and exposure to risk factors like diet. Such research is motivated by the fact that bioactive agents found in everyday foods have enormous potential in oncology due to their ability to regulate coding or non-coding genes, and as adjuvants in cancer therapy [9, 16].

Nutritional genomics is the study of how diet may influence the expression of genetic information and how an individual's genetic makeup influences response to nutrients, metabolism, and the bioactive compounds in food. It aims to identify genetic variants associated with a genetic response to diet and with diet-related diseases, to develop disease treatment and prevention strategies, and to improve dietary guidelines [17]. Its principles are based on the concept that diet is a critical predisposing factor for certain diseases in certain people under certain conditions. Variations in individual genotype can help explain the balance between health and disease. Dietary ingredients alter gene expression and/or gene structure, and therefore the human genome. Genes regulated by dietary factors may play a role in the onset, severity, progression and development of chronic diseases [18].

Nutritional genomics includes nutrigenetics and nutrigenomics [17]. These two disciplines have acquired importance in clinical research and practice: they explore the two-way interaction between diet and the human genome.

Nutrigenetics seems to have its origins in classical genetics. It concerns the interaction of nutritional and genetic factors that may play a role in disease progression. Its primary goal is to investigate the effects of genetic variations, specifically single-nucleotide polymorphisms (SNPs), on the metabolic response to diet. It is therefore concerned with how the body responds to nutrients in relation to genotype [19].

The methylenetetrahydrofolate reductase gene (*MTHFR*), which is involved in folic acid metabolism and maintenance of normal homocysteine levels in the blood, is a well-known example of gene-nutrient interaction. A specific *MTHFR* SNP is related to elevated homocysteine levels in the blood, particularly in the presence of folic acid deficiency [20], which is linked to increased predisposition to colon cancer [21]. Nutrigenetics connects nutrition, human genes and environmental exposure, with the focus on genes. It can be used to personalize diet in order to maintain health, prevent onset of disease and aid treatment.

Nutrigenomics provides a more comprehensive view of how nutrients affect gene expression, and the transcribed characteristics associated with those genes and with direct effects manifested by metabolomic and proteomic activities [22]. Nutrigenomics originated from the Human Genome Project and is concerned with the impact of nutrition on gene expression and the epigenomic, proteomic, transcriptomic and metabolomic effects of dietary intake [17]. One example to illustrate nutrigenomics is the single nucleotide change that determines phenylketonuria. Carriers should avoid foods rich in phenylalanine. Many Asian populations lack the aldehyde dehydrogenase necessary to metabolize ethanol and develop skin irritation after consuming alcohol. Another example is galactosemia, a disease caused by an inherited genetic deficiency in one of the enzymes involved in metabolizing galactose [21].

Nutrients may affect many cell processes, some associated with tumorigenesis. One concern is therefore how specific nutrients influence the development and growth of cancer. Natural nutrients can disrupt tumor progression at many levels while also increasing chemotherapeutic effectiveness and reducing the side-effects associated with these treatments [16]. Proper nutrition is especially important for cancer patients because the illness and its treatments can impair appetite. Cancer and its treatments can also impair the body's capacity to endure and use dietary nutrients. Cancer treatment generally requires treating the tumor as well as the patient, and nutrition is an essential component of the treatment plan [23].

Several studies have documented the epigenetic impacts of nutrition on phenotype and predisposition to disease throughout life. Dietary nutrients interact with genes, the dietary and environmental factors have an impact on epi-

genetics. Certain bioactive food components or micronutrients are known to play a role in DNA methylation, histone modifications, gene expression, and biological and metabolic regulatory pathways [24]. Deficiency of any key nutrient, a lack of methyl donors, or inhibition of methyltransferases can result in gene mutations by activating promoter genes and DNA hyper or hypomethylation, which with age and cell proliferation silence tumor suppressor genes, allowing cancer to develop. Moreover, nutrients can either inhibit epigenetic enzymes like DNMT, HDAC and HAT or change the substrate accessibility required for such enzyme reactions. They can also alter the expression of specific genes, affecting health and longevity [24].

Since epigenetic marks can be altered, they provide an additional explanation for how external factors, such as diet, can impact biological processes and phenotypes. Many nutritional components, including folate, choline, methionine, selenium and retinoic acid, have been shown to affect DNA methylation patterns [25].

Nutrigenomic studies indicate that macronutrients and micronutrients like certain vitamins, minerals and dietary fibre are not only beneficial for cancer prevention but also in treatment, particularly regarding the major characteristics of cancer cells, such as uncontrolled proliferation possibly leading to metastasis [19]. Flaxseed diets, for example, have been shown to aid in the treatment of breast cancer. The mechanism includes a flaxseed lignan that is converted into a compound that binds to estrogen receptors and thus inhibits cell growth [26].

A recent study provided evidence that diet matters for breast cancer survival. While high blood glucose and circulating insulin levels are related to breast cancer prognosis, shreds of statistical of evidence show that women with increased glycemic load have a 31% higher risk of death from breast cancer and a 26% higher risk of all-cause mortality [27]. Chronic hyperinsulinemia can be caused by a high glucose intake, which reduces the production of insulin-like growth factor-binding proteins while increasing insulin-like growth factor 1 [28]. This increase causes a reduction in sex hormone-binding globulin production and inhibits apoptosis, stimulating synthesis of sex hormones and proliferation of ovarian cells. All of these alterations can promote development of ovarian cancer [28, 29].

Another study showed a strong relationship between risks of ovarian cancer and dietary glycemic index in overweight women, non-diabetics, non-users of oral contraceptives and alcohol consumers, and in women without a family history of ovarian or breast cancer, demonstrating a significant increase in the risk of ovarian cancer in all women with a high glycemic diet [30].

According to some pre-clinical studies, nutrients such as carbohydrates, saturated fat, red and processed meat, increase endogenous estrogen levels and could be risk factors for breast cancer [14]. Cholesterol is the precursor of the steroid hormones estrogen and progesterone. A high-fat diet leads to over-synthesis of estrogen, which stimulates cell proliferation in the female genital tract [31]. The polyphenols found in vegetables and fruit and are known as polyhydroxy phenols. They are common in items such as green tea, cinnamon and curcumin, and they number more than 8000 in the human diet. Polyphenols have an epigenetic role in the prevention of cancer through gene silencing and chromatin remodeling [32]. This may be due to their ability to modify histones and to inhibit DNA methyltransferase.

Research into the effects of curcumin, a known antiproliferative and apoptotic, in the experimental breast cancer alpha-model, showed that curcumin inhibits cell proliferation and invasion, metastasis and angiogenesis in various cancers through interaction with many cell signaling proteins [33].

Manganese superoxide dismutase (MnSOD) is a mitochondrial enzyme involved in the detoxification of reactive oxygen species. Since MnSOD contains manganese in its active site, factors that affect manganese availability can also have a marked effect on lipid peroxidation. This emphasizes the importance of fruits and vegetables including pineapple, acai and spinach as well as nuts and legumes, which are rich manganese sources. Increased risk of breast cancer has been linked to a polymorphism (valine to alanine substitution) in the mitochondrial targeting sequence of the MnSOD gene, which is thought to alter transport of the enzyme into the mitochondria. Women who eat less fruit and vegetables have a stronger correlation between breast cancer indidence and reduced fruit and vegetable intake [34, 35].

The World Cancer Research Fund/American Institute for Cancer Research, the American Society for Clinical Oncology and the American Cancer Society have published collective dietary guidelines for breast cancer survivors which recommend increased intake of fruit, vegetables, legumes and whole grains and reduced intake of sugar, sugary drinks and calorie-dense foods [36].

Various studies indicate that a diet rich in vitamins and fibre, low in meat and milk, and with moderate intake of wine is the best combination for cancer prevention. Putting these pieces of evidence together, the Mediterranean diet seems to be qualified for use as a prescribed medication [5]. It reflects the traditional eating habits of people from Mediterranean countries. In general, it is high in fruit, beans, nuts, fish, green vegetables, legumes, cereals, grains and virgin olive oil. Although its main feature is considered to be low meat and dairy intake, the eating habits of Mediterraneans vary by country and region. Being low in saturated fats and high in minimally processed plant-based foods, the Mediterranean diet is considered an ideal nutritional model. Observational and epidemiological studies suggest that it may have protective effects against cardiovascular disease and cancer, because Mediterranean countries show lower rates of these diseases than other countries [37, 38]. Olive oil contains about 30 phenol compounds, including hydroxytyrosol and oleuropein, and is the principal source of fat in the Mediterranean region. These compounds are powerful antioxidants that have been shown to protect cells from free radical damage induced by normal metabolism [39]. Antioxi-

dant-rich foods may postpone the accumulation of cell damage in the body and protect against cardiovascular disease, diabetes and the consequences of aging. A number articles in the literature have suggested that olive oil consumption is inversely related to risk of breast and ovarian cancer [40].

Another important feature of the Mediterranean diet is the diversity of seafood from the Mediterranean Sea. Long-chain omega-3 (n-3), eicosapentaenoic and docosahexaenoic fatty acids in seafood help improve cardiovascular health by decreasing risk factors such as blood pressure, triglyceride concentrations, heart arrhythmias and platelet aggregation [41]. These acids are thought to inhibit breast cancer [42, 43]. Fish like sardines and mackerel are an important part of the Mediterranean diet and are rich in omega-3 fatty acids. Other foods rich in omega-3 are nuts like walnuts, almonds, pumpkin and other seeds. These help prevent cancer progression by moderating cell proliferation, angiogenesis, metastasis and inflammation [44].

One or two glasses of wine per day, taken with food, are usually part of the Mediterranean diet. Although alcohol generally has negative effects on health, The Copenhagen Prospective Population Studies (2000) demonstrated that moderate wine intake may have a beneficial effect on human health as it contains various polyphenols. Resveratrol in particular increases expression of the sirtuin 1 (*SIRT1*) gene [45]. Changes in *SIRT1* expression are critical in diseases such as neurodegeneration, metabolic syndrome, cardiovascular disease and cancer. Moderate intake of red wine can be protective and decrease proliferation of breast cancer cells, while the antioxidant polyphenols modify ROS production and target steroid receptors [46]. There are studies indicating an inverse relationship between vegetable consumption and hormone-related tumors, including breast, ovarian and endometrial cancer (Pelucchi et al., 2009). The abundance of fruit and vegetables in the Mediterranean diet, such as apples, citrus, onions, broccoli, rich in flavonol polyphenols and fibre, is suggested to protect against carcinogenesis [14]. The vegetable component of the Mediterranean diet, specifically non-starchy vegetables, regulates steroid hormone concentrations and metabolism, activating antioxidant enzymes, stimulating the immune system and protecting against cancer [47]. The Mediterranean diet contains high concentrations of phytoestrogens, estrogen-like agents which may compete with estrogens for estrogen receptors, with antiestrogen effects [48]. Some studies demonstrate an association between significantly close observance of the Mediterranean diet and lower risk of endometrial cancer, providing evidence of a protective effect [49].

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

Literature review: G.T., Q.H.; Collected the data: G.T., Q.H., G.M., M.C.E.; Contributed data: G.T., Q.H., G.M., M.C.E.; Performed the analysis: G.T., Q.H.; Wrote the paper: G.T., Q.H., revised the paper: G.T., Q.H., G.M., M.C.E; supervised the project: M.C.E.

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Review

The role of Mediterranean diet and gut microbiota in type-2 diabetes mellitus associated with obesity (diabesity)

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Keywords

Mediterranean diet • Gut Microbiota • Type-2 Diabetes Mellitus • Obesity • Diabesity

Summary

The human body is made up of 10^{14} human cells and 10^{15} bacterial cells, forming a combined structure that is described as a "superorganism". Commensal, symbiotic, and pathogenic microorganisms in the human body, many of which are located inside the intestine, affect health conditions and diseases. An important factor contributing to the development of chronic diseases is dysbiosis, which occurs when the number of pathogenic microorganisms increases. Dysbiosis is associated with increased intestinal permeability, endotoxemia (increased LPS), pro-inflammatory cytokine release, energy harvest, and adiposity, thus being involved in the pathogenesis of disorders like diabetes and obesity. Nutritional habits are the most important environmental factor that affects intestinal microbial composition. A dietary pattern that was proven successful in regulating gut microbiota is the renowned Mediterranean diet, which is characterized by high plant-based foods consumption, moderate fish and dairy products consumption, and low red meat consumption. There is an inverse relationship between adherence to the Mediterranean diet and chronic diseases like obesity and diabetes. In addition to the direct effects of the Mediterranean diet on the pathogenesis of these diseases, it can also be effective in preventing these diseases due to its effects on the intestinal microbiota. It is noted that the number of Bifidobacterium and Bacteroides increases the longer one's eating habit adhere to the Mediterranean diet, and the number of Firmicutes decreases, accordingly, thus supporting the symbiotic distribution in the intestinal microbiota.

Introduction

Relman and Falkow reawakened the project named "second human genome project", in 2011. This project attracted attention to the necessity to analyze the microbial genome in the determination of microbial colonization in the gastrointestinal (GI) system. Hence, it provided knowledge about endogenous flora in GI and its differences in disease and health status [1]. There are about 10^{14} bacterial cells in the human body. Along with bacteria, other microorganisms, viruses, and archaea use the human body as a host and can be found in different anatomical parts of the body such as skin, urogenital region, respiratory tract, oral cavity, digestive system etc. Amongst the different regions of the body; the digestive system possesses a large part of the microbiota within the human body [2, 3]. All the anatomical parts of the digestive tract, from mouth to anus are involved in digestion and absorption processes. Also, commensal bacteria colonized in the digestive tract is a modulator for the host's health via influencing different physiological processes and gene expressions. Nutritional habits are the most important environmental factor that affects the gut microbiota composition [4]. It is emphasized in the literature that the Mediterranean diet model supports the composition of the gut microbiota for the benefit of human health [5]. The Mediterranean diet model is described as one of the plant-based nutritional models. The traditional Mediterranean diet model involves rich consumption of vegetables, beans, nuts and seeds, fruits, whole intact grains, fish and other seafood; olive oil, and dairy products (mainly yoghurt and cheese). Whereas on the other hand, it involves low consumption amounts of red meat; sugars or honey and low to moderate consumption amounts of wine [6].

Food environment, diet and physical activity are some of the significant players in the development of diabetes and obesity [7]. Both of the diseases are in the characteristics of an epidemic according to the global drastic increase in their prevalence [8]. According to World Health Organization (WHO) statistics 2021, 463 million people were diagnosed with diabetes and 650 million people were diagnosed as obese, worldwide [8, 9]. Type-2 diabetes is more frequent than Type-1, such that 95% of all diabetes are diagnosed to be Type 2. A range of complications including Type-2 diabetes, arthritis and cardiovascular disease (CVD) are related to obesity. The reasons behind this consist of the building of excess adipose tissue, insulin resistance and chronic inflammation. Noninsulin-dependent diabetes, namely Type-2 diabetes is a frequent condition found in obese individuals. Recently, a new unique term referred to as "diabesity" is used to describe the situation

of Type-2 diabetes mellitus associated with obesity [10]. WHO states that Type-2 diabetes and obesity are the two preventable pathological disorders. In these cases, modifiable risk factors such as dietary habits and physical activity are effective in both its prevention and management [9, 11]. Epidemiological studies have revealed that the Mediterranean diet has a positive effect on preventing both obesity and Type-2 diabetes [12, 13].
The Mediterranean diet directly interacts with diabesity and the gut microbiota. In this chapter, the interaction between the Mediterranean diet, Type-2 diabetes - obesity (diabesity) and gut microbiota will be studied.

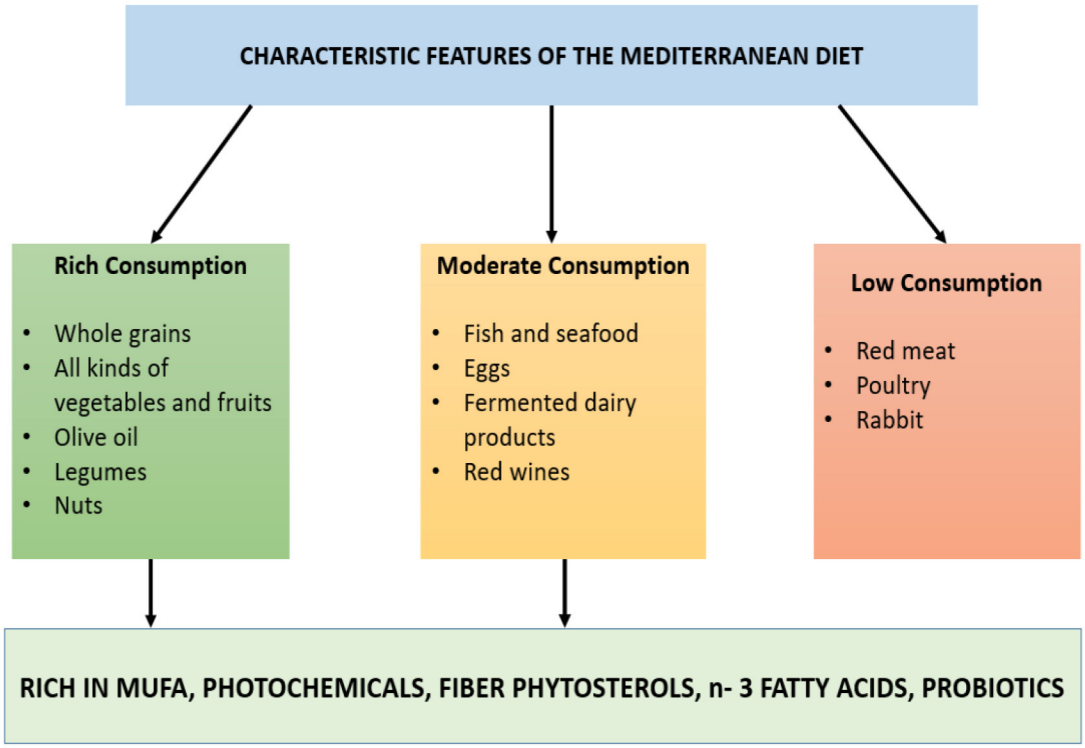
Definition and components of the mediterranean diet model

The Mediterranean diet reflects the taking of balanced and adequate nutrients (carbohydrate, protein, fat, vitamins and minerals) which are rich in plant-based proteins, complex carbohydrates and fiber, monounsaturated fatty acids (MUFA) (n-9) and polyunsaturated fatty acids (PUFA) (n-3) and poor in animal-derived foods. Thus, it positively affects the prevention and management of non-communicable chronic diseases [14, 15]. Figure 1, represents the Mediterranean diet model. This eating model is also environmentally and sustainability friendly, since it involves the consumption of seasonal and local foods [16].
Balanced nutrition and food diversity are the key players in protecting and maintaining a healthy life. Each food group contains different nutrients and bioactive nutrients. The Mediterranean diet is an eating pattern that provides all the food diversity that fulfils the nutritional requirements and keeps the consumers healthy [18, 19]. Nutrient/non-nutrient components are found in high amounts in plant-derived foods and flavouring spices. Consuming these foods known as containing bioactive nutritional components has a potentially positive effect on human health. The Mediterranean model recommends for consumption of high amounts of bioactive food components [20, 21].
Due to the food groups involved; this model also recommends a high to moderate daily consumption of MUFA, PUFA, phytochemicals, fiber, phytosterols and probiotics [22]. The bioactive nutrient components of the Mediterranean diet and its correlation of potential positive effects on health are summarized in Table I.

Gut microbiota

Microbiota is defined as a community of microorganisms living on/in the host whereas microbiome is the entire genome of the microbiota [28]. In a healthy state of the human body, in the concept of microbiota, commensal, symbiotic and pathogenic microorganisms are living within the body in a homeostatic fashion. The balance of the microbiota composition is a key determining factor for the disease and health conditions of the host.

Fig. 1. Mediterranean diet model: Consumption distribution of some food products in the Mediterranean diet (The figure has been created in PowerPoint using the information in [14, 16, 17]).



Tab. I. Bioactive component of Mediterranean Diet (MD).

Component of MD	Bioactive food component/s	Reference(s)
Olive oil	Hydroxytyrosol, Tyrosol, Oleuropein, Oleocanthal, Oleacein, oleic acid.	[23]
Fruits and Vegetables	Carotenoids, Quercetin, Fiber, Vitamin A- C- E, Folate, Se, Lycopene etc.	[17]
Red wine	Resveratrol, Quercetin	[24]
Fish	n- 3 fatty acid	[25]
Seeds	Linolenic acid, vitamin E	[17, 26]
Culinary Herbs and Spices	Quercetin, Securenet, Rosmarinic acid, Chlorogenic acid, Davidigenin	[27]
Dairy products	Calcium, vitamin D, linoleic acid, lactoferrin, lactic-acid-producing bacteria	[24]

There are 10% human and 90% bacterial cells within the human body. This composition together is called the superorganism, with optimal living status [29]. Microbial content varies throughout life in response to environmental and non-environmental factors and is host-specific [30]. Symbiont and pathobiont indicate a balanced distribution in the microbiota of healthy individuals. Dysbiosis is defined as the deterioration of the balance between the symbiont and the pathobiont composition and the increase in pathogenic microorganisms [31]. The most important factors that affect microbial composition can be summarized as phenotype, age, type of birth delivery, physical inactivity, smoking, alcohol consumption and dietary habits [32]. In recent years, the scientific world has focused on studies to clarify the role of gut microbiota in health and diseases. It is believed that the development of dysbiosis causes an increase in intestinal permeability, endotoxemia, energy production (energy harvest), adiposity and pro-inflammatory cytokine production. Thus plays a role in the etiopathogenesis of diseases such as CVD, obesity, diabetes, some cancer types, rheumatoid arthritis, and non-alcoholic fatty liver disease, which are based on systemic inflammation [33, 34]. The human gut microbiota, constitutes 10-100 trillion microbial cells making the largest symbiotic relationship within the host [35].

As well as maintaining intestinal homeostasis, gut microbiota also influences the metabolism, physiology, and immune function within the host [34, 36]. Although, the composition of gut microbiota has a very rapid turnover; in terms of species composition it is fairly stable [37]. Its composition consists of more than 500 species within 6 phyla which are *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria* and verrucomicrobia. Of these bacterial population, 90% accounts for *Bacteroidetes* and *Firmicutes*. On the other hand, proteobacteria and *Actinobacteria* and other pylas of verrucomicrobia, *Cyanobacteria*, *Fusobacteria*, *Spirochaetes*, are scarce compared to *Firmicutes*, *Bacteroidetes* in the colon [38, 39]. Several human diseases are associated with the *Fusobacterium*. Hence, it is generally considered as a pathogenic bacterium. Also, *Firmicutes* and proteobacteria are considered as pathogenic since they negatively affect the glucose and fat metabolism within the gut. In contrast, verrucomicrobia, *Actinobacteria*, and *Bacteroidetes* influence gut health positively by providing a host to become resistant to infectious disease, involving in glucose homeostasis and generation of the short-chain fatty acids (SCFAs) which are known to decrease inflammation [38]. Symbiotic or dysbiotic distribution of intestinal microbial distribution is closely associated with increased disease risk or optimal health. Studies have shown that symbionts such as *Bacteroidetes thethaiotamicron, Bifidobacteria, Lactobacilli, and Faecalibacterium prausnitzii* are dominant in the symbiotic microbiota, while pathobionts such as *Bacteroides spp., Clostridium difficile* are dominant in the dysbiotic microbiota [32]. A dysbiotic distribution of the gut microbiota is closely related with:

- increased intestinal permeability;
- increased endotoxemia (increased LPS production);
- increased pro-inflammatory cytokine secretion;
- increased adiposity;
- increased insulin resistance;
- increased energy harvest.

There is a positive correlation between dysbiosis and increased risk of inflammatory disease due to these metabolic changes [4, 40].

As mentioned earlier, dietary habits are one of the important environmental risk factors that affect microbiota composition. For this reason, recent studies have focused on studies related to gut microbiota - healthy nutrition habits - and decreased prevalence of chronic diseases.

Triple interaction; Gut Microbiota - Diabesity- Mediterranean Diet

Obesity is defined as the increase of adipose tissue in the body to a degree that impairs health [9]. Also, it is the most important risk factor contributing to the development of Type-2 diabetes [41]. Studies have shown that approximately 80% of individuals with Type-2 diabetes are obese. This close relationship between Type-2 diabetes and obesity is related to the lipid overflow, inflammation and adipokine hypothesis. The pathological condition consisting of the combination of these two diseases is called diabesity [10]. According to this hypothesis, a high serum concentration of free fatty acids (FFA) causes increased oxidative stress on pancreatic β-cells and both β-cell apoptosis and defects in insulin receptor signalling. Moreover, the insulin signalling pathway is inhibited by the products of fatty acid metabolism such as diacylglycerols (DAGs), long-chain acyl-CoA esters (LCAEs) and ceramides. It is argued that adipocytokine levels increases due to increased adipose tissue causing the antilipolytic activity of insulin to be inhibited, as well as the coexistence of lipotoxicity and glucotoxicity [10]. The components of the Mediterranean diet described in the previous section (See: Title 2) affect the composition

Tab. II. Effects of Mediterranean Diet Model versus Western Diet Model on Microbial Diversity.

Diet Type	Metabolic Effect	Microbiota Diversity
Adherence to Mediterranean Diet [54, 55]	↓ Oxidative stress, inflammation, immune system function, Endotoxemia, a pro-inflammatory cytokine, Obesity, Type 2 Diabetes Mellitus	↑ *Bacteroides, Lactobacilli, Bifidobacteria, Roseburia* ↓ *Firmicutes, proteobacteria*
Adherence to Western Type Diet [32]	↑Oxidative stress, inflammation, immune system function, Endotoxemia, a pro-inflammatory cytokine, Obesity, Type 2 Diabetes Mellitus	↓ *Bifidobacterium, Eubacterium* ↑ *Firmicutes*

of the gut microbiota. Adaptation to the Mediterranean diet has the potential to prevent the development of obesity and type 2 diabetes by increasing the diversity of the intestinal microbiota and modulating its composition (increased *Bacteroidetes, Lactobacilli*, *Bifidobacteria*, *Faecalibacterium* and decreased *Firmicutes*, proteobacteria). These changes lead to increased microbiota-mediated metabolites, intestinal homeostasis, decreased dysbiosis and decreased intestinal permeability [42].

The role of the microbiota undoubtedly is a key determinant in diabesity. Many animal studies have revealed that gut microbiota composition in healthy individuals compared to diabesity is different [43]. Studies have shown that obese and type 2 diabetic individuals have lower microbial diversity compared to healthy individuals, as well as an increase in the number of *Firmicutes* and a decrease in the number of *Bacteroidetes* [43, 44]. Symbiotic change in the dysbiotic microbial composition of individuals diagnosed with obesity and type 2 diabetes draws attention as a potential treatment method for improving diabesity-related biomarkers. *Sergeev et al.*, revealed that individuals diagnosed with obesity and type 2 diabetes after prebiotic and galacto oligosaccharide supplementation were affected in parameters such as HbA_{1c}, waist circumference, BMI and body weight in parallel with the abundance of *Bifidobacterium* and *Lactobacillus* in their intestinal microbiota [45].

Effects of Mediterranean Diet Model on Gut Microbiota

The potential positive effects of the Mediterranean diet on health are well established. Furthermore, increased adherence to the Mediterranean diet has been associated with the suppression of the growth of pathobionts such as proteobacteria and *Bacillaceae* phyla. Whereas on the other hand promotes the growth of *Bacteroidetes* and beneficial *Clostridium* species in the intestinal microbiota through Mediterranean diet components (MUFA, PUFA, polyphenols, phytosterols and fiber). In contrast to the western-style diet, the Mediterranean diet model (Tab. II) decreases the *Firmicutes*: *Bacteroidetes* in the gut supporting the prevention of chronic diseases such as Type-2 diabetes, obesity, CVD and cancer [46, 47]. There is an inverse relationship between the consumption of MUFAs and PUFAs and the prevalence of diseases such as obesity, Type-2 diabetes, CVD, cancer and hypertension [48, 49]. In addition, high amounts of MUFAs and PUFAs consumption can modulate human health by affecting intestinal microbial composition. It has been reported that MUFAs have a potentially positive effect on the intestinal microbiota by supporting the growth of Lactic acid-producing bacteria (*Bifidobacterium* and *Lactobacillus*) [46]. Similarly, PUFAs can positively affect the intestinal microbiota and human health via a potential suppressive effect on the growth of *Enterobacteria* and support the growth of *Lachnospiraceae* and *Bifidobacteria* and modulate the *Firmicutes*: *Bacteroides* ratio [50]. There is a bidirectional relationship between polyphenols and microbiota, bioactive nutritional components commonly found in vegetables and fruits and one of the main components of the Mediterranean diet, due to the conversion of polyphenols to metabolites that positively affect human health by colonic bacteria and the effects of these metabolites on the colonic microbiota [51]. Effects of polyphenols on microbiota composition: I) stimulating the growth of beneficial bacteria living in the colon, II) inhibiting pathogenic bacteria growth, and III) having positive effects on enterocyte development and integrity [52]. In the study conducted by *Wang et al.*, it was reported that the number of *Bacteroides* increased after the red wine polyphenol resveratrol supplementation, and the symbiotic distribution was supported by this effect [25]. According to *Etxeberria et al.*, the supplementation of quercetin, which is common in vegetables and fruits, causes a change in the composition of the intestinal microbiota. This change was reported as decreasing the *Firmicutes*/*Bacteroidetes* ratio and inhibiting the growth of bacterial species (*Erysipelotrichaceae, Bacillus, Eubacterium cylindroides*) that contribute to the development of obesity [53].

Due to the positive potential effects of polyphenols on the intestinal microbiota composition, studies on this subject need to be continued to provide more precise statements about their potential to be used in the treatment and prevention of Type-2 diabetes and obesity through reduced endotoxemia and inflammation.

Conclusion

The gut microbiota plays an important role in body homeostasis; such that it can modulate, the enteric nervous system and central network system via producing neurotransmitters. Hence the link between human microbiota and diet has led to the development of a "second brain" reputation. Undoubtedly, a bidirectional communication network – the "gut-brain axis" is a bridge between the enteric and central nervous systems [56]. Also, the habit of consuming an unhealthy diet in a way of not favouring the microbiota in the gut is associated with many diseases such as diabetes and obesity; which was the main subject of this chapter. Thus,

to keep the gut microbiota healthy consuming a healthy diet such as the Mediterranean diet is significant - and within the scope of the "second brain" reputation, it is not wrong to state that "you are what you eat".

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Conflicts of interest

The authors declare no conflict of interest.

Author's contributions

S.O.and N.S. have written the chapter under the supervision of T.S. All authors have contributed on literature research. Figure 1 in the manuscript have been designed by S.O. and proofread has been done by N.S. Design of the study have been conducted by T.S.

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Review

Main nutritional deficiencies

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Keywords

Nutrient inadequacies • Nutritional biomarkers • Micronutrient deficiencies • Vitamins • Dietary supplements

Summary

Nutrition is the source of energy that is required to carry out all the processes of human body. A balanced diet is a combination of both macro- and micronutrients. "Nutritional inadequacy" involves an intake of nutrients that is lower than the estimated average requirement, whereas "nutritional deficiency" consists of severely reduced levels of one or more nutrients, making the body unable to normally perform its functions and thus leading to an increased risk of several diseases like cancer, diabetes, and heart disease. Malnutrition could be caused by environmental factors, like food scarcity, as well as disease conditions, like anorexia nervosa, fasting, swallowing inability, persistent vomiting, impaired digestion, intestinal malabsorption, or other chronic diseases. Nutritional biomarkers – like serum or plasma levels of nutrients such as folate, vitamin C, B vitamins, vitamin D, selenium, copper, zinc – could be used for the evaluation of nutrient intake and dietary exposure. Macronutrients deficiencies could cause kwashiorkor, marasmus, ketosis, growth retardation, wound healing, and increased infection susceptibility, whereas micronutrient – like iron, folate, zinc, iodine, and vitamin A – deficiencies lead to intellectual impairment, poor growth, perinatal complications, degenerative diseases associated with aging and higher morbidity and mortality. Preventing macro- and micronutrient deficiency is crucial and this could be achieved through supplementation and food-based approaches.

Introduction

Nutrition is considered as one of the strongest and most adjustable environmental factors that could be used to reduce the burden of disease during an individual's entire life [1]. Appropriate and balanced nutrient's intake and metabolism provide the substrates for the normal physiological functions of the human body [2]. A balanced human diet is based on both macronutrients (e.g. essential fatty acids, carbohydrates, essential amino acids, etc.), which are the major source of energy, and micronutrients (e.g. vitamins, essential minerals, etc.), which are needed for almost all developmental and metabolic processes [3]. Poor nutrition results in an increased risk of several diseases like cancer, diabetes, and heart disease.

Nutritional requirements change during the course of life. For example, during fetal development, infancy, and childhood, the recommended amount of macro- and micronutrients intake is relatively higher with respect to body size. In older individuals, the requirement of certain nutrients (like vitamin D) increases, while other nutrient requirements considerably decrease (like iron and energy) [4].

Other causes of malnutrition can be diseases like anorexia nervosa, fasting, swallowing inability, persistent vomiting, impaired digestion, intestinal malabsorption, or other chronic diseases. Several formulations of food blends are introduced in the diet in order to overcome malnutrition. Malnutrition could also be caused by bad food choices or insufficient food intake [5-9].

Nutritional diseases can cause nutrients deficiencies or excesses, eating disorders, obesity, chronic diseases like cardiovascular diseases, hypertension, diabetes mellitus, cancer, inherited metabolic disorders, developmental abnormalities, food intolerances and allergies [5]. The most prominent nutritional disorder is chronic undernutrition, which affects over 925 million people around the world. Undernutrition is the condition caused by taking insufficient food to fulfil the energy requirement; its main symptoms include weight loss, muscle wasting, body fat wasting, and failure to thrive [8, 9].

Nutrient inadequacies and deficiencies

"Micronutrient inadequacies" are defined as the intake of nutrients in lesser quantity than the estimated average requirement and are prevalent in the United States and other developed countries. These inadequacies mostly occur when the intake of a nutrient is above the deficiency level but below the recommended dietary intake. Unlike micronutrient deficiencies, which lead to clinically evident symptoms, micronutrient inadequacies might cause hidden symptoms that are quite difficult to be identified [10]. Furthermore, an energy-rich and nu-

trient-poor diet might lead to "hidden hunger" condition, which is characterized by micronutrient inadequacies despite having sufficient or excessive amount of calories. These micronutrient inadequacies could ultimately cause various chronic diseases like cancer, osteoporosis, and cardiovascular diseases. In addition, subclinical or marginal micronutrient deficiencies have also been associated with impaired immunity, general fatigue, and cognitive deficits [11, 12].

Several factors contribute to marginal or lower nutrient status, such as poor quality or quantity of food, increased dietary requirements, greater metabolic losses, or decreased gastrointestinal digestion and absorption. Thus, the continuous consumption of reduced nutritional quantity, because of loss of appetite, or poor quality, like unbalanced, restrictive, or low-nutrients diets, increases the risk of developing poor nutritional status [13, 14]. Other factors contributing to the increased nutritional deficiencies risk include: 1) decreased capacity of nutrient absorption caused by gastrointestinal disorders like coeliac disease, inflammatory bowel disease, reduced absorption of vitamin B12 among elderly, etc., 2) poor bioavailability of nutrients, such as low zinc and iron absorption from the plant-based diets, and 3) reduced bioconversion, such as lower provitamin A carotenoids bioconversion into vitamin A from the plant-based diets. In addition, various genetic polymorphisms and the use of certain medication could also increase the risk of specific nutrient deficiencies [15-17].

Nutritional biomarkers

Accurately estimating the nutrients intake is considered a major challenge. Several countries lack their own food composition databases; therefore, they have to rely on data obtained from the food composition databases of other countries, like the U.S. Department of Agriculture (USDA) Nutrient Database [18].

Nutritional biomarkers can be used for evaluating nutrient intake and dietary exposure [19]. Serum or plasma levels of nutrients like folate, vitamin C, B vitamins, vitamin D, selenium, copper, zinc are reported in the National Health and Nutrition Examination Survey (NHANES) [20].

For instance, body iron status cannot be accurately evaluated through a single biomarker. Therefore, several different analyses calculating serum iron concentration, transferrin saturation levels, serum ferritin concentrations, transferrin receptor, and total iron-binding capacity, are used to estimate the real iron status of the body. It is important, however, to recognize the limitations of the biomarkers used: in fact, due to the homeostatic regulation of the blood concentration of nutrients, the circulatory levels of nutrients are not good indicators of their body status. Additionally, not all nutrients have available biomarkers, and they can change in response to infections, inflammation, age, or kidney function [1, 10].

Thus, nutritional biomarkers and dietary surveys are the two most commonly used methods for assessing the population's dietary exposure. Each of these has its own limitations and advantages, but combined they could work well to estimate dietary intake and nutritional status [10].

Macronutrients Deficiency

Protein-energy malnutrition (PEM)

Protein-energy malnutrition (PEM) is a condition in which individuals have a very little dietary intake of proteins, energy or both; it is thus prevalent in developing countries because of insufficient dietary intake. The two major diseases linked with this condition are marasmus, which is complete food deprivation with exceptionally limited quantities of protein and energy, and kwashiorkor, which is characterized by extreme protein deficiency [5, 21, 22]. The World Health Organization (WHO) Global Database on Child Growth and Malnutrition (from 1980-1993) has shown that PEM has affected almost one-third of the children around the world, 80% of which are living in Asian countries [2, 23].

Infants with marasmus are exceptionally underweight, as they have lost almost all their subcutaneous fat. Their body, appearing to be a combination of only bones and skin, is extremely weak and they have higher susceptibility to infections. The major cause of this condition is an extremely low calorie diet intake from all the sources, including protein. If not treated properly, marasmus could lead to death because of starvation [5, 21, 23].

Kwashiorkor is a condition that usually appears in children that, after being weaned from breast milk (containing high-protein), are fed carbohydrate-rich diet sources without sufficient protein intake. The main characteristic of kwashiorkor is the swollen belly, caused by the fluid retention (edema), and affected children are mostly weak, wasted, poorly grown, and more susceptible to infectious diseases [5]. Kwashiorkor suppresses the production of insulin, causing a reduced protein synthesis which leads to hypoproteinemia, immunosuppression, edema, and diarrhea [23].

Marasmus-kwashiorkor could also appear in institutionalized or hospitalized patients that get intravenous glucose for long time like during recovery from illness or surgery, or with diseases causing appetite loss or nutritional malabsorption [5].

Carbohydrates deficiency

Specific cells of human body, like neurons, need high amounts of glucose. In the absence of adequate dietary carbohydrates, gluconeogenesis depends upon the breakdown of amino acids that are obtained from dietary proteins, body proteins, and glycerol obtained from fats [21]. Gluconeogenesis mostly takes place in the liver. Long-term insufficiency of carbohydrate intake could lead to a condition called ketosis (increased ketones production), that is characterized by the peculiar sweet odor of the patient's breath. Ketosis and other complications linked with low carbohydrate intake can be prevented by consuming daily 50 to 100 g of carbohydrate; although, for a healthy and balanced diet, at least about half of the

daily calorie intake should be obtained from carbohydrates, which means a minimum of 250 g of daily carbohydrate intake. The most common sources of carbohydrates in human diet are fruits, vegetables, whole-grain cereals, and legumes, which also give necessary dietary fibers intake [5, 21].

Essential fatty acids deficiency

Omega-3 and Omega-6 are polyunsaturated and essential fatty acids (EFA). Clinical symptoms of EFAs deficiency include diminished growth in children and infants, scaly dry rash, reduced wound healing, and increased infection susceptibility. Omega- 3, 6 and 9 fatty acids compete for the same fatty acid desaturases. One of the prominent biomarkers for essential fatty acid deficiency is omega-9. Furthermore, among the main indicators of EFA deficiency is the plasma eicosatrienoic acid and arachidonic acid (triene:tetraene) ratio higher than 0.2. This condition has been reported in patients suffering from chronic fat malabsorption and cystic fibrosis [24-26].

Several observational and research studies have reported the association of lower omega-3 index with higher risk of mortality by coronary heart disease. Furthermore, in 2016, a meta-analysis established that the supplementation of omega-3 PUFA during pregnancy decreases the risk of premature births and increases the birth weight and gestational age at delivery. Several preparations of omega-3 fatty acid have been approved and recommended by US Food and Drug Administration for hypertriglyceridemia treatment [24, 27-29]. Similarly, replacing other dietary saturated fatty acids (SFAs) with omega-6 PUFA decreases the total blood cholesterol [24, 30, 31].

Micronutrient deficiencies

Micronutrients are crucial for sustaining life. A lower micronutrient consumption than the current Recommended Dietary Allowance Important might lead to chronic metabolic disorders [3]. The inadequacy of any constituent of the metabolic system directly affects both individuals and societies by causing poorer health, reduced work capacity, decreased educational accomplishment, and lower earning potential [1, 32].

In industrialized and developing countries micronutrient deficiencies affect more than 2 billion people of all ages [21], especially pregnant women and children below 5 years of age [1]. Micronutrient deficiencies are linked with almost 10% of children deaths [5, 22]. Iron, folate, zinc, iodine, and vitamin A are among the most occurring micronutrient deficiencies in the world, and all of these contribute to intellectual impairment, poor growth, perinatal complications, and higher morbidity and mortality [1]. In addition, micronutrient deficiencies accelerate mitochondrial decay and degenerative diseases associated with aging [5].

Preventing micronutrient deficiencies is thus crucial and it can be achieved through supplementation and food-based approaches. Micronutrient deficiencies should be determined through reliable and validated biomarkers [1].

Vitamin A deficiency

Vitamin A deficiency is a relatively frequent nutrient deficiency in developing countries and it primarily causes ophthalmologic diseases. In fact, vitamin A is crucial for preserving the integrity of epithelial tissues in the eye and in the urinary, intestinal, and respiratory tracts. The initial clinical symptoms of vitamin A deficiency include xerophthalmia, Bitot spots development, and night blindness. With the progression of the vitamin A deficiency, keratomalacia and permanent blindness may take place. Furthermore, children with vitamin A deficiency may also exhibit protein energy malnutrition [33].

According to the estimation of the WHO, almost 70-80 million children around the world suffer from subclinical vitamin A deficiency, apparently without clinical symptoms. In subclinical vitamin A deficiency, children have higher infection susceptibility and reduced physical growth [33, 34].

B vitamins deficiencies

Vitamin B6

Vitamin B6 is water-soluble and can be obtained from different foods and supplements. It mainly exists in three forms (pyridoxine, pyridoxamine, and pyridoxal), which are biologically active in their phosphorylated forms. Vitamin B6 acts as a coenzyme for several enzymes, such as those essential for amino acids decarboxylation and transamination, neurotransmitter synthesis, fatty acid metabolism, and tryptophan conversion to niacin. Vitamin B6 deficiency is not very common, but can occur due to insufficient dietary intake, malabsorption, and use of certain medications. Malnourished, elderly, and anorexic individuals are at higher risk of developing vitamin B6 deficiency. Alcoholics are also at higher deficiency risk, since they have poor vitamin B6 dietary intake and alcohol increases its catabolism. Vitamin B6 deficiency causes anemia, peripheral neuropathy, seborrheic dermatitis, glossitis, cheilosis, depression, celiac disease, Crohn disease, and seizures. Medications can bind vitamin B6 and increase its excretion or reduce its enzymatic activity [2].

Vitamin B12

For humans, animal products like dairy and meat are the only dietary source of vitamin B12. Although currently the incidence of vitamin B12 deficiency in the United States is not fully known, the NHANES III (1991-1994) reported a frequency of 1 in 200 children of 4-19 year age with decreased levels of vitamin B12 (< 200 pg/mL). Clinically, vitamin B12 deficiency is uncommon among infants or children without any predisposing factors. In early stages, cobalamin deficiency is secondary to the maternal deficiency among breast-feeding mothers following strict or moderate vegan diets. The main causes of vitamin B12 deficiency mostly are inadequate intake, inborn errors of metabolism or transport, and malabsorption. Elder individuals, people with psychiatric illnesses,

vegans, and their breastfed infants are at higher risk of vitamin B12 deficiency because of the insufficient intake [2, 35].

Folate deficiency

Vitamin B9 (folic acid, folate) and Vitamin B12 have several closely linked functions. Folate is crucial for purines and thymidylate synthesis and plays a significant role in DNA synthesis, repair and stability. Folate is also involved in carbon metabolism and changes in DNA methylation pattern. Folic acid deficiency can be caused by excessive consumption of alcohol, since it impairs vitamin absorption. Individuals with folate deficiency exhibit fatigue and weakness caused by megaloblastic anemia. In pregnancy, folate deficiency is linked with preterm delivery, low birth weight, retardation of fetal growth, and neural tube defects. Moreover, folic acid supplementation during the periconceptional period decreases the incidence of neural tube defects [1, 5, 21].

Vitamin C deficiency

Vitamin C (ascorbic acid) is considered an essential nutrient and is mainly derived from diet; its deficiency causes scurvy and could also lead to behavioral and mood changes [2]. In the first 3 months of ascorbic acid deficiency, the symptoms are bleeding gums, petechiae, ecchymoses, hyperkeratosis, coiled hairs, impaired wound healing, and arthralgias [33]. Vitamin C plays a significant role also in osteoblast and osteodentin formation, carnitine synthesis, catecholamines synthesis, reductions of urinary folic acid excretion, and higher dietary iron absorption.

Humans are incapable of synthesizing vitamin C, therefore they totally depends upon dietary vegetables and fruits for its sufficient intake and storage. Good vitamin C sources can be citrus fruits, tomatoes, strawberries, potatoes and green leafy vegetables [2]. Furthermore, vitamin C deficiency is observed in growing infants exclusively fed with cow milk or formula and in children with neurodevelopmental disabilities. Pharmacological doses of vitamin C are known to resolve the symptoms [33, 36].

Vitamin E deficiency

Vitamin E comprises a group of eight fat-soluble compounds, the most important of which is tocopherol. Vitamin E protects against the free radical damage associated with chronic diseases. Vitamin E deficiency-associated disorders are not very common. Vitamin E deficiency can occur in individuals with fat malabsorption or specific genetic disorders, like Friedreich ataxia and abetalipoproteinemia. Research data have shown that vitamin E may prevent diseases like diabetes, atherosclerosis, ischemic heart disease, Alzheimer, cataracts, and Parkinson. Studies have also reported the protective effect of vitamin E against cancer [33, 37].

The major characteristics of vitamin E deficiency include ataxia, myopathy, and pigmented retinopathy, like retinitis pigmentosa with vision loss. At the later stages of vitamin E deficiency, sensory motor neuropathy takes place, with loss of position, vibration sense and reflexes, and generalized weakness [33, 38].

Vitamin K deficiency

Vitamin K deficiency leads to coagulation disorder, which manifests by increased prothrombin time and international normalized ratio, with normal levels of fibrinogen and platelets. In newborns, vitamin K deficiency is called “hemorrhagic disease of the newborn”. Early vitamin K deficiency at birth (VKDB), which manifests within the first 24 hours after birth, usually affects infants whose mothers during pregnancy have been taking medications that inhibit vitamin K metabolism. Usually, at-risk infants have 6-12% chances of developing VKDB without being administered vitamin K at birth. Late VKDB, on the other hand, is linked with babies fed only with breast milk and it starts between 8 days to 6 months of age. Usually, the clinical manifestation of late VKDB is quite severe, with a mortality rate ranging between 20 and 50% of infants undergoing intracranial hemorrhage. Infants with malabsorptive syndrome or cholestasis are at higher risk. A single intramuscular dose of vitamin K at birth in wholly breast-fed infants seems to prevent the development of late VKDB, but in order to prevent this condition it is advisable to also repetitively add vitamin K oral administration [33, 39].

Vitamin D deficiency

Vitamin D, also called calciferol, is a fat-soluble secosteroid that is essential for the intestinal absorption and metabolism of calcium, magnesium and phosphorous. Vitamin D stimulates the osteoclasts to release calcium and phosphorus, and activates the synthesis of enterocyte calcium channel, which enhances the absorption of calcium. Vitamin D can be acquired through dietary sources, like ergocalciferol/vitamin D2, or endogenously synthesized, like cholecalciferol/vitamin D3. Vitamin D sources are fish and fish oils (which contain the highest proportion of available vitamin D), eggs, shiitake mushrooms, liver, and fortified foods (like orange juice and milk) [2]. Vitamin D deficiency causes hypocalcemia and hypophosphatemia, which lead to osteomalacia among adults and rickets among children. Furthermore, vitamin D deficiency is associated with immunomodulatory disorders, cardiovascular diseases, hypertension, and insulin resistance in adults [2, 40].

Vitamin D deficiency depends upon various dietary and environmental factors, such as body mass index, sun exposure, and milk ingestion. Usually, vitamin D deficiency occurs due to its malabsorption, decreased synthesis and reduced dietary intake [41]. In several countries, including North America, cow milk, infant formulas, and different cereals are supplemented with vitamin D [33, 42]. Long-term and short-term therapy of vitamin D deficiency mainly involves vitamin D supplementation and implementation of vitamin D- and calcium-rich diets. In order to solve vitamin D deficiency, for most people over 1 year of age it is considered sufficient to supplement 50,000 IU of ergocalciferol for at least eight weeks [2, 43].

Calcium deficiency

Calcium deficiency or hypocalcemia is characterized by low levels of serum calcium. A long-standing calcium deficiency could lead to cataracts, dental changes, brain alterations, osteoporosis, and rickets. Sufficient calcium intake is crucial throughout life to maintain bone health [44]. In several parts of the world, rickets caused by calcium deficiency is still present. A double-blind, randomized controlled study of 123 Nigerian children suffering from rickets showed that the baseline calcium intake of these children was low, almost 200 mg/d. Moreover, these children reacted better to calcium treatment alone or combined with vitamin D rather than vitamin D alone [45, 46]. In addition, certain diseases and specific diets, like vegetarian diets, might cause calcium deficiency. Calcium supplementation is required also in inflammatory bowel disease patients, particularly those administered with corticosteroids/glucocorticoids [33, 47].

Iron deficiency

Iron deficiency is the most prevalent nutritional deficiency, with young children and premenopausal women at the highest risk of iron deficiency [21, 22]. Being iron a major contributor in hemoglobin synthesis, depletion of its reserves leads to microcytic hypochromic anemia, characterized by smaller-size red blood cells containing a smaller amount of hemoglobin than normal red blood cells. Symptoms of anemia include fatigue, apathy, paleness, weakness, breathing difficulty upon exertion, and decreased resistance for cold temperatures [21]. Iron deficiency could affect development, behavior, learning abilities, and growth during childhood. Severe anemia due to iron deficiency may also increase risks of complications during pregnancy and maternal death. Iron deficiency is mostly caused by insufficient dietary iron intake, blood loss during menstruation, intestinal blood loss, or blood loss due to hookworm, tumors, hemorrhoids, and regular use of drugs like aspirin [5, 21, 22, 48, 49].

Iodine deficiency

Iodine is a trace element that plays a major role in thyroid hormone synthesis. Thyroid hormone is essential for regulating human development and growth. Iodine is naturally present in some foods and is also used as a dietary supplement, added to salt, or used in organic form. Physiologically, iodine reserves are estimated to be 60 μg; on the other hand, during deficiency, the reserves are as low as 10-20 μg [50]. Absorption and utilization of iodine can be impaired due to goitrogens presence or exposure to disulfides, thiocyanates, and percolate [51]. Reduced dietary intakes of iodine (10-20 μg daily) may result in hypothyroidism, followed by goiter. Thyroid hormone is necessary for optimum fetal and postnatal development and growth of the central nervous system [52, 53]. During the early stages of pregnancy, maternal iodine deficiency may lead to iodine deficiency disorder, which causes permanent neurological damage and mental retardation in the offspring [53]. From the embryonic stage to adulthood, iodine deficiency disorder includes diminished mental functions, goiters, cretinism, and hyper- or hypothyroidism. Infants and pregnant women are at the highest risk of developing iodine deficiency [1]. Universally, salt iodization is the most efficient and practical strategy used to reduce global iodine deficiency [1, 54].

Zinc deficiency

Zinc is a trace mineral that is essential for health and is associated with cellular metabolism. Zinc is needed for the proper functioning of over 200 enzymes and is crucial for normal growth and development, immune system function, DNA and protein synthesis, and cell division [55]. Since human body cannot store zinc for long, constant dietary intake of zinc is required to maintain normal functions. Primarily, zinc is found in seafood, animal products, and human breast milk. Zinc absorption is greatly impaired by lignins, phytates and fiber, which reduce the bioavailability of zinc from non-animal sources [1, 56]. Severe zinc deficiency has been reported in patients fed with intravenous solutions lacking zinc and in people suffering from hereditary zinc metabolic disorders like acrodermatitis enteropathica. Zinc deficiency symptoms can include skin lesions, increased susceptibility to infection, diarrhea, poor appetite, night blindness, reduced taste and smell acuity, hair loss, low sperm count, impotence, and slow wound healing [5, 22]. In the developing countries, zinc deficiency is considered one of the main causes of morbidity [57]. Furthermore, zinc supplementation during pregnancy is linked with a substantial decrease in the number of preterm births without affecting the infants' birth weight [58, 59].

Magnesium deficiency

In humans, magnesium deficiency is linked with colorectal cancer, osteoporosis, hypertension, metabolic syndrome, and diabetes. In human primary cell cultures, magnesium deficiency results in mitochondrial DNA damage, increased telomere shortening, activation of cell-cycle arrest proteins, and premature senescence. Common magnesium sources include beans, green leafy vegetables, nuts and whole grains. In rats, magnesium deficiency can cause chromosome breakage and cancer [3, 60, 61]. Magnesium blood levels are strongly controlled and therefore could not be used for the assessment of the nutritional status of magnesium [62, 63].

Selenium deficiency

Selenium is an essential trace mineral that is mostly found in certain foods, soil and water. Selenium acts as a cofactor of many enzymatic reactions, also playing role in redox function, production of active thyroid hormone, and immune function. Selenium also constitutes selenoproteins such as glutathione peroxidase, thioredoxin reductase, and selenoprotein-P. Nutritional selenium deficiency happens in regions with reduced selenium content in soil. Serious selenium deficiency could lead to Keshan disease, an endemic cardiomyopathy, and Kashin-Bek disease, a deforming arthritis. Moreover, selenium deficiency has a negative impact on spermato-

genesis, immunocompetency, thyroid function, cardiovascular diseases, and mood swings [32, 64].

Potassium deficiency

According to US Dietary Guidelines 2015 to 2020, potassium is considered as a nutrient of public health concern, since it is not sufficiently consumed by the US population. Furthermore, US national surveys have reported that the majority of American population do not take the recommended amount of potassium. NHANES survey 2011-2012, concerning 4,730 subjects, has revealed that less than 3% of US adults had a potassium intake over the required amount, 4,700 mg/day [44]. The main potassium sources include vegetables and fruits [65].

Fluoride deficiency

Fluoride plays a significant role in the mineralization of teeth and bones and protects them from tooth decay. Several US epidemiological studies (1930s to 1940s) have reported an inverse relationship of the amount of natural fluoride in water with dental caries rate. Areas with low fluoride levels in drinking water are prescribed to add fluoride supplements for children over 6 months of age; in those regions dentists recommend fluoride rinses or gels to be periodically applied to their patients' teeth. Fluoridated toothpastes are significant fluoride source for both children and adults, providing a continuous fluoride intake [5, 21, 22]. Antacids containing aluminum could decrease fluoride absorption. Therefore, it is recommended to take these antacids two hours before or after fluoride supplementation [66].

Biotin

Biotin is an essential cofactor of four carboxylases: pyruvate carboxylase, acetyl-CoA carboxylase, 3-methylcrotonyl-CoA-carboxylase, and propionyl-CoA-carboxylase. Biotin is both synthesized by the intestinal bacteria and obtained from dietary sources. Sources of dietary biotin are eggs, walnuts, liver, mushrooms, peanuts, soybeans, and cow milk. Since biotin is both available from dietary sources and synthesized by the human microbiota, acquired biotin deficiency is very rare. Most of the acquired biotin deficiencies are linked with excessive intake of raw egg whites, gastrointestinal malabsorption, extended parenteral nutrition, and extended use of anticonvulsants. Biotinidase deficiency leads to metabolic acidosis, conjunctivitis, ataxia, organic aciduria, developmental delay, encephalopathy, sensorineural hearing loss, seizures, periorificial dermatitis, and alopecia [2, 67].

Causes of micronutrient deficiencies

Micronutrient deficiencies can be caused by either insufficient intake or impaired absorption, which ca be due to infections or chronic inflammation. In infants, micronutrient deficiencies are caused by maternal micronutrient deficiencies in utero or due to rapid postnatal growth [1, 68, 69].

Low- or middle-income countries have the highest micronutrient deficiencies burden; however, some micronutrient deficiencies can also exist in certain subpopulations of high-income countries [1]. Lactation and pregnancy also increases the requirements of macro- and micronutrients. An inadequate nutritional intake of pregnant mothers may lead to insufficient nutritional levels in infants and children, thus causing stunting, infection susceptibility, and developmental delays [70, 71]. Nutrient deficiency can also be caused by selective diets: any diet that completely excludes a specific food group is potentially inadequate for macro- and micronutrient intake. For example, vegan diets exclude all animal-based foods, thus leading to an increased risk of causing B vitamins deficiency. Similarly, various energy-limiting diets used for weight loss may cause an increased micronutrient deficiency risk [66, 72]. Chronic alcoholism causes depletion of the liver reserves of vitamin A and might contribute to alcohol-induced cirrhosis [66, 73]. To break the malnutrition cycle, it is critical to make an intervention during the first malnutrition 1,000 days; however, a well-coordinated and sustainable commitment is required to increase the global nutrition levels. To achieve this goal, it is critical to better understand the epidemiology of micronutrients deficiencies and to select the best-suited interventional strategies [1].

Nutrient deficiency screening test

An important requirement for screening individuals that are at risk of specific nutrient deficiency or inadequacy is the availability of an accurate and suitable test, with sufficient specificity and sensitivity. In order to determine the nutritional status of the majority of minerals and vitamins, blood, saliva, and urine-based biomarkers are available, using nominally invasive sampling. These biomarkers can detect particular nutrient deficiencies at an early stage, before symptoms development. Thanks to technology advancement, sensitive methodologies are being developed that measure the nutritional status of omega-3 polyunsaturated fatty acid by simple blood draw, whereas in some countries only finger-prick blood tests are available [74].

Several advanced screening tools and techniques have been established for the identification of patients or elderly at risk of protein or calorie malnutrition. Some of the biomarkers that reveal general malnutrition include body mass index, total cholesterol, and hemoglobin. Another approach to evaluate the risk of certain nutrients insufficient intake is using validated dietary questionnaires. However, usually these dietary questionnaires are time-consuming and not sensitive enough [75].

Importance of dietary supplements

Mineral and vitamin supplements are the most frequently used kinds of dietary supplementation worldwide. [76] National Health and Nutrition Examination Survey

(NHANES) data revealed that, despite their micronutrient intake from all sources (like enriched and fortified foods), almost 90% of the US adult population takes less than the estimated average requirement for vitamins D and E, 51% for vitamin A, 43% for vitamin C, 61% for magnesium, and 49% for calcium. Besides, only 39% and 2% of the adult US population had respectively potassium and vitamin K intakes over the recommended adequate intake. Low micronutrients intake is also prevalent in children of 2 to 18 years of age, particularly for minerals like magnesium and calcium, and for vitamins D, E and K [77, 78]. Usage of multivitamin/multimineral supplements is frequent in the USA. Data from NHANES 2011 to 2012 establish that almost 31% of adults from USA take multi-vitamin/multi-mineral supplements because they contain at least 10 micronutrients [79]. Generally, dietary supplements usage is more common in non-Hispanic whites, females, elderly people, and educated individuals [80]. Several research studies have reported that using multi-vitamin/multi-mineral supplements is linked with greater micronutrient intake, thus suggesting they could help filling nutritional gaps and improving the nutrient adequacy among populations. In comparison with the micronutrient intake from food source alone, using multi-vitamin/multi-mineral supplements is also associated with decreased prevalence of several "shortfall" nutrients inadequacies, such as iron, magnesium, calcium and vitamins A, C, D and E (NHANES 2009-2012) [81]. Afshin et al. in their research study established that diet improvement could potentially prevent one fifth of deaths worldwide [80]. Furthermore, the American Academy of Pediatrics proposed that all adults, infants, and children should take 400 IU of vitamin D supplementation, 400 μg/day of vitamin B supplementation and 400 mg of vitamin C supplementation daily [77, 82].

Conclusion

Nutritional deficiencies not only cause developmental failure, loss of various body functions, and several other diseases such as diabetes, vision loss, immunity loss, and cancer, but it also has several long-term effects on economic productivity. The major causes of nutritional deficiencies are insufficient intake of food, inability to absorb nutrients, and consumption of diets that lack some of the essential nutrients. Micronutrient deficiencies are the most prevalent type of nutritional deficiencies, usually caused by the insufficient intake of one or more of the micronutrients that are essential to maintain optimal health. Some of these essential micronutrients are iron, iodine, calcium, zinc, magnesium, fluoride, and vitamins A, B6, B12, C, D, E and K. Macro- and micronutrient deficiencies may cause several serious diseases, like goiter, mental retardation, acute respiratory infections, decreased cognitive function, cancer, vision loss, rickets, pellagra, beriberi, and diarrhea. Dietary supplementation is one of the major solutions for managing micronutrient deficiencies, as it can increase the under-consumed nutrients intake within a population and fill the nutritional gaps.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; AKK, Kristjana D, Kevin D, BA, VV, GM, AI, STC, FB, PG: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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Review

Clinical assessment for diet prescription

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Keywords

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Summary

Accurate nutritional assessment based on dietary intake, physical activity, genetic makeup, and metabolites is required to prevent from developing and/or to treat people suffering from malnutrition as well as other nutrition related health issues. Nutritional screening ought to be considered as an essential part of clinical assessment for every patient on admission to healthcare setups, as well as on change in clinical conditions. Therefore, a detailed nutritional assessment must be performed every time nutritional imbalances are observed or suspected. In this review we have explored different techniques used for nutritional and physical activity assessment. Dietary Intake (DI) assessment is a multidimensional and complex process. Traditionally, dietary intake is assessed through self-report techniques, but due to limitations like biases, random errors, misestimations, and nutrient databases-linked errors, questions arise about the adequacy of self-reporting dietary intake procedures. Despite the limitations in assessing dietary intake (DI) and physical activity (PA), new methods and improved technologies such as biomarkers analysis, blood tests, genetic assessments, metabolomic analysis, DEXA (Dual-energy X-ray absorptiometry), MRI (Magnetic resonance imaging), and CT (computed tomography) scanning procedures have made much progress in the improvement of these measures. Genes also plays a crucial role in dietary intake and physical activity. Similarly, metabolites are also involved in different nutritional pathways. This is why integrating knowledge about the genetic and metabolic markers along with the latest technologies for dietary intake (DI) and physical activity (PA) assessment holds the key for accurately assessing one's nutritional status and prevent malnutrition and its related complications.

Introduction

In the present advanced world, nutritional research mainly focuses on improving individual as well as population health through diet management. Health- and Nutrition-linked researcher have established that, along with their essential functions, both the nutrients and the non-nutrient food components interact with various metabolic pathways, thus influencing health and increasing or decreasing the risk of various diseases [1].

The precise assessment of the dietary intake (DI) as well as of physical activity (PA) is crucial for quality research in the areas of nutrition, public health, and exercise science [2]. Nutritional screening ought to be considered as an essential part of clinical assessment for every patient on admission to healthcare setups, as well as on change in clinical conditions. Therefore, a detailed nutritional assessment must be performed every time nutritional imbalances are observed or suspected. However, the differentiation between such procedures is quite subtle, especially because of the identification of significant prognostic clinical processes that are interlinked both with each other as well as to the nutritional status, like sarcopenia and frailty [3].

Several nutritional assessment and screening tools have been proposed, but none of them are truly comprehensive. However, among those assessment tools, the multidimensional ones seem to be more informative. Some of these tools are age-specific; like certain assessment tools that have been tailored specifically for older people. Moreover, in certain cases applying biochemical parameters (i.e. blood tests, genetic assessments and metabolomic analysis) might be considered significant and their extra costs should be compensated by the useful information they provide as nutrition marker for different perspectives, like nutritional status assessment, malnutrition grading, prognosis and refeeding effectiveness (Tab. I) [4].

Dietary Intake (DI) assessment in healthy adult population is a multidimensional and complex process that makes an accurate quantification somewhat challenging. Traditionally, DI is assessed via self-report techniques including diet records, FFQs (food frequency questionnaires), and recalls [5]. These self-assessment or self-reporting techniques have been known to underestimate the caloric intake by almost 11 to 35% (mostly in obese people) as compared to direct measuring techniques, such as doubly labeled water [6]. Reporting er-

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Tab. I. Biochemical values to detect malnutrition and monitor nutritional status [8].

Biochemical values	Nutrition Independent Factors	Half-Life	Appropriateness to Detect Malnutrition
Albumin	Increased dehydration, Decreased inflammation, Infections, Trauma, Heart failure, Edema, Liver dysfunction, Nephrotic syndrome	20 d	+/++ Not appropriate in case of anorexia and acute illness
Transferrin	Increased renal failure, Iron status, Acute hepatitis, Hypoxia	10 d	+
Prealbumin/Transthyretin (TTR)	Increased renal dysfunction, Dehydration, Corticosteroid therapy Decreased inflammation, Hyperthyreosis, Liver disease, Overhydration	2 d	Not appropriate to detect anorexia Subnormal values within one week when fasting
Retinol bindingprotein (RBP)	Increased kidney failure, Alcohol abuse, Decreased hyperthyreosis, Chronic liver diseases, Vitamin A deficiency, Selenium deficiency	12 h	Idem prealbumin
Insulin-like growth factor 1 (IGF-1)	Increased kidney failure, Decreased liver diseases, Severe catabolic status, Age	24 h	++ Rapid decrease in fasting periods
Urinary creatinine	Increased collection time > 24h, Infection, Trauma, Decreased and insufficient collection time, Acute kidney failure	-	1 mmol of creatinine is derived from 1.9 kg of skeletal muscle mass
Lymphocytes	Increased healing phase after infection, Hematologic diseases, Decreased sepsis, Immune suppressants, Steroids	-	+ Very unspecific

rors including biases (also called "systematic errors"), random errors, mis-estimations, and nutrient databases-linked errors are the source of some of the current criticisms, which leads to questioning the adequacy of self-reporting dietary intake procedures for scientific conclusions about the relationship between dietary intake and health [7]. Additionally, the Malnutrition Universal Screening Tool (MUST) (Tab. IIa, IIb) was developed to identify malnourished individuals within all care settings (like nursing homes, hospitals, home care, etc.). MUST was the basis of the NRS-2002; however, since it did not include the last food intake, the weight loss percentage calculations might be tedious and create an obstacle for the busy staff of healthcare wards [8]. The most recent studies have thus suggested that dietary intake should be assessed using novel and improved methods, suitable to apply in independently living individuals (like biomarkers, digital photography, or remote sensing devices), instead of solely relying on self-reporting methods [2].

Tab. IIa. The Malnutrition Universal Screening Tool (MUST).

BMI (kg/m^2)	Unintentional weight loss in the past 3–6 months	Acute illness with reduced food intake (estimated) for ≥ 5 days
≥ 20 0	≤ 5% 0	No = 0
18.5-20.0 1	5-10% 1	Yes = 2
≤ 18.5 2	≥ 10% 2	

Tab. IIb. Overall Risk for Malnutrition.

Total	Risk	Procedure	Implementation
0	Low	Routine clinical care	**Clinic:** weekly **Nursing home:** monthly **Outpatient:** yearly in at-risk patient groups, e.g., age > 75 years
1	Medium	Observe	**Clinic, nursing home, and outpatient:** Document dietary intake for 3 days. If adequate: little concern and repeat screening (hospital weekly, care home at least monthly, community at least every 2-3 months). If inadequate: clinical concern. Follow local policy, set goals, improve and increase overall nutritional intake, monitor and review care plan regularly.
≥ 2	High	Treat	**Clinic, nursing home, and outpatient:** Refer to dietitian, Nutritional Support Team, or implement local policy. Set goals, improve and increase overall nutritional intake. Monitor and review care plan (hospital weekly, care home monthly, community monthly).

Additionally, the availability of various diagnostic tools is another important issue or limiting factor to overcome in clinical practice. Particularly for muscle mass assessment, in spite of the precise result of DEXA (Dual-energy X-ray absorptiometry), MRI (Magnetic resonance imaging), and CT (computed tomography) scanning procedures, noninvasive, bed-side and low-cost techniques like BIA (bioelectric impedance analysis) are still considered as an ideal solution for the routine usage and extensively used. Besides, in the absence of these instrumental methods, anthropometric measurements like calf or mid upper-arm circumference could be adequate substitutes [9].

Moreover, many subjective as well as objective methods of dietary intake (DI) and physical activity (PA) assessment exist, each of which has its own biases and limitations (Fig. 1) [2]. Besides, even though nutritional assessment and screening should be easy and quick procedures, increasing evidences are suggesting that more time should be devoted to them [3].

Assessment of nutritional status by dietary intake

Dietary intake assessment has been performed using several objective as well as subjective tools, each having its own inherent limitations and strengths. Hence, the selection of the appropriate tool for the research mostly depends upon nutrients of interest, study design, target population, availability of time and economic resources. Some limitations hinder the capability of self-report dietary intake (DI) measures to reach scientific conclusions about the relationship between dietary intake (DI) and the health outcomes. However, the traditional DI assessment methods like diet records, FFQs and recalls remain the main choice because of their familiarity, cost efficiency and the lack of consensus upon other objective methods that are capable of producing the complex required outcomes [10].

Latest advancements in technology have resulted in the development of many automated tools for dietary assessment that can overcome some of the limitations of traditional subjective tools, in addition to strive for time and cost efficiency. Such advanced assessment methods include automated self-administered 24-hour dietary assessment tool (ASA24) and food records [11], photo-assisted dietary assessments (PADAs) [12], image-based dietary assessments (IBDAs), and graphic and automated food frequency questionnaires (FFQs) [2, 13].

Automated Self-Administered 24-hour (ASA24)

The National Cancer Institute (NCI) introduced an upgraded version of the USDA's (U.S. Department of Agriculture) Multiple-Pass 24-Hour Recall Method that enables automated self-administered 24-hour recalls (ASA24) by the respondent and could be used for several days to maintain food record. Automated self-administered 24-hour recalls (ASA24) overcome the limitations of traditional 24-hour recalls like reduced time, independence from trained interviewers, reduced respondent burden and reduced economic burden for researchers. In order to automate the ASA24 tool, several novel self-administered web-based food frequency questionnaires (FFQs) have been developed, like Nutrition Quest's NCIB lock questionnaire, Fred Hutchinson Cancer Research Center FFQs, and NCI's Diet History Questionnaire (DHQ) III. All of these are web-based

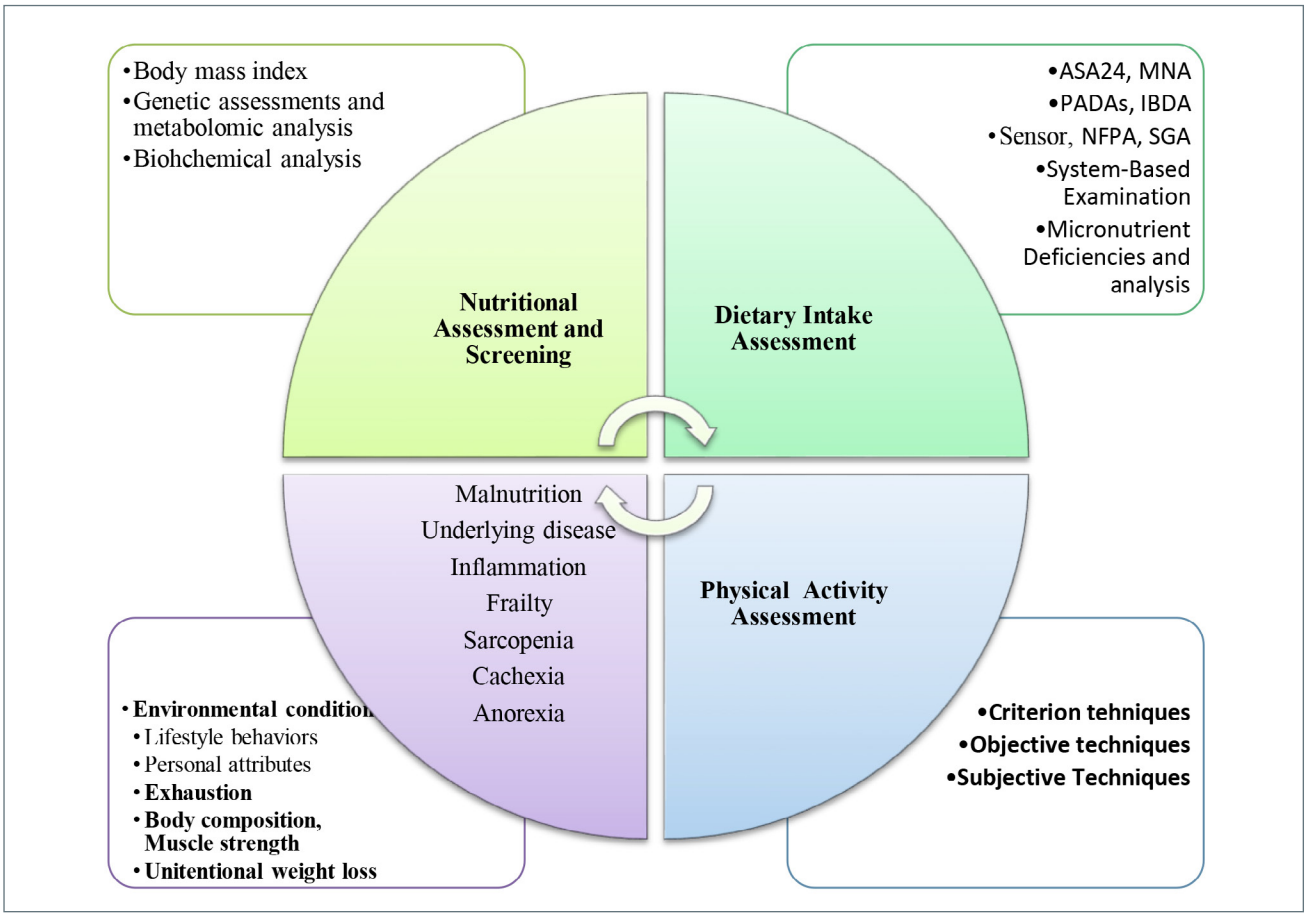

Fig. 1. Steps and Factors involved in Nutritional Assessment and Screening.

questionnaires and comprise over 100 questions concerning food items and their purchasing and preparation, with different layout designs and analytical techniques. Some of these, like NCI's Diet History Questionnaire (DHQ) III, are also freely available for researchers. Another novel alternative, VioScreen, provides a graphical FFQ option, hence addressing the limitations of the traditional FFQs [2, 14, 15].

Nutritional risk screening (NRS 2002)

Globally, one of the most commonly used nutritional risk screening and evaluating tools for hospitals is the NRS-2002 (Tab. III), developed by Kondrup et al. as a generic tool for the hospital setup to be used for detecting patients who could benefit from nutritional therapy [16]. Recently, NRS-2002 was presented in large multi-centric randomized controlled study involving medical inpatient population; the results of the study established a decrease of significant clinical outcomes like mortality in patients that were evaluated by NRS-2002 and found to be at malnutrition risk. NRS-2002 is a well-validated and simple tool, incorporating the preliminary screening via four questions. If the answer to any of these questions is positive, the patient will undergo a complete screening that includes alternate measures of their nutritional status, with static as well as dynamic parameters along with data about the severity of the disease [8].

Mini Nutritional Assessment (MNA)

For other care settings – such as outpatient, community, institutions, rehabilitation and subacute – their evaluation should be carried out using Mini Nutritional Assessment (MNA), on the bases of the amount of data collected. Even the short-version MNA has been validated and optimized as full-assessment procedures, that identifies three categories of nutritional status even in patients without possibility of BMI measurement (measuring circumference of calf as an alternative) (Tab. IV). Hence, this nutritional assessment tool is designed to be faster and easier to complete, minimizing the requirement of patient's participation as well as the quantity of unanswered questions, also enabling a wider distribution between healthcare professionals. Thus, all at-risk patients must undergo a complete nutritional assessment to evaluate the presence of any malnutrition [3, 17].

Photo-Assisted Dietary Assessments (PADAs)

Photo-Assisted Dietary Assessments (PADAs) involve images of the food selections and any remaining food after the meal for dietary intake estimation and might be an efficient and unobtrusive method of DI assessment among large groups of independent individuals. Mostly, they have been used to assess the DI of military recruits during their basic training, younger adults, disabled individuals, and overweight or obese females.

Tab. III. Nutritional Risk Screening (NRS-2002).

Sr. No	Preliminary Screening	Yes	No
1	Is the BMI of the patient < 20.5 kg/m^2	-	-
2	Did the patient lose weight in the past 3 months?	-	-
3	Was the patient's food intake reduced in the past week?	-	-
4	Is the patient critically ill?	-	-

If yes to one of those questions, proceed to screening. If no for all answers, the patient should be re-screened weekly.

Screening					
Impaired nutritional status		Score	Severity of the disease		Score
Normal nutritional status	Absent	0	Normal nutritional requirements	Absent	0
Weight loss > 5% in 3 months OR 50-75% of the normal food intake in the last week	Mild	1	Patient is mobile Increased protein requirement can be covered with oral nutrition Hip fracture* Chronic patients, in particular with acute complications: cirrhosis*, COPD*, chronic hemodialysis, diabetes, oncology	Mild	1
Weight loss > 5% in 2 months OR BMI 18.5-20.5 kg/m^2 AND reduced general condition OR 25-50% of the normal food intake in the last week	Moderate	2	Patient is bedridden due to illness Highly increased protein requirement, may be covered with ONS Major abdominal surgery* Stroke* Severe pneumonia Hematologic malignancy	Moderate	2
Weight loss > 5% in 1 month OR BMI < 18.5 kg/m^2 AND reduced general condition OR 0-25% of the normal food intake in the last week	Severe	3	Patient is critically ill (intensive care unit) Very strongly increased protein requirement can only be achieved with (par)enteral nutrition Head injury* Bone marrow transplantation* Intensive care patients (APACHE > 10)	Severe	3
-		Total A	-		Total B

Age: < 70 years: 0 pt; ≥ 70 years: 1 pt

Grand Total = (A) + (B) + Age
≥ 3 points: patient is at nutritional risk, a nutritional care plan should be set up.
< 3 points: repeat screening weekly.
NRS-2002 is based on an interpretation of available randomized clinical trials.
* indicates that a trial directly supports the categorization of patients with that diagnosis.

PADAs methods include both the traditional methods as well as advanced technologies of digital photography and remote food photography plus recalls, both of which validate direct energy measurement in different population and environment extremities. Their major limitations include lack of the fully automated nutrient analysis after capturing the photo as well as the nutrient database quality used for analysis [2, 18, 19].

Image-based dietary assessment (IBDA)

Image-based dietary assessment (IBDA) is a technique that also uses images of the food selections as well as any remaining food after the meal to estimate the patient's dietary intake (DI) but, unlike PADAs, IBDA captures the image passively (i.e. the images, automatically captured by the device, are the main information source the user provides for verification). IBDA's updated versions have combined the automated food identification, a software for portion size estimation, and user prompts for accurate DI assessment. Some examples of these assessment techniques include the Technology-Assisted Dietary Assessment system, the Nutricam Dietary Assessment Method, and the eButton [13, 20].

Sensors and informatics research tools

Early studies have observed the use of smart kitchen equipment, like tables, plates, and bowls that can measure and record the weight of the food (either with or without the plates) before as well as after the consumption of the meal. Similarly, wearable sensors provide an automated record of food consumption by hand-to-mouth gestures or chewing modality (like microphones that detect the crushing of food), electro-myographic sensors for the detection of muscle activation or acceleration, and strain sensors to

Tab. IV. The Mini Nutritional Assessment (MNA) Screening Short-Form.

A	Has food intake declined over the past 3 months due to loss of appetite, digestive problems, or chewing or swallowing difficulties?	0 = severe loss of appetite 1 = moderate loss of appetite 2 = no loss of appetite
B	Weight loss during the last 3 months	0 = weight loss over 3 kg 1 = does not know 2 = weight loss between 1 and 3 kg 3 = no weight loss
C	Mobility	0 = bedridden or chairbound 1 = able to get out of bed/chair but does not go out 2 = goes out
D	Has the patient suffered psychological stress or acute disease in the past 3 months?	0 = yes 2 = no
E	Neuropsychological problems	0 = severe dementia or depression 1 = mild dementia 2 = no psychological problems
F1	Body mass index (BMI)	0 = BMI under 19 1 = BMI 19 to under 21 2 = BMI 21 to under 23 3 = BMI 23 or higher
If BMI is not available, replace question F1 with F2. Do not answer F2 if F1 is already completed.		
F2	Calf circumference (CC) in cm	0 = CC less than 31 3 = CC 31 or greater

12-14 points: normal nutritional status. 8-11 points: at risk of malnutrition. 0-7 points: malnourished

detect the chewing motion or the frequency of swallowing [21, 22]. Chewing monitors are considered as reliable ingestion indicators for people that live in the community. Interestingly, chew counts present a significant correlation with ingested food mass. Still, these chewing monitors might also lead to false detections, for example due to gum-chewing movements, or they might be unable to detect liquids consumption, even though the intake of some liquids also cause jaw motions that are similar to chewing movements (like sucking through a straw) and therefore they might possibly be detected. On the other hand, swallowing is considered a reliable DI indicator, as all food needs swallowing to be a part of nutrition. Moreover, the intake of solid as well as liquid food could be detected as an increased swallowing frequency over spontaneous non-nutritive swallowing. Swallowing sensors are made up of microphones, motion and electrical sensors. [23]
Other informatics- and sensor-based assessment tools have been developed to determine the food type as well as its nutritional composition, such as food classification based on acoustic sensors, miniaturized portable (near infrared) spectrometers that can scan food items and determine their matrix characteristics, miniaturized tooth mounted sensor that can detect nutrients as well as wirelessly communicate to the user's mobile. Research and developmental studies are still going on these technologies and devices, several of these require comprehensive nutrient databases to support their mechanism and technology to assess accurately the portion size [24].

Nutrition-focused physical assessment (NFPA)

The application and utility of the nutrition-focused physical assessment (NFPA) could cover various settings for supporting the best practice in patient care. Moreover, NFPA is a part of the nutrition care process and model (NCPM), which is a framework of the nutritional care planning in four distinct and consecutive steps, including nutrition assessment, diagnosis, intervention, as well as monitoring and evaluation. Nutrition-focused physical assessment (NFPA) is considered as an essential part of nutritional assessment, as it could be used to identify the physical outcomes linked to micronutrient deficiencies. Historically, interest in using physical assessment skills within clinical settings is higher when an increased morbidity as well as mortality rate is reported in the hospitalized patients of surgical and medical intensive care units (ICUs), linked with poor nutritional status either prior to or during hospitalization [25].

Subjective Global Assessment (SGA)

The awareness of the harmful effects of "malnutrition" led to the requirement of assessment and screening tools in order to identify patients at risk or suffering from malnutrition. Thus, this medical challenge brought to the development of the bedside nutrition assessment tool, the Subjective Global Assessment (SGA), which was among the first assessment tools that included a patient-generated subjective scoring system, calculating the nutrition status on the basis of physical examination as well as patient history. Unlike other traditional assessment techniques that are solely based on anthropometric and biochemical markers, SGA outlines a rating scale that is based upon the variations in dietary intake (DI), in gastrointestinal signs linked with nutrition, weight, functional capacity, subcutaneous fat loss assessment, disease severity, edema, and muscle wasting. SGA has

been endorsed in several diseases due to its sensitivity and specificity in detecting nutrient deficiencies as well as malnutrition risk [25, 26].

System-Based examination

Surrogate biochemical markers, formerly used for nutrition status assessment, are found to be unreliable nutrition markers; however, they indicate disease severity, inflammation, morbidity as well as mortality risks (this is the case of serum albumin, prealbumin, and transferrin, for example). Besides, according to the latest etiology-based definition of malnutrition, physical parameters depicting changes in body composition – like subcutaneous fat loss, fluid accumulation, and muscle mass wasting – are included in the six malnutrition characteristics. Clinicians are therefore required to do a brief physical examination of their patients to identify body regions that are linked to macronutrient deficiencies; the findings should be rated as normal, mild to moderate depletion, or severe depletion. These physical indicators could be integrated into the nutrition-focused physical assessment (NFPA) by performing the full head-to-toe assessment by the clinicians, along with the thorough evaluation and examination of all body systems for those physical findings associated with nutrition-linked problems. Moreover, micronutrient deficiencies could have a multifactorial etiology, including inadequate intake, enhanced nutrient requirement, malabsorption, disease processes, natural disasters (e.g. famine), or drug interaction/shortage [25, 27, 28].

According to the Academy of Nutrition and Dietetics, nutrition assessment requires critical observational and analytical skills to identify physical indications through system-based examination. The main constituents of system-based examination and evaluation of the whole body involve the general inspection of vitals, nails, skin, eyes, nose, head, hair, neck, chest, mouth, musculoskeletal, and abdomen. Different inspection techniques are used to carry out the basic examination, involving both critical eye – to observe the shape, color, texture, size of the individual – as well as palpation – that requires touching with the pads and fingertips for the evaluation and assessment of texture, tenderness, size, temperature and mobility. Consequently, data obtained from all these examinations along with other parameters could be used for nutrition assessment as well as for critical interpretation and identification of nutrition-related problems [25].

Micronutrient Deficiencies and analysis

Often micronutrients deficiencies are stated as a single nutrient or multiple nutrients deficiency, on the bases of the region, phase of life cycle, or disease state. Micronutrient deficiencies universally affect over 2 million people worldwide; the predominant single-nutrient deficiencies include iodine, iron, and vitamin A. Vitamins are the essential organic micronutrient and only a small amount is required in the diet for them to play their role in many specific chemical reactions, such as growth, metabolism, and the preservation of cellular integrity [29].

Moreover, micronutrient deficiencies could also play a significant role in the development and progression of certain acute and chronic disorders, and they also could be linked to harmful changes in overall health [30]. Today the percentage of elderly individuals is much higher than in the past, thanks to the advancement in medical technology (like organ transplantation, noninvasive surgeries, obesity treatments, cancer treatment options, nutrition support modalities, etc.) and to the wider possibilities to have access to it [31]. Howeverm in spite of all these medical advances, micronutrient deficiencies are still predominant, even in the absence of malnutrition and insufficient caloric intake. Biochemical lab tests could be used to assess micronutrient status through the evaluation of metabolites or nutrient levels in urine, blood, or body tissues. However, biochemical lab tests only provides a quantitative and qualitative measurement of the micronutrient in a specific tissue or in some fluid sample like blood, urine, or plasma, but these results might fail to reveal the overall storage of that micronutrient in the body in terms of deficiency or excess [25].

Changes in skin color are mostly related to deficiencies of iron or B-complex vitamins or both, as these micronutrients are involved in several hematologic processes. Vitamin A deficiency (VAD) causes impairment of cell differentiation and maturation, leading to changes in the mucosal membranes and skin. Furthermore, protein and/or iron deficiencies could result in pallor, spoon-shape, clubbing, transverse banding, or ridging of nails. Whereas vitamin C deficiency leads to coiled and corkscrew hair, vitamin A deficiency affects the vision and can cause night blindness. The depletion of iodine, protein, and energy causes thyroid enlargement as well as fat and muscle wasting, with noticeably bony chest [25].

Nutrition-Focused Physical Assessment (NFPA) techniques analyze the obvious physical signs to assess macro- or micronutrients deficiencies during a head-to-toe physical examination and assessment. Thus, identifying the physical and clinical changes in different regions of the body caused by the unavailability of nutrient could be a cost-effective alternative approach to recognize micronutrient deficiencies (Tab. V) [25].

Nutritional assessment in older people

In older people, another significant aspect to be considered is the functional status impairment, evaluated by analyzing muscle strength and physical performance. Various factors are involved in functional status evaluation via screening procedures in older people; specifically, the relationship between muscle atrophy and decreased physical functioning acts as an independent diagnostic factor. Certainly, impaired functioning mostly results from muscle loss that is linked to disease-related malnutrition or immobility [32].

To maximize general health with aging, older individuals should undergo a complete geriatric assessment, including multidisciplinary diagnostics as well as treatment processes that identify medical, functional, and psychosocial capabilities. Similarly, nutrition status is mostly assessed due to its associations with functional status and disabilities. Therefore, the evaluation of

Tab. V. Clinical signs and symptoms of micronutrient deficiencies [8].

Affected organs	Symptoms	Micronutrient deficiencies
Skin	Petechiae Purpura Pigmentation Edema Pallor Decubitus Seborrheic dermatitis Unhealed wounds	Vitamins A and C Vitamins C and K Niacin Protein, vitamin B1 Folic acid, iron, biotin, vitamins B12 and B6 Protein, energy Vitamin B6, biotin, zinc, essential fatty acids Vitamin C, protein, zinc
Nails	Pallor or white coloring, Clubbing, Spoon-shape, Transverse ridging/banding, Excessive dryness, Darkness in nails, Curved ends	Iron, protein, vitamin B12
Head/Hair	Dull/lackluster, Banding/sparse, Alopecia, Hair depigmentation, Scaly/flaky scalp	Protein and energy, biotin, copper, essential fatty acids
Eyes	Pallor conjunctiva Night vision impairment Photophobia	Vitamin B12, folic acid, iron Vitamin A Zinc
Oral cavity	Glossitis Gingivitis Fissures, stomatitis Cheilosis Pale tongue Atrophied papillae	Vitamins B2, B6, B12, niacin, iron, folic acid Vitamin C Vitamin B2, iron, protein Niacin, vitamins B2 and B6, protein Iron, vitamin B12 Vitamin B2, niacin, iron
Nervous system	Mental confusion Depression, lethargy Weakness, leg paralysis Peripheral neuropathy Ataxia Hyporeflexia Muscle cramps Fatigue	Vitamins B1, B2 and B12, water Biotin, folic acid, vitamin C Vitamins B1, B6 and B12, pantothenic acid Vitamins B2, B6 and B12 Vitamin B12 Vitamin B1 Vitamin B6, calcium, magnesium Energy, biotin, magnesium, iron

body composition in screening phase, particularly muscle mass and its functioning, appears to be mandatory. While considering the problems related to muscle mass and its functioning, systematic estimation of inflammation, vitamin D status, and protein intake should be included in nutritional assessment [33].

Older people are often unable to cooperate with the assessment, thus sometimes limiting the extent of collected information. Malnutrition in older people is not always related to a disease condition, but it could also be caused by psychological or socioeconomic problems. Besides, older people also usually have the so-called "inflammaging" (also spelled "inflamm-aging"), a chronic condition with low-grade inflammation mostly prevalent in the elderly and frequently overlapping with disease-linked inflammation. The key element in this situation is therefore the presence of an already established disease, even though older people are likely to have co-occurrence of many aging-related diseases [34, 35].

Moreover, age-related factors cause muscle mass loss and conditions like sarcopenia, that recently has been recognized by the International Classification of Disease-10 (ICD-10) as an independent condition, which is clinically significant and identified via systemic screening [36]. Additionally, vitamin D deficiency is quite prevalent in older people. Vitamin D levels decrease with aging because of multiple factors, such as sun exposure, decreased synthetic activity of the skin, reduced gastrointestinal absorption, and reduced dietary intake. It is known that vitamin D also has anti-inflammatory characteristic and an increasing amount of literature supports the involvement of vitamin deficiencies in the reduced synthesis of muscle protein and muscle strength. In order to age healthily, it is essential to begin implementing effective strategies early on, so that any additional functional decline or disability could be prevented, especially in healthy older people [3, 37].

Assessment of nutritional status by physical activity

Physical activity (PA) can be defined as the bodily movements that are produced by the skeletal muscles and results in caloric expenditure. According to this comprehensive concept, the amount of energy expenditure (EE) is directly proportional to the size of the muscle mass involved. In the last few decades, technology usage for the personalized dietary intake (DI) assessment along with PA has been expanding rapidly. Typically, both self-report techniques and mechanical devices are used for PA assessment. Self-report measures for PA assessment include usage of questionnaires and completion of comprehensive diaries and logs. On the other hand, device-based techniques include motion sensors like accelerometers, heart rate monitors, pedometers, and other multisensory devices. Although these novel technologies have exhibited some advantages in the methodology of dietary intake and physical activity assessment, there are still many challenges and limitations [2, 38].

PA-related energy expenditure of an individual is affected by their body weight as well as their movement efficiency. Evidently, activity energy expenditure (AEE) involves a broad range of activities, including physical activity during leisure time, occupation, sports, household activities, transportation, home and personal care. In 1992, the American Heart Association published a report identifying physical inactivity as the fourth most significant and treatable risk factor of coronary heart disease (CHD) [39, 40].

Therefore, an accurate quantification of PA becomes essential in determining how much PA is of importance for a specific health outcome, in monitoring temporal events of PA, in evaluating the effectiveness of intervention programs, and in studying dose-response relationships. There are three main types of physical activity assessment methods/techniques, namely criterion, objective, and subjective [39].

Criterion techniques

Calorimetry

Physical activity is defined as the body movement that results in the expenditure of energy. The so-called "direct calorimetry", which measures energy expenditure (EE) by quantifying the heat production or heat loss, is considered as the gold standard of the physical activity measurement and other methods should be validated against it. However, its feasibility is not likely because of practical reasons. Hence, the mostly used criterion for assessment validation is by indirect calorimetric method, which involves the quantification of energy expenditure or heat production by calculating oxygen consumption or carbon dioxide production [39].

Direct behavioral observation

The initial methods for physical activity assessment include direct behavioral observation of motor activities by some skilled observers. Although now there are many assessment techniques to evaluate different physical activity (PA) settings, like sport classes, physical education, or independent living conditions, the main goal is to classify PA behaviors into separate categories that can be analyzed and quantified using different codes. However, the strength of this technique mostly relies on its access to contextual information.

Another important factor that influences physical activity is environmental conditions. This relationship is very significant for cognitive behavior research, as it could suggest change in sedentary behavior. The direct behavioral observation method is mostly used to assess children's physical activity patterns, while other assessment techniques like questionnaires or pedometers are not useful for them. Unfortunately, this method is a very time-consuming and tiresome method and therefore it is not suitable for larger studies [39, 41].

Doubly labelled water method (DLW)

The doubly labelled water method (DLW) is an isotope-based technique for the assessment of daily energy expenditure and average daily metabolic rate of an organism over a period of time and could be used for both field and lab studies. DLW measures metabolic processes that are directly linked to physical activity. The DLW principle involves the ingestion of two stable isotopes, i.e. 2H and 18O, in the form of water (2H2 18O) in standard amount. These isotopes are then evenly distributed in the body water, as observed from urine samples. Elimination of Deuterium (2H) from the body takes place in the form of water (2H2O), whereas 18O is removed from the body in the form of water (H2 18O) as well as carbon dioxide (C18O2). The elimination rates difference (over 5 to 14 days) between isotopes presents the quantity of CO2 produced, which leads to the assessment of energy expenditure (EE) [42]. In adults, the accuracy of this method is almost 3-10% of the calorimeter values and the variation of DLW within a subject is 8%; moreover, DLW is also applicable in children and provides precise measurements for free living conditions because it is does not influence PA patterns [43]. Still, DLW also has some limitations. The production as well as the analysis of isotopes is quite expensive, which is why this method is not suitable for larger studies; also, it could only calculate the TEE, therefore not distinguishing between physical activity energy expenditure (AEE), diet-induced energy expenditure (DEE), and basal metabolic rate (BMR) [39, 44].

Objective techniques

Pedometers

Motion sensors can register body motion. Pedometers are small electromechanical devices that have a spring mechanism to register the vertical movements and are generally worn on the waist. They are used for counting steps during a certain time period, mostly from morning to night. Then, these steps are converted into distance by entering the individual's average stride length. As a result, pedometers can only register physical activities related to running or walking, but it cannot monitor correctly movements of the upper body, cycling, carrying a load, swimming, or even movements on land or soft surfaces. Yet, as walking and running is a major part of our physical activity pattern, pedometer use remains highly valuable for estimating total daily movements. Hence, pedometers are considered as very helpful instruments for various health campaigns, such as "10,000 steps a day". In his study, Crouter et al. assessed the validity of 10 different pedometers and found out that the accuracy of pedometers is excellent for step counts, whereas they are less accurate for the assessment of distance and the accuracy of kilocalories assessment is even less [39, 45].

Accelerometers

An accelerometer is a sophisticated monitor that records the person's movements on several different planes. Instead of through a mechanical lever, as in pedometers, accelerometers function with piezoelectric transducers, along with microprocessors for the quantification of the magnitude as well as the direction of acceleration, which

is also considered as the dimensionless "counts". Tri-axial accelerometers are considered the best available accelerometer to date because, theoretically, they have the ability to record all movements; however, like pedometers, they still have some limitations in recognizing complex movements, such as upper body movements, cycling, graded terrain, etc. Also, studies have showed that there is a linear relationship between accelerometer counts and energy expenditure (EE). Subsequently, the EE of physical activities could be estimated by using linear regression equations along with body weight, height, gender, and age as co-variables. However, most studies have revealed that accelerometers provide a sufficiently accurate estimation of the overall PA, but its accuracy level for EE is relatively low, specifically for the point estimation of specific activities. Still, accelerometry is one of the most popular techniques used in PA research [46, 47].

Heart Rate monitoring (HR)

Another objective PA assessment method is the heart rate monitoring (HR). The heart rate indicates the intensity of the relative stress applied to the cardio-respiratory system by the movement, therefore indirectly measuring physical activity. This method basically relies upon the linear relationship of heart rate with oxygen consumption during moderate to intense PA range. While in resting state or during low-intensity physical activities, this heart rate/oxygen consumption relationship might not be linear and it is also affected by many other factors in addition to energy demands, like smoking, stress, caffeine, and body position. After establishing this relationship, the heart rate calculation could lead to the estimation of oxygen consumption, which in turn helps estimating energy expenditure in free living conditions. The heart rate records are usually maintained minute-by-minute and can be stored for many hours or even for days, hence providing information about the frequency, duration, and intensity of certain activity in addition to total energy expenditure (TEE). For the estimation of energy expenditure (EE) from the heart rate values, the FLEX HR methodology is a comprehensively examined approach. The HR data can show a great variability because of many confounding factors, thus making the EE estimation quite unreliable at individual level; still, this method shows significant epidemiological validity [39, 48, 49].
The next generation assessment PA in free-living conditions combines both HR monitoring and movement sensor, which might improve the precision an accuracy of activity energy expenditure (AEE) assessment [50].

Subjective techniques

Questionnaires/Surveys

Traditionally, physical activity questionnaires and surveys are an inexpensive tool of PA assessment that can be used efficiently for larger populations. However, this technique mostly depends on subjective analysis of the questions as well as observation related to the PA behavior of the individual. When dealing with very young or elderly people, extra care and attention should be taken, because their memory could be compromised [51]. Over- or under-estimation of the physical activity could be influenced by various factors like age, social desirability, questionnaire complexity, seasonal variation, as well as the span of the surveyed period [52]. Surveying techniques can be divided into four categories: interviewer-assisted questionnaires, self-report questionnaires, diaries, and proxy-report questionnaires. All of these questionnaires must undergo validation against the criterion methods (direct observation, DLW, or indirect calorimetry) or against objective techniques (HR, pedometers, or accelerometers). During their research, Philippaerts et al. evaluated the reliability as well as the validity of the three most frequently used PA questionnaires against doubly labelled water method (DLW) [53] and established that the Baecke Questionnaire [54], the Tecumseh Community Health Study Questionnaire [55], and the Five City Project Questionnaire [56] provided the most valid as well as reliable physical activity data. Whereas, Racette and colleagues [57] made the comparison of seven-day physical activity recall questionnaire for obese women against DLW, as well as two other physical activity questionnaires, like PA scale for elderly and Zutphen physical activity questionnaire, and their results validated that method against DLW in elderly people. Also, the results of these validation studies have shown that in general questionnaires classify the population into various distinctive categories of physical activity behavior such as low, moderate or highly active categories, but they are still not suitable for EE assessment at individual level [39, 58].
The latest developments in information technology (IT) – like computer networking, internet, and multimedia software – leads to the development of electronic surveys that are useful for PA research. Information technology facilitates the researcher in simultaneously administering the questionnaires to great number of people. In addition, in electronic surveys the subjects directly enter their answers or response on the computer, eliminating all the coding errors that could occur in interviews or traditional paper-pencil surveys. Moreover, in electronic surveys the subjects could not omit any question. Additionally, depending upon the subject's answers, the computer program could skip the unnecessary questions, which results in brief administration time. Lastly, certain studies have also indicated that people might be relatively more honest about any objectionable behavior to a computer rather than a researcher or paper-pencil questionnaires [39, 59].

Assessment of nutritional status by metabolomics

To overcome the limitations of self-reporting dietary assessment methods, nutritional epidemiologists have started to examine the biomarkers as measures of the nutritional status and dietary intake. Dietary biomarkers

were proven to be a more accurate and objective measure for DI in comparison with traditional questionnaires because they also consider the nutrient bioavailability as well as its metabolism [1].

The human genome initiative has introduced new visions for biological research as well as its translation into human health [1], and metabolomics is one of the most significant tools for its implementation [60]. Metabolomics uses different approaches than analytical chemistry and provides a comprehensive picture of all the metabolites that are present in the bio-fluids at a certain time [61]. The analysis of metabolites in blood – like glucose, cholesterol, and triglycerides – is already employed to diagnose monitor diabetes risk and heart diseases. Metabolomics provides the potential to magnify the intrinsic capability of urine and plasma metabolites to evaluate the human health status (Tab. VI) [60]. Researchers strongly believe that metabolites are highly sensitive to dietary exposure because diet is not only a significant source of the variation in metabolites, but it also induces metabolic responses. The two major approaches applied in metabolomics are MS (mass spectroscopy) and NMR (nuclear magnetic resonance) spectroscopy. The use of metabolomics in characterizing habitual dietary exposure as well as in identifying nutritypes have proven it to be a very exciting and emerging field that has many potential applications in the field of nutrition epidemiology [61].

The mechanisms that drive these metabolic and nutritional pathways are intricate and multi-factorial. The latest advancements of the large-scale metabolite profiling for larger epidemiological studies not only provide insights of molecular mechanisms causing age-linked diseases, but they also help in the assessment of metabolites that could predict the risk factors for cardio-metabolic disorders [61]. Metabolite profiling might identify and estimate such metabolites, like acylcarnitines, sphingolipids, and glycerophospholipids, that could not be estimated by the HDI (Healthy Diet Indicator) score. Moreover, these metabolites are known to be associated with greater risks of insulin resistance, fatty acid oxidation, cardiovascular diseases (CVD), and type 2 diabetes (T2D) [31, 32]. Several metabolites, like phosphatidylcholine and acylcarnitines, are associated with gut microbial-dependent pathways that are involved in the hepatic production of TMAO (trimethylamine-N-oxide) from choline; TMAO is subsequently converted into trimethylamine (TMA) within the microbiota, which might increase atherosclerosis risk [37] as well as glucose metabolism [36, 38]. TMAO plays a role in cardiovascular disease, as it promotes accumulation of macrophage foam cells that lead to reverse cholesterol transport inhibition and affect bile as well as sterol metabolism, which subsequently enhances the hyperactivity of platelets along with the initiation of atherosclerotic plaque formation [39, 40] (M2).

Several studies have investigated the association of overall diet with metabolites and mostly these investigations have evaluated the metabolites through mass spectrometry. For instance, in a large prospective cohort study like The European Prospective Investigation into Cancer and Nutrition (EPIC-Potsdam), which included 2,380 adults, the dietary intake patterns were analyzed by the methods of reduced rank regression, and the results showed maximum metabolites variations as well as the weak association of habitual diet with serum metabolites [62]. Similarly, in the ARIC (Atherosclerosis Risk in Communities) study, 1,977 participants samples were assessed for 336 metabolites; the results have revealed an association between a high-sugar (both in food and beverages) dietary pattern and seven long-chain unsaturated fatty acids, two sex steroids, five 2-hydroxybutyrate-linked metabolites, five γ-glutamyl dipeptides, as well as 4 metabolites involved in other pathways [63]. Likewise, the Women's Health Initiative study exhibited the association of Prudent dietary pattern with 85 metabolites (most of them are lipids). Another study examined 502 participants from a Lung, Prostate, Ovarian and Colorectal Cancer Screening Trial and established the correlations of 412 metabolites with food groups as well as the Healthy Eating Index score [64]. The researchers established the association of 39 metabolites with 13 different dietary groups, thus confirming the usefulness of metabolomics in identifying biomarkers and thus endorsing the nutritional intake effects on human metabolic system [61].

Identifying the strong associations of dietary habits with metabolites might thus provide a better prospect to understand the pathways through which nutritional intake mediates the protection against various chronic diseases, like CVDs [61].

Tab. VI. Metabolites, their Function and associated Disease Condition.

Disease Condition	Metabolites	Sample type	Function	Analyzing Technique
Dysbiosis	Skatole	Urine Plasma	Pulmonary toxin that induces the expression of AhR regulated genes	HPLC
	Indican	Urine	Stimulated vascular smooth muscle cell proliferation in vitro	Liquid chromatography/ electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS)
	Propionate	Serum	Lower lipogenesis, serum cholesterol levels, and carcinogenesis	HPLC

Tab. VI. *Continues.*

Disease Condition	Metabolites	Sample type	Function	Analyzing Technique
Oxydative stress	Homocysteine	Blood	Lipid peroxidation, free radical formation, inflammation, and endothelial dysfunction	HPLC
	Vitamin D	Blood	Promotes calcium absorption in the gut	Liquid chromatography (LC), Liquid chromatography-mass spectrometry (LC-MS), Tandem mass spectrometry, Radioimmunoassays (RIA), Chemiluminescence immunoassays (CLIA)
	Thioglycolic acid	Urine	Present as cysteine-thioglycolic acid	HPLC, Gas chromatography
Amino acid profile	Histidine	Serum Urine		NMR spectroscopy
	Isoleucine	Plasma Urine	Associated with both lower AHEI score and increased incident CVDs risk	Mass spectrometry
	Leucine	Plasma Urine	Protein synthesis stimulation and reduction of muscle protein breakdown after a physical trauma	Mass spectrometry
	Lysine	Serum Plasma Urine	Calcium absorption and collagen formation	Mass spectrometry
	Methyonine	Plasma	Angiogenesis, overconsumption is related to cancer growth	High performance liquid chromatography
	Cysteine	Plasma Urine	Antioxidant	Mass spectrometry
	Phenylalanine	Blood Plasma Urine	Gluconeogenesis, lower chronic inflammation	HPL, Tandem mass spectrometry
	Tyrosine	Urine Plasma	Production of neurotransmitters	Mass spectrometry
	Threonine	Serum Plasma	Keeping connective tissues and muscles throughout the body strong and elastic	Peptide microarray technology
	Tryptophan	Urine Serum	Part of melatonin and serotonin production	HPLC, Liquid chromatography- tandem mass spectrometry
	Valine	Plasma	Muscle growth and tissue repair	NMR spectroscopy, Mass spectrometry
	Alanine	Plasma Urine	Source of energy for muscles and central nervous system	NMR spectroscopy Mass spectrometry,
	Aspartic acid	Urine Plasma	Fatigue, athletic performance, and muscle strength	Mass spectrometry
	Glutamic acid	Urine Plasma	Sugars and fats metabolism, neurotransmitter	Mass spectrometry
	Glycine	Urine Plasma	Acts as neurotransmitter, proteins production	NMR spectroscopy
	Asparagine	Urine Plasma	Production of body's proteins, enzymes and muscle tissue	Mass spectrometry
	Proline	Urine Plasma	Role in protein synthesis, metabolism, nutrition, wound healing, antioxidative reactions, and immune responses	NMR spectroscopy
	Glutamine	Urine Plasma	Substrate for protein synthesis, anabolic precursor for muscle growth, acid-base balance in the kidneys	Mass spectrometry
	Arginine	Urine Plasma	Important role in cell division, wound healing, removing ammonia from the body, immune function, and hormone release	Mass spectrometry
	Serine	Urine Plasma	Biosynthesis of proteins, purines pyrimidines, enzymes, and muscle tissue	Mass spectrometry

Tab. VI. *Continues.*

Disease Condition	Metabolites	Sample type	Function	Analyzing Technique
Non-protein amino acid	Carnitine	Urine	Energy production support, general brain function maintenance	NMR spectroscopy
	Taurine	Urine Plasma	Nerve growth support, blood pressure lowering, calming the nervous system	Mass spectrometry
	Glutathione	Urine Plasma	Antioxidant, involved in nutrient metabolism, cellular events regulation	Mass spectrometry NMR spectroscopy
Inflammation and lipidomics	Fatty acid amides	Urine Plasma	Signaling lipids, modulation of neurobehavioral processes in mammals, including pain, sleep, feeding, and locomotor activity	Gas chromatography, Mass spectrometry
	Leukotrienes	Urine Plasma	Potent inflammatory mediator	Liquid chromatography, Mass spectrometry
	Prostaglandin	Blood Serum Urine	Regulation of smooth muscle tissue contraction and relaxation	Liquid chromatography, Mass spectrometry
	Thromboxane	Blood	Blood clotting and constriction of blood vessels	Liquid chromatography, Mass spectrometry, Thin layer radio-chromatography
Steroids	Aldosterone	Serum Urine	Increased sodium and water retention, increased potassium excretion, blood pressure regulation	Liquid chromatography/ tandem mass spectrometry
	Cortisol	Blood Urine Saliva	Metabolism and immune response regulation	Liquid chromatography/ tandem mass spectrometry
	Corticosterone	Blood	Involved in metabolism, energy balance, stress and adaptation	Mass spectrometry HPLC ELISA Radioimmunoassays
	Dehydroepiandrosterone sulfate	Blood Plasma	Involved in the development of male sexual characteristics at puberty	Liquid chromatography/ tandem mass spectrometry
	11-Deoxycortisol	Serum Plasma	Metabolic intermediate within the glucocorticoid pathway	Mass spectrometry
	21- Deoxycortisol	Blood Plasma Urine	Marker of congenital adrenal hyperplasia due to 21-hydroxylase deficiency	Liquid chromatography/ tandem mass spectrometry, HPLC
	Androstenedione	Serum Urine	Increasing the production of the hormone testosterone to enhance athletic performance, build muscle, reduce body fat, increase energy	Liquid chromatography/ tandem mass spectrometry
	Testosterone	Blood	Sexual development regulation, muscle mass, and red blood cells production	Liquid chromatography/ tandem mass spectrometry
	17-OH-Progesterone	Blood	Marker for congenital adrenal hyperplasia (CAH)	Liquid chromatography/ tandem mass spectrometry
	Dehydroepiandrosterone	Blood	Endothelial function modulation, inflammation reduction, improvement of insulin sensitivity, blood flow, cellular immunity, body composition, bone metabolism,...	Gas chromatography-mass spectrometry (GC-MS), Liquid chromatography-mass spectrometry (LC-MS)
	Progesterone	Serum Plasma Urine	Menstruation regulation and pregnancy support	Liquid chromatography-mass spectrometry (LC-MS)
	Estradiol	Blood Saliva	Development and maintenance of female reproductive system	HPLC, Liquid chromatography-mass spectrometry (LC-MS)
	Estrone	Serum Urine	Involved in female sexual development and function	Gas chromatography-mass spectrometry (GC-MS), Liquid chromatography-mass spectrometry (LC-MS)
Toxoma	More than 5,000 toxins	-	-	-

Assessment of nutritional status by genetic biomarkers

Genetic biomarkers play a crucial role in determining the association between intermediate biomarkers like fasting glucose, inflammation markers, plasma lipids, oxidative markers, etc. and the occurrence of diseases like type 2 diabetes, cardiovascular diseases, neurodegenerative diseases, cancer, etc. Currently, hundreds of SNPs are known to be persistently associated with various phenotypes of nutrition-linked diseases (Tab. VII); hence, nutritional epidemiological studies require the knowledge of most pf the genetic polymorphisms that are linked to the phenotypes of interest to establish reliable associations between the diet and the disease. This phenomenon is especially relevant to understand individual variations associated with certain gene variants that might influence the correct evaluation of the nutritional status [65].

Lactase 13910C>T polymorphism /rs4988235 located on MCM6 gene is one example of the effect of genetic polymorphism on nutritional status, as it effects the lactase gene (LCT). It strongly affects the persistence of lactase synthesis, which in turn influences the individual's intolerance or tolerance to lactose [66]. Usually, those who have a CC genotype exhibit a physiological decrease of lactase activity within the intestinal cells because of the difficulty in lactose metabolism. Therefore, CC genotype variant of Lactase 13910C>T polymorphism has been proposed to act as proxy for the low consumption of milk [67]. Similarly, genetic variants also affect the intake biomarkers concentration, like phylloquinone/vitamin K1, which is the major circulating vitamin K form and reflects the intake of vitamin from plants. Circulating phylloquinone acts as a biomarker associated with a healthy lifestyle, while its lower concentrations are considered to be associated with an enhanced risk of different chronic diseases. Thus, understanding the gene variants that might affect phylloquinone concentration might explain the individual variability in the response of phylloquinone intake from the diet or supplements [68].

Additionally, genetic variability also plays significant role in accurately assessing the micronutrient status, which might have a small safety range between the toxic and safe dosage, as well as regulate the bioavailability of these micronutrients. For instance, zinc homeostasis is usually regulated by the zinc transporter genes, and the zinc transporter SLC-30A8 polymorphism is found to be associated with an increased risk of type 2 diabetes, as zinc is required for insulin metabolism within the pancreatic beta-cells. Empirically, the total zinc intake is inversely related with the level of fasting plasma glucose in people having the glucose increasing A allele. Moreover, many studies have evidently proposed that zinc levels might be considered at individual basis [65, 69].

In addition to dietary intake (DI), genes also affect physical activity (PA). Since physical activity is one of the major factors contributing to the total energy expenditure (TEE), it plays a vital role in regulating energy balance. It is commonly established that the training-associated metabolic changes are mostly influenced by the individual's genetic background. Moreover, identifying the genetic markers that enhance the beneficial effects of training might be helpful in assessing various training programs that, along with dietary intervention, could improve the body weight reduction among obese individuals. Therefore, personalized interventions for obesity reduction would be significant in the clinical management of obesity and obesity-related diseases, such as lymphedema [70-73].

In the last two decades, genome-wide association studies (GWAS) and the development of new technologies in the fields of molecular biology and human genetics have enable scientists to easily perform hundreds of genomic analyses, as well as high throughput DNA sequencing techniques. Such broad-range techniques have facilitated the identification of novel genes and established the correlations between different SNPs related with training capabilities (collectively, such genetic factors are known as performance-enhancing gene polymorphisms, or PEPs) [74]. Recently, a scientific review has identified the association of 5,147 genes with training and physical activity (PA). However, 51% of these genes have up-regulatory effect by training and PA, while 42% of the genes have shown down-regulatory effects by PA [74,75].

For instance, MYBPH gene encodes the structural component of the muscle sarcomeres, which is a myosin-binding protein that might be involved in myosin interaction with the thick A-band myofilaments [76]. Similarly, PDK4 gene encodes the protein kinase (PTK) enzymes, which are located in the mitochondrial matrix and are involved in the inhibition of pyruvate dehydrogenase complex (PDC), which reduces glucose usage and increases free fatty acid (FFA) catabolism [77]. Likewise, ACE (angiotensin-converting enzyme) gene is a reliable candidate for the genetic predisposition to athletic physical activity. Several studies have shown that insertion (allele I) or deletion (allele D) polymorphism of 287bp Alu repetitive sequence located in intron 16 have an association with increased performance as well as duration of exercise in many subjects [78]. A common single nucleotide polymorphism (SNP) rs1801282 C>G (Pro12Ala) in PPARG (Peroxisome Proliferator Activated Receptor Gamma) gene is known to be associated with various muscle changes linked with exercise. Also, the Ala variant of the SNP rs1801282 could enhance the positive effects on increased muscle mass related with training resistance [70, 79].

Conclusion

Food is required to maintain activities of life. The nutrients and the non-nutrient food components interact with various metabolic pathways, thus influencing health. Nutrient deficiencies could play a significant role in the development and progression of several acute and chronic disorders, and they also could be linked to harmful changes in overall health. For nutritional assessment or screen-

Tab. VII. Genes and SNPs involved in Nutritional Assessment.

Sr. No.	Gene	SNP	Alleles	Gene function
1	*LTB4R2*	rs1950504	A/G	Chemotaxis mediation of granulocytes and macrophages
2	*ALOX5*	rs4987105,	C/T	Catalyzes the first step in leukotriene biosynthesis and has a role in inflammatory processes
		rs59439148	del$(GGGGGC)_{4/3/2}$ /del$(G)_5C$ /dup$(G)_5C$ /dup$(GGGGGC)_{2/3}$	
		rs4769874	G/A	
3	*LTA4H*	rs17525495	C/T	Epoxide hydrolase that catalyzes the final step in the biosynthesis of leukotriene B4
		rs1978331	C/T	
4	*MMP2*	rs1030868	G/A	Metalloproteinase involved in vasculature remodeling, angiogenesis, tissue repair, inflammation
		rs2241145	G/C	
5	*CEACAM1*	rs8110904	G/A	Cell-cell adhesion molecule with roles in angiogenesis and immune response modulation, reduction of inflammasome activity, blood vessel remodeling through endothelial cell differentiation and migration, vascular permeability regulation
		rs8111171	G/T	
6	*FOXC2*	rs199772307,	G/A	Transcriptional activator. Involved in mesenchymal tissue formation
		rs34221221,	A/G	
7	*TNF*	rs1800629	G/A	Cellular responses to cytokines and stress, regulates the immunological response to infections
8	*TLR2*	rs121917864	C/T	Key role in the innate immune system. It is expressed in macrophages, B lymphocytes, mast cells
9	*TLR4*	rs4986791	C/T	Key role in the innate immune system. It is expressed in macrophages, B lymphocytes, mast cells
10	*VEGFA*	rs699947	C/A	Growth factor active in angiogenesis, vasculogenesis, and endothelial cell growth. Induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and induces blood vessels permeabilization
		-1154	G>A	
		-460	C>T	
		+405	G>C	
		+936	C>T	
11	*HGF*	rs5745652	C/T	Role in angiogenesis, tumorigenesis, tissue regeneration
		rs2074725	C/A	
12	*CYP26B1*	rs2241057	A/G	Involved in the retinoic acid metabolism
13	*PROX1*	rs340874	T/C	Critical role in neurogenesis and in the development of the heart, eye lens, liver, pancreas, and lymphatic system
14	*RORC*	rs11801866	A/T	Essential for lymphoid organogenesis
		rs12128071	G/A	
		rs12045886,	A/G	
15	*LCP2*	rs572192	C/T	T-cell antigen receptor-mediated signaling
		rs6866733	C/G,T	
		rs315721	A/G	
16	*NRP2*	rs849530	G/T	Interaction with vascular endothelial growth factor (VEGF)
		rs849563	T/A,G	
		rs16837641	G/A,C,T	
17	*SYK*	rs158689	T/A	Regulation of innate and adaptive immunity, vascular development. Crucial role in the innate immune response to fungal, bacterial and viral pathogens. Activates the inflammasome and NF-kappa-B-mediated transcription of chemokines and cytokines in presence of pathogens. Involved in vascular development, where it may regulate blood and lymphatic vascular separation
18	*VCAM1*	rs3176861	C/T	Pathophysiologic role in immune responses and leukocyte emigration to inflammation sites

Tab. VII. *Continues.*

Sr. No.	Gene	SNP	Alleles	Gene function
19	*miR499*	rs3746444	A/C,G	miR-499 gene targets are involved in remodeling and inflammation-related signaling pathways, including fibrogenic and immune-modulator pathways
20	*CDKN2B-AS1*	rs1333048	A/C,G	Interacts with polycomb repressive complex-1 and -2, leading to epigenetic silencing
21	*CALCRL*	rs185008808	C/T	Receptor for calcitonin-gene-related peptide together with RAMP1 and receptor for adrenomedullin together with RAMP3 and RAMP2
		rs61739909	A/G	
		rs10177093	G/C,T	
22	*VEGFC*	rs2333496	C/T	Growth factor active in angiogenesis of veins and lymphatic vessels, and in endothelial cell growth, stimulating their proliferation and migration, and permeability of blood vessels
		rs7664413	C/T	
23	*EPHB4*	rs314313	T/A,C,G	Cell adhesion and migration regulation, angiogenesis, blood vessel remodeling and permeability
		rs314311	T/G	
24	*PLA2G4A*	rs10798069	G/T	Hydrolyzes arachidonyl phospholipids for releasing arachidonic acid. Implicated in the initiation of the inflammatory response
25	*IL1R1*	rs949963	C/T	Mediator involved in cytokine-induced immune and inflammatory responses
26	*IL4*	rs2227284	T/C,G	B-cell activation, DNA synthesis stimulation, expression induction of MHC-II on resting B-cells, secretion enhancement and cell surface expression of IgE and IgG, expression regulation of CD23 IgE receptor on lymphocytes and monocytes, expression induction of IL31RA in macrophages, autophagy stimulation in dendritic cells
27	*IL6*	rs2066992	G/A,C,T	Inducer of the acute phase response, final differentiation of B cells into Ig-secreting cells, lymphocyte and monocyte differentiation, generation of Th17 cells, myokine, increased fats breakdown, improved insulin resistance
28	*IL10*	rs1518111	T/C	Cytokine produced by monocytes, lymphocytes, pleiotropic effects in immunoregulation, inflammation, down-regulation of Th1 cytokines expression, MHC-II, macrophages stimulator, B cell survival enhancement, proliferation, antibody production
		rs1518110	A/C,G,T	
29	*NFKB2*	rs1056890	G/A,C	Pleiotropic transcription factor ubiquitously expressed involved in inflammation, immunity, differentiation, cell growth, tumorigenesis, apoptosis
30	*ANGPT2*	rs6990020	C/A,T	Endothelial cell migration and proliferation
31	*SOX17*	rs12541742	C/G,T	Embryonic vascular development, postnatal angiogenesis
32	*FLT4*	rs75614493	C/T	Lymphangiogenesis and lymphatic endothelium maintenance
		rs10464063	A/G	
		rs307814	G/A	
		rs307811	C/T	
		rs11960332	C/T	
		rs11739214	G/C	
33	*KDR*	rs2239702	G/A	Endothelial proliferation, survival, migration, tubular morphogenesis, sprouting
		rs4576072	A/G	
		rs10020464	C/A,T	
		rs11133360	C/T	
34	*CYP2A6*	rs1801272	T/A	High coumarin 7-hydroxylase activity

Tab. VII. *Continues.*

Sr. No.	Gene	SNP	Alleles	Gene function
35	*PLIN1*	rs228948	A>G/A>T	Modulators of lipolysis and triglyceride levels; protection of lipid storage droplets from hormone-sensitive lipases
		rs894160	C>T	
		rs230479	A>C	
		rs105270	-	
		rs2304794	T>A	
36	*ADRB2*	rs1042713	G>A	Induction of thermogenesis in response to cold and diet, lipolysis induction
		rs1042714	G>A	
37	*ADRB3*	rs4994	A>G	Induction of thermogenesis in response to cold and diet; induction of lipolysis
38	*PPARGC1A*	rs8192678	C>T	Transcriptional regulation of white adipocyte differentiation, insulin, and adipocytokine pathways
39	*TFAM*	rs1937	G>C	Maintenance of normal levels of mitochondrial DNA
40	*PPARA*	rs4253778	G>C	Stimulating the expression of genes required for fatty acid oxidation in mitochondria
41	*PPARD*	rs2016520	C>A/T	Regulation of the peroxisomal beta-oxidation pathway of fatty acids in mitochondria
42	*GABPB1*	rs7181866	A>G	Activation of cytochrome oxidase expression and nuclear control of mitochondrial function
		rs12594956	C>A/G	
		rs8031031	C>T	
		rs12594956	C>A/G	
43	*ACE*	rs4646994	-	Regulation of energy expenditure, lipolysis and glucose incorporation into lipids in adipocytes
44	*AMPD1*	rs17602729	-	Critical role in energy metabolism
45	*CKM*	rs8111989	-	Central role in energy transduction in tissues with large fluctuating energy demands (skeletal muscles, heart)
46	*ADRB2*	rs1042713	-	Induction of thermogenesis in response to cold and diet, lipolysis induction
47	*IL6*	rs1800795	-	Increase in fat breakdown, insulin resistance improvement
48	*UCP3*	rs1800849	-	Uncoupling of oxidative phosphorylation, thermogenesis
49	*AGT*	rs699	-	Activation of lipogenic enzymes, induction of lipid transport into adipocytes, increase in delivery of fatty acids to adipocytes
50	*KCNJ11*	rs5219	-	Insulin secretion
51	*COL5A1*	rs12722	-	Ubiquitous connective tissue component that also binds insulin
52	*HIF1A*	rs11549465	-	Activation of glucose transporter transcription under hypoxic conditions, encodes glycolytic enzymes
53	*PPARG*	rs1801282	-	Regulator of adipocyte differentiation
54	*GABPB1*	rs12594956	-	Activation of cytochrome oxidase expression and nuclear control of mitochondrial function
		rs7181866	-	
55	*SOD2*	rs4880	-	Destroys toxic superoxide anion radicals normally produced in cells
56	*ACTN3*	rs1815739	-	Structural component of sarcomeric Z line in skeletal muscle
57	*BDKRB2*	rs1799722	-	Mediators of pain and inflammation
58	*AQP1*	rs1049305	-	Passive transport of water across osmotic gradient
59	*MTHFR*	rs1801131	-	Conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate
60	*NOS3*	rs2070744	-	Vasodilation in response to training
61	*FTO*	rs9939609	-	Stimulation of food consumption
		rs1558902	-	
		rs8050136	-	

Tab. VII. *Continues.*

Sr. No.	Gene	SNP	Alleles	Gene function
62	*ADIPOQ*	rs1501299	-	Energy expenditure
63	*LEP*	164160	-	Appetite regulation
64	*LEPR*	rs1805094	-	
65	*INSIG2*	rs7566605	-	Regulation of cholesterol and fatty acid synthesis
66	*MC4R*	rs17782313	-	Energy homeostasis, appetite regulation
		rs17066866	-	
		rs1943226	-	
		rs11875096	-	
		rs1943224	-	
		rs7235242	-	
		rs11872992	-	
		rs8093815	-	
		rs17066856	-	
		rs17066836	-	
		rs1943227	-	
		rs1943218	-	
		rs17066829	-	
		rs9966412	-	
		rs17066859	-	
		rs9965495	-	
		rs12970134	-	
		rs17700633	-	
		rs11873305	-	
		rs8091237	-	
		rs7240064	-	
67	*PCSK1*	rs236918	-	Insulin resistance
68	*PPARG*	rs1801282	-	Increased BMI
69	*ADBR2*	rs1042714	-	Adaptive thermogenesis
		rs1042713	-	
70	*ADBR3*	rs4994	-	
71	*GHRL*	rs696217	-	Appetite regulation
72	*FABP2*	Ala54Thr-polymorphism	-	Fatty acid uptake
73	*APOA5*	rs964184	-	Lipoprotein metabolism
		rs662799	-	
74	*APOA1*	rs670	-	
75	*LIPC*	rs2070895	-	
76	*CETP*	rs3764261	-	
77	*MTNR1B*	rs10830963	-	Appetite regulation
78	*NPY*	rs16147	-	
79	*GIPR*	rs2287019	-	Insulin signaling
80	*IRS1*	rs1522813	G>A/G>C	
		rs2943641	T>A/T>C	
81	*TCF7L2*	rs12255372	G/T	Blood glucose homeostasis
		rs7903146	C>G/C>T	
82	*PCSK1*	rs6232	T>C	Energy metabolism
		rs6234	G>C	
83	*MCM6 (*601806)*	rs4988235	C>T (C)	Lactose intolerance, adult type (#223100)
		rs182549	G>A (G)	
		rs145946881	G>C (G)	
84	*HLA-DQA1 (*146880)*	rs2187668	C>T (C)	Susceptibility to celiac disease 1 (#212750)
		rs2395182	G>T (G)	
		rs4639334	G>A (G)	
		rs4713586	A>G (G)	
		rs7454108	T>C (C)	
85	*HLA-DQB1 (*604305)*	rs7775228	T>C (C)	

Tab. VII. *Continues.*

Sr. No.	Gene	SNP	Alleles	Gene function
86	*HJV (*608374)*	rs74315323	C>A	Hemochromatosis, type 2A (#602390)
		rs74315324	G>A	
		rs74315325	A>T	
		rs74315326	A>G	
		rs28940586	A>C,G	
		rs74315327	A>G	
		rs121434374	G>C,T	
		rs786205063	$(GA)_3G>GAG$	
		rs121434375	T>A	
87	*SLC40A1 (OMIM *604653)*	rs104893662	T>A,G	Hemochromatosis, type 4
		rs28939076	G>T	
		rs878854984	$(CAA)_4>(CAA)_3$, $(CAA)_5$	
		rs104893663	T>A,C	
		rs104893670	C>A,T	
		rs104893671	C>A	
		rs104893672	T>A	
		rs104893673	C>A	
		rs104893664	C>T	
88	*HFE (*613609)*	rs1800562	G>A	Hemochromatosis
		rs1799945	C>G,T	
		rs1800730	A>T	
		rs1800758	G>A	
		rs28934889	G>A	
		rs111033557	G>A	
		rs28934595	A>C	
		rs111033558	G>C,T	
		rs28934596	T>C	
		rs28934597	G>C	
		rs111033563	A>C	
89	*TFR2*	rs80338880	G>C	Hemochromatosis, type 3
		rs80338877	$(G)_5>(G)_6$	
		rs80338879	A>T	
		rs41303501	C>T	
		rs80338889	T>C,G	
90	*FTH1*	rs387906549	T>A	Hemochromatosis, type 5
91	*ADH1B (*103720)*	rs1229984	A>G	G/C, higher alcohol consumption
		rs2066702	C>T	A/T, acetaldehyde accumulation
92	*ADH1C (*103730)*	rs1693482	C>T (T)	Type II alcoholism
		rs698	T>A,C (C)	
93	*ALDH2 (*100650)*	rs671	G>A (A)	Acute alcohol sensitivity (#610251)
94	*CYP1A2 (+124060)*	rs762551	C>A (C)	Higher risk of nonfatal myocardial infarction
95	*ADORA2A (*102776)*	rs5751876	T>C (C)	Greater caffeine sensitivity, sleep impairment, increased beta activity during non-REM sleep
		rs35320474	delT (T)	Greater caffeine-induced anxiety
96	*DRD2*	rs1110976	T>G (G)	Greater caffeine-induced anxiety
97	*COMT*	rs4680	G>A (A)	Higher risk of acute myocardial infarction
98	*ALDOB (*612724)*	rs1800546	C>G (G)	Fructose intolerance (#229600)
		rs76917243	G>T (T)	
		rs118204425	AAGdel (del)	
99	*UGT1A1 (*191740)*	rs6742078	G>T (T)	Bilirubin serum level (#601816)
100	*G6PD (*305900)*	rs1050829	T>A,C (A)	Nonspherocytic hemolytic anemia (#300908)
		rs1050828	C>T (T)	
101	*BCO1*	rs12934922	A>T (T)	Reduced conversion of beta-carotene to retinol
		rs7501331	C>T (T)	

Tab. VII. *Continues.*

Sr. No.	Gene	SNP	Alleles	Gene function
102	*GC*	rs2282679	T>G (G)	Lower vitamin D levels
		rs4588	G>T (T)	
		rs842999	C>G (C)	
103	*SLC23A1*	rs33972313	C>T (T)	Reduction of circulating levels of vitamin C
104	*SLC30A8*	rs11558471	A>G (G)	Susceptibility to diabetes mellitus
105	*SLC5A6*	rs1395	G>A (A)	Reduced intestinal uptake, cellular delivery, and transplacental transport of pantothenate and biotin
106	*TCN2*	rs1801198	C>G (G)	Decreased serum vitamin B12, increased homocysteine
107	*TTPA*	rs4501570	G>A (A)	Vitamin E deficiency
		rs4587328	T>C (C)	
		rs4606052	C>T (T)	
108	*VDR*	rs731236	A>G (G)	Immune weakness, increased cancer risk, early bone loss, increased cognitive decline risk, mood disorders
109	*CYP2R1*	rs10741657	A>G (G)	Lower vitamin D levels
		rs10766197	A>G (A)	
110	*LPA*	rs10455872	A>G (G)	Coronary artery disease
		rs3798220	C>T (C)	Cardiovascular events risk
111	*CDKN2B-AS1*	rs10757274	A>G (G)	Heart disease risk
		rs2383206	A>G (G)	
		rs2383207	A>G (G)	
112	*Intergenic*	rs10757278	A>G (G)	Heart attack risk
113	*MC4R*	rs17782313	C>T (C)	Increased BMI
114	*APOA2*	rs5082	C>T (C)	Higher total energy, fat, protein intake
115	*PCSK1*	rs6232	A>G (G)	Higher risk of obesity and insulin sensitivity
116	*APOA5*	rs662799	A>G (G)	Higher risk of early heart attack, less weight gain on high-fat diets
117	*SH2B1*	rs7498665	A>G (G)	Obesity, type-2 diabetes
118	*SLC2A2*	rs5400	C>T (T)	Higher sugar consumption
119	*F2*	rs1799963	A>G (A)	Higher risk of thrombosis and cerebral stroke
120	*F5*	rs6025	A>G (A)	Higher risk of thrombosis
121	*FUT2*	rs602662	A>G (G)	Lower vitamin B12 levels
122	*ALPL*	rs4654748	C>T (C)	Lower Vitamin B6 blood concentration
123	*CYP2R1*	rs10741657	A>G (G)	Lower vitamin D levels
		rs10766197	A>G (A)	
124	*GC*	rs4588	G>T (T)	
		rs842999	C>G (C)	
125	*MTHFR (*607093)*	rs1801133	G>A (A)	Homocystinuria (#236250)
126	*CBS (*613381)*	rs121964962	C>T (T)	Homocystinuria (#236200)
127	*FOXO3*	rs2802292	C>T (T)	Longer lifespan
		rs2802288	A>G (A)	
		rs2802292	T>G (G)	
128	*SIRT1*	rs3740051	-	Higher basal energy expenditure
		rs2236319	-	
		rs2272773	-	
129	*PEMT*	rs12325817	G>C (C)	Low choline
130	*CHDH*	rs12676	G>T (T)	

ing, precise evaluation of the dietary intake (DI) as well as of physical activity (PA) is crucial. Nutritional screening ought to be considered as an essential part of clinical assessment for every patient on admission to healthcare setups, as well as on change in clinical conditions. Therefore, a detailed nutritional assessment must be performed every time nutritional imbalances are observed or suspected. Dietary Intake (DI) assessment is a multidimensional and complex process. Traditionally, DI is assessed through self-report techniques including diet records, FFQs (food frequency questionnaires), and recalls. But due to reporting errors such as biases, random errors, misestimations, and nutrient databases-linked errors, questions arise about the adequacy of self-reporting dietary intake

procedures for scientific conclusions. Therefore many objective methods are proposed for dietary intake (DI) and physical activity (PA) assessment such as biomarkers analysis, blood tests, genetic assessments, metabolomic analysis, DEXA (Dual-energy X-ray absorptiometry), MRI (Magnetic resonance imaging), and CT (computed tomography) scanning procedures as well as bed-side and low-cost techniques like BIA (bioelectric impedance analysis). Although most of these methods have their own biases and limitations.

In the future, thanks to the presence of such nutritional, genetic, and metabolic status indicators along with intelligent interventions, like healthier and more aware choices in food and supplements and lifestyle modifications, the individual metabolic phenotype will be developed in a beneficial and individually designed direction. Such vision will bring a deeply different prospect to the management of human diet and will enable a very interactive, detailed, and significantly valuable system to provide health.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; AKK, MCM, Kristjana D, Kevin D, PC, FF, MAP, MRC, PM, SN, MC, TB: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript

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Review

Polymorphisms, diet and nutrigenomics

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Keywords

Nutrigenomics • Nutrigenetics • Metabolomics • Precision nutrition • Physical activity

Summary

Every human being possesses an exclusive nutritional blueprint inside their genes. Bioactive food components and nutrients affect the expression of such genes. Nutrigenomics is the science that analyzes gene-nutrient interactions (nutrigenetics), which can lead to the development of personalized nutritional recommendations to maintain optimal health and prevent disease. Genomic diversity among various ethnic groups might affect nutrients bioavailability as well as their metabolism. Nutrigenomics combines different branches of science including nutrition, bioinformatics, genomics, molecular biology, molecular medicine, and epidemiology. Genes regulate intake and metabolism of different nutrients, while nutrients positively or negatively influence the expression of a number of genes; testing of specific genetic polymorphisms may therefore become a useful tool to manage weight loss and to fully understand gene-nutrient interactions. Indeed, several approaches are used to study gene-nutrient interactions: epigenetics, the study of genome modification not related to changes in nucleotide sequence; transcriptomics, the study of tissue-specific and time-specific RNA transcripts; proteomics, the study of proteins involved in biological processes; and metabolomics, the study of changes of primary and secondary metabolites in body fluids and tissues. Hence, the use of nutrigenomics to improve and optimize a healthy, balanced diet in clinical settings could be an effective approach for long-term lifestyle changes that might lead to consistent weight loss and improve quality of life.

Nutrigenomics

Nutrigenomics is an emerging field where advanced genomics tools are used to analyze the effects of nutrients on the genome and gene expression, and the effects of genetic variants on the intake of nutrients. The term "Nutrigenomics" was created to describe the interaction between nutrients and genes. Therefore, nutrigenomics links genetics to nutrition, physiology, biochemistry, metabolomics, proteomics, transcriptomics, and bioinformatics [1].

Nutrigenomics relies on three fundamental tenets:

- Genomic diversity in ethnic groups, which can affect bioavailability of nutrients and their metabolism;
- Choice of food and its availability based on cultural, geographical, and socio-economic factors;
- Malnutrition, which affects gene expression and poses a serious threat to genome stability by causing mutations in the DNA sequence or even chromosomal instability, that result in abnormal gene dosage and adverse phenotypes [2].

Therefore, nutrigenomics is the field of nutritional study that applies molecular techniques to exploring, analyzing, and understanding the physiological responses of particular populations or individuals to specific diets[3]. It further explains how dietary components might affect gene expression at pre-transcriptional, post-transcriptional, and translational levels, resulting in gain or loss of function of those particular proteins [3]. These, gene-nutrient interactions depend on the capacity of particular nutrients to bind with transcription factors, eventually regulating RNA polymerase recruitment to gene promoters and the ensuing transcript levels. For example, research on vitamin A, vitamin D and fatty acids indicate that these vitamins directly trigger the activation of nuclear receptors and induce gene transcription [4]. Furthermore, compounds like soy genistein and resveratrol from wine indirectly affect various molecular signaling pathways through nuclear factor kappa B, thereby activating and regulating major molecules linked with disease [1, 5].

Recently, nutrigenetic studies have identified genetic variants associated with susceptibility to various diseases secondary to interaction with dietary factors. Theses scientific advancements will greatly contribute to the treatment and prevention of chronic disease, as they could potentially predict an individual's risk, explain the etiology of the disease, and enable the personalization of nutritional management [6]. This scientific approach

may have caveats, as certain genes might preferentially favor the intake of some nutrients and adversely affect the consumptions of other beneficial nutrients [2, 7].

Nutrigenetics

Nutrigenetics encompasses the genetic variation effects on nutritional responses and nutrient function [2, 6]. Although nutrigenetics and nutrigenomics are closely related, these terms are not interchangeable. Nutrigenetics explores the effect of hereditary genetic variants on the uptake and metabolism of micronutrients, whereas nutrigenomics studies the interconnection between genome and diet with reference to nutritional effects on the metabolic, proteomic, transcriptional, and translational changes along with dietary variation due to an individual's genetic background [8]. Recently, nutrigenetic research studies have enabled identification of genetic variants associated with disease susceptibility through interaction with specific dietary factors. For example, various genetic variants in genes involved in metabolic pathways affect the intake and usage of different micronutrients [2, 7, 9]. Nutrigenetic studies may be used to predict the risk of various chronic diseases, and, with the help of personalized nutritional management, these diseases could be prevented or better managed.

Gene-diet interactions are also involved in the response to nutritional interventions when limiting the total energy intake or altering the relative proportion of carbohydrates, proteins and fats. Studies have been performed in different populations to further explore the effects of genetic polymorphisms located near or within genes regulating food intake, lipoprotein and lipid metabolism, glucose homeostasis, insulin signaling, circadian cycles, inflammatory responses and amino acid metabolism on metabolic improvement, weight gain/loss, insulin resistance, and serum lipid levels. Most nutrigenetic tests analyze the effect of multiple polymorphisms on eating behavior changes. For instance, diets tailored to people with polymorphisms in the apolipoprotein E gene should decrease the intake of saturated fats compared to the standard dietary advice, because carriers of such polymorphisms are at increased risk of myocardial infarction (MI) [6, 10].

It is worth noting that not only DNA sequence variants are important, but also copy number variants. Some studies have reported the association between copy number variants (CNVs) for small genome sections and the risk of metabolic diseases, as illustrated in the following three examples: 1) copy number variants of the leptin receptor gene are linked with metabolic traits and with type 2 diabetes mellitus risk [11]; 2) lower copy number of the salivary amylase alpha 1A gene has been associated with obesity predisposition, thereby linking obesity to carbohydrate metabolism [12]; 3) a pentanucleotide (CTTTA) deletion/insertion in the 3′-untranslated region of the leptin receptor gene has been associated with type 2 diabetes mellitus risk [13]. Additional studies are needed to further explore the many levels of gene-diet interactions in relation to disease risk and dietary response [6].

Nutritional epigenetics

Epigenetics involves reversible and heritable processes that regulate the expression of genes without associated changes in the coding sequence of DNA. In fact, epigenetic dysregulation may underlie the onset of various chronic diseases and their progression [14]. Complex interactions between nutrients and DNA methylation, noncoding RNAs, and covalent histone modifications contribute to obesity, type 2 diabetes mellitus, dyslipidemia, cardiovascular diseases, non-alcoholic fatty liver disease, and cancer. For example, diets rich in fats and sugar are associated with abnormal methylation patterns of neuropeptide genes that control food intake and could be involved in obesity development [15]. Similarly, low-protein diets could alter lipid and glucose levels by disrupting histone modifications within major regulatory genes [16]. Moreover, deficiency of various micronutrients – like vitamin A, group B vitamins, selenium, potassium, and iron – are linked with hypermethylation of tumor suppressor genes that play a crucial role in cancer [6, 16].

Nutriepigenetics is the study of nutritional interventions that alter epigenetic changes which significantly impact treatment and prevention of chronic diseases. For example, it has been demonstrated that the anti-inflammatory effects of the Mediterranean diet are linked to inhibitory hypermethylation of proinflammatory genes [17, 18]. Furthermore, polyunsaturated fatty acid administration positively regulates expression of specific miRNAs that inhibit lipogenic and oncogenic genes [19]. Curcumin is also an important epigenetic regulator that exerts protective effects against heart failure and liver injury through the regulation of specific DNA methylation and histone modification patterns. These data suggest that introducing specific dietary compounds to an individual's diet, that modulate epigenetic patterns, could be an efficient strategy for reducing the prevalence of obesity and associated comorbidities [6, 20].

Nutritional transcriptomics

Transcriptomics is the process that evaluates the sequence and abundance of all RNA transcripts at a specific time point. RNA levels are tissue-specific and time-specific. During the process of transcription, activated transcription factors move to the nucleus, where they bind to a specific DNA sequence within the promoter region of a particular gene and inhibit or facilitate that gene's transcription. Transcription factors can also be stimulated by physiological signals triggered by bioactive food components, nutrients or their metabolites, hormones, diseases, and pharmacological treatments. Therefore, transcription factors act like sensors and thereby modulate transcription. Transcriptomics can provide information on the mechanisms

related to a specific nutrient or diet. Transcriptomics also helps the identification of genes, metabolites, or proteins that alter pre-disease states and assists in distinguishing and characterizing bioactive food components or nutrient-regulated pathways [1, 21, 22].

Nutritional proteomics

Proteomics identifies the complex array of proteins involved in biological processes, i.e. the proteome. Various pathological or physiological states can alter the proteome [21, 22].

Proteomics uses a variety of technologies designed to analyze protein expression including electrophoresis, organelle proteome analysis, high throughput extract pre-fractionation screening and mass spectrometry [3, 21]. Proteomics serves as a biological tool to fully understand genome activation in response to specific nutrients. For example, butyrate can change the expression of different proteins belonging to the ubiquitin proteasome system. This suggests that butyrate regulates major proteins that control cell differentiation, cell cycle, and apoptosis by proteolysis [1, 22, 23]. Proteomics can thereby identify pathways that are important in various disease states including those related to nutrition.

Nutritional metabolomics

Metabolomics is the branch of functional genomics that identifies primary and secondary metabolites in bodily fluids and can be used to understand alterations in metabolites and the mechanisms to isolate and characterize them. Metabolomics is a significant tool for investigating the effect of food on the health of individual. Identification of the food-derived biomarkers helps in understanding the variability among individual to metabolize the same foods during healthy as well as in diseased states. Nutritional metabolomics identifies the metabolic changes caused by specific nutrients or diets [21, 24, 25]. It also involves the study of metabolism under various genetic and environmental stresses [1, 21, 26, 27]. Food components and nutrients interact and alter metabolic pathways in different ways. Many cohort studies have identified the intake biomarkers like red meat, fish, walnuts and whole-grain bread. Under specific organic stimulations the monoterpene called perilla alcohol, extracted from strawberries, could behave as an anticancer molecule [24]. Similarly, Wittenbecher et al. [28], applied serum metabolomics to reveal the significant association of various red meat intake biomarkers with type-2 diabetes risk.

Precision nutrition

Nutrigenetics can be used to personalize diets by modifying them according to individual genetic variation. Precision nutrition is an important part of precision medicine, which consists of establishing guidelines for nutritional requirements of particular subgroups of people [6, 29, 30]. For example, lactose intolerance, phenylketonuria, or celiac disease are managed via tailored nutritional instructions based upon the genetic background [29].

Numerous SNPs are linked with chronic diseases because of their interaction with the intake of micro- and macronutrients or by specific foods or diets. For instance, polymorphisms of taste perception genes, including the sweet taste receptor *TAS1R2* (Taste 1 Receptor Member 2) gene and *CD36* gene, were reported to be linked with dyslipidemia among research participants in Mexico with high consumption of carbohydrates and fats, respectively [31, 32]. Similarly, common variants of homocysteine metabolism-regulating genes, such as *MTHFR* (methylenetetrahydrofolate reductase) and *MTR* (methionine synthase), have been associated with increased breast cancer risk in individuals with reduced intake of vitamin B6, vitamin B12, and folate [33]. Interestingly, SNPs in the *VDR* (vitamin D receptor) gene affect the availability of vitamin D and are known to be associated with osteoporosis predisposition in postmenopausal females with reduced calcium intake [6, 34].

In clinical practice, nutrigenetics is currently being used to evaluate the genes involved in the transport and metabolism of nutrients, toxins removal, and protection against oxidative stress. Therefore, polymorphisms in these genes are included in nutrigenetic tests to evaluate their effects on eating habits. For instance, personalized diets designed according to specific ACE (angiotensin I converting enzyme) genotypes may recommend higher sodium intake compared to the standard population-based dietary advice [6, 10, 35].

Nutritional effects on gene expression profiles

Nutrition influences health outcomes by affecting expression of genes that regulate crucial metabolic pathways. Western dietary patterns – rich in processed grain products, processed meats, sweets, and desserts – have a gene expression profile typical of cancer signaling and inflammatory response. This is not the case in individuals that eat whole grain products, fruits, and vegetables. Pathway analyses have shown that higher meat consumption is linked to genetic networks associated with colon cancer [36]. Moreover, higher saturated fatty acid consumption results in a gene expression profile that is typical of glucose intolerance, liver lipid accumulation, inflammation, and increased neuropeptide expression, leading to development of obesity. On the contrary, lower protein diets increase the expression of hepatic gluconeogenic genes, with subsequent glucose intolerance. Furthermore, diets lacking folate and choline are linked with dysregulation of lipid metabolism genes, thus predisposing to non-alcoholic fatty liver disease [37]. Similarly, chromium deficiency induces downregulation of insulin signaling genes, which may lead to type 2 dia-

betes mellitus. Selenium, vitamin A, and vitamin B12 deficiencies increase the susceptibility to cardiovascular diseases by upregulating lipogenic and proinflammatory genes [6].

Research studies have also reported favorable effects of bioactive food components and nutrients on gene expression profiles; for example, people consuming the Mediterranean diet have lower postprandial expression of genes encoding proteins involved in inflammation, oxidative stress, atherogenesis, and endoplasmic reticulum stress-related activation. Furthermore, a higher intake of monounsaturated fatty acids through olive oil consumption is linked with reduced expression of inflammatory and lipid storage genes. Consumption of higher polyunsaturated fatty acid-containing diets positively regulates the expression of neuropeptide genes that modulate energy homeostasis [38, 39].

Bioactive food components like theaflavin, epigallocatechin-3-gallate, genistein, curcumin and sulforaphane exhibit anticancer properties by upregulating tumor-suppressor genes and downregulating proto-oncogenes. In addition, resveratrol and curcumin have antiatherogenic effects by downregulating the expression of matrix metalloproteinases that cause the formation and progression of plaques. Finally, apple polyphenols prevent diet-induced obesity by regulating genes involved in fatty acid oxidation, lipolysis, and adipogenesis [15, 40].

Genetic polymorphism effect on dietary intake

Genome-wide association studies have evaluated genetic polymorphisms associated with various metabolic pathways [2]. Epidemiological and interventional studies have also explored the associations of genetic variants with dietary intake [41]. For example, clinically significant associations have been reported between: 1) the *APOA2* (c.2265T>C) variant and intake of saturated fatty acids and body mass index, 2) MTHFR variants and homocysteine levels, and 3) CYP1A2 variants and caffeine-related hypertensive response [2, 42, 43].

Inborn errors of metabolism are caused by mutations in specific genes encoding key metabolic enzymes. These pathogenic variants lead to gene-diet interactions altering nutritional requirements and metabolism: classical examples are lactose intolerance and phenylketonuria. The T>C-13910 variant upstream of the lactase gene (*LCT*) results in non-persistence or absence of the lactase enzyme after infancy, therefore individuals with this variant do not digest lactose. On the other hand, phenylketonuria is an autosomal recessive disorder caused by mutations in the phenylalanine hydroxylase (*PAH*) gene, a major hepatic enzyme that is responsible for the conversion of phenylalanine to tyrosine [2, 44, 45].

Other genetic-food interactions are much more complex, such as polygenic interactions underlying the multifactorial etiology of cancer, obesity, type 2 diabetes, and cardiovascular disease. Such diseases derive from the interaction among several genes and environmental factors, and respond to numerous dietary exposures. For example, a number of genetic variants are associated with an increased obesity risk, such as those found in the *FTO* gene, *UCP1* and *UCP3* genes, the *PPAR* (peroxisome proliferator-activated receptor) encoding genes, the melanocortin 4 receptor (*MC4R*), and the leptin receptor (*LEPR*) gene [2, 46, 47], as detailed in Table I.

In coronary artery disease, variants in genes associated with lipid metabolism, such as *LPL* (lipoprotein lipase), *CETP* (cholesteryl ester transfer protein), *LDLR* (low density lipoprotein receptor), and *APOE* (apolipoprotein E), affect the intake and catabolism of cholesterol and other lipids, resulting in atherosclerosis (Tab. I) [2, 48, 49]. Further studies evaluated the role of the genetic variants in the *CYP1A2* (Cytochrome P450 1A2) gene, which encodes the main caffeine-metabolizing enzyme, in cardiovascular disease. A higher consumption of caffeine might be linked with increased cardiovascular disease risk in subjects with genetic variants associated with "slow" caffeine metabolism. On the other hand, people that have genetic variants associated with fast caffeine metabolism are protected from the effects of moderate caffeine consumption [2, 50].

Genetic variations of the *APOA2* (apolipoprotein A2) gene are associated with obesity via alterations in energy intake. Chinese and Asian-Indian populations with a specific *APOA2* variant are at a greater risk of developing obesity when consuming food rich insaturated fatty acids, but with lower saturated fatty acids intake, such risk was not observed. Similar studies were performed among Mediterranean populations of Southeastern Spain. Moreover, polymorphisms of genes associated with iron, vitamin C, vitamin D, and vitamin B12 metabolism have been reported to affect the risk of deficiency or reduced levels of these nutrients [51, 52].

Other genetic loci were analyzed for their associations with the intake of macronutrients. Merino et al. [53] identified two genetic loci, *DRAM1* (DNA damage regulated autophagy modulator 1) and *RARB* (retinoic acid receptor beta), which exhibited a genome-wide significant association with macronutrient intake. Additionally, they also confirmed the association of *FGF21* (fibroblast growth factor 21) genetic variant (rs838133) with the intake of macronutrients [41, 53].

Genetic polymorphisms associated with body weight

Research studies have identified significant associations between genetic variants and body weight. Numerous genetic loci have been linked to weight loss following hypocaloric diets and physical activity. These genes encode important enzymes regulating adipogenesis, lipid metabolism, the circadian clock, carbohydrate metabolism, appetite control, energy intake and expenditure, cell differentiation, and thermogenesis [54, 55]. Moreover, genetic variants associated with taste- and texture-related, and olfactory genes could affect individual preferences and sensitivity towards certain foods, influ-

Tab. I. Genetic polymorphisms, their related genes, and involved dietary factors if known, and putative disease risks.

Gene	Polymorphism	Putative disease risks	Effect
TAS1R2	rs35874116	Hypertriglyceridemia	Carbohydrate responsiveness
	Ile191Val		
cSHMT	L474F	Colon cancer Neural tube defects	Folate degradation
MTHFR	rs1801133	Breast cancer Homocystinuria Cardiovascular diseases Diabetes Neural tube defects	Increased folic acid intake Macronutrient intake High levels of homocysteine Folate metabolism
	C677T		
	A1298C		
	A222V		
MTHFD1	R653Q	Neural tube defects	Higher folate intakes
MTR	rs1805087	Breast cancer	Lower folate concentration
	A2756G		
MTRR	A66G	Neural tube defects in offspring	Lower folate concentration
VDR	rs1544410	Osteoporosis Prostate cancer	Affects vitamin D levels
	T>C		
	rs11568820		
APOA1	rs670 rs5069	Metabolic syndrome	-
APOA2	rs5082	Cardiovascular diseases Obesity	Higher total energy, fat, and protein intake
APOA5	rs964184	Higher risk of early heart attacks Lipid metabolism disturbances Less weight gain on high fat diets	Greater reduction in TC and LDL-c Macronutrient intake
	rs662799		
APOB	rs512535	Metabolic syndrome	Low fat
APOC3	rs5128	Metabolic syndrome	Cholesterol metabolism
	C 3175G		
APOE	rs429358	Lipid metabolism disturbances	Macronutrient intake
	rs7412		
PNPLA3	rs739409	NAFLD	-
CYP1A1	TMsp1C	Breast and prostate cancer	Oxidative metabolism of estrogens
	Ile462Val		
CYP1A2	A>C	Heart diseases	Reduced ability to metabolize caffeine
CYP1B1	C194G	Congenital glaucoma	
CYP2R1	rs10741657 rs10766197	Lower vitamin D levels	Increased consumption of food rich in vitamin D Increased sun exposure
CYP17A	T34C	Congenital adrenal hyperplasia	Increased estrogen level
FTO	rs9939609	T2DM	Macronutrient intake
		Obesity	
FTO	rs8050136	Obesity	-
FTO	rs1558902	Obesity	Greater weight loss
			Less reductions in insulin and HOMA-IR
MC4R	rs17782313	T2DM	Increased BMI
MC4R	rs12970134	Metabolic syndrome	Macronutrient intake
TCF7L2	rs7903146	T2DM	Smaller weight loss and HOMA-IR
		Metabolic syndrome	
LCT	rs4988235	Obesity	-
PPARA	rs1800206	Lipid metabolism disturbances Hypercholesterolemia	Macronutrient intake Low n–6 fatty Acid
	rs6008259		

▶

Tab. I. *Continues.*

Gene	Polymorphism	Putative disease risks	Effect
PPARG	rs1801282	Obesity Insulin Sensitivity	Macronutrient intake
TXN	rs2301241	Abdominal obesity	-
GIPR	rs2287019	Cardiovascular diseases	Greater weight loss Greater decreases in glucose, insulin and HOMA-IR
DHCR7	rs12785878	Vitamin D insufficiency	Greater decreases in insulin HOMA-IR
LIPC	rs2070895	Lipid metabolism disturbances	Higher decreases in TC and LDL-c Lower increase in HDL-c
	rs1800588l		
PPM1K	rs1440581	Maple syrup urine disease	Less weight loss Lower decreases in insulin and HOMA-IR
TFAP2B	rs987237	Non-familial congenital heart disease Char syndrome	Higher weight regains
IRS1	rs2943641	Autism spectrum disorder Hepatocellular carcinoma	Greater decreases in insulin, HOMA-IR, weight loss
PCSK1	rs6232	Higher obesity and insulin sensitivity risk	-
PCSK7	rs236918	Metabolic disorders Liver diseases	Higher decreases in insulin and HOMA-IR
MTNR1B	rs10830963	Type 2 Diabetes Impairment of early insulin response	Lower weight loss in women
IL-1A	G4845T	Chronic inflammatory diseases Periodontitis Coronary artery disease A few autoimmune diseases and cancers	Increased IL-1 plasma concentrations
	C-889T		
IL-1B	C 3954T	Chronic inflammatory diseases Periodontitis Coronary artery disease A few autoimmune diseases and cancers	Increased IL-1 plasma concentrations
	A -511G		
IL-1RN	C 2018T	Chronic inflammatory diseases Periodontitis Coronary artery disease A few autoimmune diseases and cancers	Increased IL-1 plasma concentrations
IL-6	rs2069827	Low-grade chronic inflammation Obesity Visceral fat deposition Insulin resistance Dyslipidemia Risk for cardiovascular diseases	Lower weight gains Tissue healing
	G -174C		
IL6R	A>C	Low-grade chronic inflammation	Tissue healing
SH2B1	rs7498665	Obesity Type 2 diabetes	Higher fat intake
SLC2A2	rs5400	Diabetes	Higher sugar consumption Insulin sensitivity
F2	rs1799963	Higher risk of thrombosis and cerebral stroke	-
F5	rs6025	Higher risk of thrombosis	
FUT2	rs602662	Lower vitamin B12 levels	Increased consumption of food rich in vitamin B12
	Gly258Ser		

Tab. I. *Continues.*

Gene	Polymorphism	Putative disease risks	Effect
ALPL	rs4654748	Lower Vitamin B6 blood concentration	Increased consumption of food rich in vitamin B6
CBS	rs121964962	Colorectal Cancer Homocystinuria Vitamin deficiency Dementia Heart disease Stroke	High RBC folate Removal of homocysteine
	rs1801181		
FOXO3	rs2802292	Longer lifespan	-
	rs2802288		
SIRT1	rs3740051	Higher basal energy expenditure	-
	rs2236319		
	rs2272773		
PEMT	rs12325817	Low choline	Increased choline intake
PLIN1	rs894160	Obesity	Macronutrients intake
GCKR	rs1260326	Lipid metabolism disturbances	Macronutrients intake
LIPG	rs4939833	Lipid metabolism disturbances	Macronutrients intake
LPL	rs328	Lipid metabolism disturbances	Macronutrients intake
	C1595G		
CELSR2	rs12740374	Lipid metabolism disturbances	Macronutrients intake
eNOS	G>T	Oxidative Stress	-
NOS3	rs1799983	Lipid metabolism disturbances	Macronutrients intake
CETP	rs1800777	Lipid metabolism disturbances	Reduced HDL-C concentrations
	G 279A		
CLOCK	rs4580704	Coronary heart disease	-
	T3111C		
CRY1	rs2287161	Type 2 diabetes Metabolic syndrome	Insulin resistance Low carbohydrate intake
T1R1	rs34160967	Dental caries	-
	rs41278020		
T1R2	rs35874116	Obesity Dental caries	High sensitivity to sweet taste
	rs9701796		
T1R3	rs307355	Dental caries	Reduced promoter activity
	rs35744813		
	rs307377		
T2R16	rs846664	Association with the aging process	Alcohol dependence
	rs978739		
TAS2R38	rs713598	Metabolic diseases Coronary heart disease	Bitter taste of PTC or PROP perception
	rs1726866		
	rs10246939		
SCNN1A	rs239345	Risk of hypertension Cardiovascular disease	-
	rs11064153		
SCNN1B	rs3785368	Risk of hypertension	-
	rs239345		
SCNN1G	rs4401050	Risk of hypertension	-
TRPV1	rs4790522	Cardiovascular risk disease	-
	rs8065080	Risk of hypertension	

Tab. I. *Continues.*

Gene	Polymorphism	Putative disease risks	Effect
CD36	rs1761667	Hypercholesterolemia Metabolic syndrome Type 2 diabetes mellitus	Ethnic-specific effects
	rs1984112	Lipid metabolism Type 2 diabetes Cardiovascular disease risk	-
	rs1527483	Obesity	-
	rs2151916	Obesity	High triglycerides levels
	rs7755	Type 2 diabetes mellitus	
	rs1049673	Obesity Hypertension Type 2 diabetes mellitus Premature coronary heart disease	-
	rs3840546	Obesity Type 2 diabetes mellitus	-
	rs3211938	Metabolic syndrome	-
	rs10499859	Metabolic syndrome	-
	rs3211867	Obesity	-
	rs3211883	Metabolic syndrome	-
	rs3173798	Obesity Metabolic syndrome	-
	rs3211892	Obesity Metabolic syndrome	-
	rs1358337	Metabolic syndrome	-
	rs1054516	Metabolic syndrome	High levels of triglyceride
	rs1049654	Metabolic syndrome	-
	rs3211909	Metabolic syndrome	-
	rs3211849	Metabolic syndrome	High levels of triglyceride
	rs13246513	Obesity Metabolic syndrome	-
	rs3211842	Obesity Metabolic syndrome	-
GNAT3	rs1194197	Metabolic syndrome	-
	rs11760281		
OR7D4	rs61729907		-
	rs5020278		
OR11H7P	rs1953558	Obesity Dental caries Diabetes Cardiovascular disease Hypertension Hyperlipidemia Cancer	-
OR6A2	rs72921001	Gestational choriocarcinoma	-
LEPR	rs3790433	Obesity Metabolic syndrome	Low n-6 PUFA High n-3 PUFA
POMC	rs713586	Obesity Early-onset type 2 diabetes	-
BDNF	rs6265	Obesity Psychological eating disorders	Carbohydrate and fat intakes
	Val66Met		
KCNB1	rs6063399	Obesity	Lower BMI
KCNC2	rs7311660	Obesity	Lower BMI

Tab. I. *Continues.*

Gene	Polymorphism	Putative disease risks	Effect
TMPRSS6	rs1421312	Anemia Damage of immune function, work performance, and damage of adolescent's psychological behavior and mental development	Iron deficiency
	rs2111833		
TUB	rs2272382	Obesity	Higher consumption of mono- and disaccharides Higher glycemic load
	rs1528133		
CAPN10	SNP-44	Type 2 diabetes mellitus	Total cholesterol
ACE	Insertion/Deletion (I/D)	Type 2 diabetes mellitus Acute myocardial infarction Hypertension	Salt sensitivity
ADRB2	Arg16Gly	Asthma Chronic obstructive pulmonary disease	Carbohydrate responsiveness
	Gln27Glu		
ADRB3	Trp64Arg	Coronary heart disease Weight gain Type 2 diabetes mellitus	-
PON1	s854549	Cardiovascular disease Atherosclerosis	Detoxification/Oxidative stress Lipid levels
	r s854552		
	r s854571		
	rs854572		
Cdx-2	G3731A	Vitamin D deficiency	Calcium intestinal absorption Increasing bone mineral density
CYP24A1		Vitamin D deficiency	-
GSTM1	Insertion/Deletion	Vitamin C deficiency Cancer Coronary artery disease Atopic asthma	Low vitamin C intake
GSTP1	A313G	Ascorbic acid deficiency	Low vitamin C intake
HFE	C282Y	Iron-storage disease Iron overload	Iron metabolism
ADH1B	47His	Alcohol dependence	Systemic ethanol clearance
	369Arg		
	rs1229984		
ADH1C	349Ile		-
ALDH2	E487K	Alcohol metabolism	Acetaldehyde accumulation Alcohol metabolism
	rs671		
FADS1	rs174537	Abnormal lipid profile	PUFA metabolism
	rs174546		
AGT	T>C	Hypertension Cardiorespiratory disorders	Salt sensitivity Increased blood flow and respiration
	M235T		
MCM6	C 13910T	Lactose intolerance	-
HLA	DQ2/DQ8	Celiac disease	Gluten intolerance
BCO1	Ala379Val	Hypercarotenemia Vitamin A deficiency Chronic lung disease	Vitamin A Higher levels of provitamin A carotenoids
GSTT1	Insertion / Deletion	Serum ascorbic acid deficiency	Free radical production
MnSOD	Ala16Val	Breast cancer	Reduced oxidation of catecholamines
	C-28T		
TNF-A	G -308A	Obesity Insulin resistance Dyslipidemia.	Whole body glucose homeostasis alteration

Tab. I. *Continues.*

Gene	Polymorphism	Putative disease risks	Effect
CRP	rs1205 G>A	Mental health disorder Depressive disorder Low-grade chronic inflammation	Higher levels of CRP
SULT1A1	G638A	Post-menopausal breast cancer	Estrogen load reduction
NQ01	C609T	Cancer	Protect from oxidative stress
FACTOR V	G1691A	Deep venous thrombosis	-
MMP1	1G/2G	Accelerated skin aging	-
COL1A1	Sp1 G>T	Accelerated skin aging	Mature connective tissue structure, essential for tensile strength
COL5A1	BstUI C>T	Achilles tendinopathy Anterior cruciate ligament rupture Tennis elbow	Increase in content of type V collagen Decrease in fibril diameter and biomechanical properties of tendons
GPX1	C>T	Premature aging Prostate cancer	Protect against oxidative stress
GPX4	rs713041	Colorectal cancer	Lymphocyte GPx activities
CAT	C -262T	Premature aging	Protect against oxidative stress
EPHX1	rs1051740	Cellular damage Accelerated aging	Process toxins and pollutants
BDKRB2	C>T	Osteoarthritis Anxiety disorders Essential hypertension	Increased blood flow and respiration
VEGF	C>G	Neovascular eye disease Age-related macular degeneration	Increased blood flow and respiration
TRHR	rs7832552 rs16892496	Non-goitrous congenital hypothyroidism	Increased lean body mass
ACTN3	R577X	Alpha-actinin 3 deficiency	-
FABP2	Ala54Thr	Metabolic disorders	Fat absorption and metabolism
ADIPOQ	G -11391A	Chronic kidney disease Chronic obstructive pulmonary disease Metabolic disease	-
DRD1	rs4532 G-94A	Addictive behavior	Regulate neuronal growth and development Mediate some behavioral responses
DRD2	rs1800497 Taq1A/2A	Compulsive and risk-seeking behaviors Increased risk for co-morbid substance use disorders (alcoholism & opioids) Binge eating behavior Addictive disorder	Carbohydrate responsiveness Reduced carbohydrate intake
DRD3	Ser9Gly	Addictive behavior	Cognitive, emotional, and endocrine functions
DRD4	C521T	ADHD Opioid dependence Novelty seeking	-
ADBR3	Trp64Arg	Obesity and bodyweight-related disorders	Exercise responsiveness
GDF-8	K153R	Skeletal muscle-related disorders	-
SEP15	rs5859	Lung cancer	-
SEPP1	rs7579	Inflammation Cancer	Selenium availability and metabolism

Tab. I. *Continues.*

Gene	Polymorphism	Putative disease risks	Effect
BCMO1	rs1293492	Vitamin A deficiency	Low vitamin A levels
	rs7501331		
SOD2	rs4880	Breast and prostate cancers	-
ACSL1	rs9997745	Metabolic Syndrome	-
DNMT3B	rs6087990	Colorectal cancer Adenoma	High folate
	rs2424913		
	rs2424909		
ADAM17	rs10495563	Obesity	Low n-6 fatty acids
FAF1	rs3827730	Alcohol dependence	-
CSK	rs1378942	Hypertension	-
Intergenic	rs2168784	Alcohol dependence	-
NADSYN1	rs75038630	Abnormal eating behavior	-
OCTN1	C 1672T	Mushroom intolerance Crohn's disease	-
NBPF3	rs4654748	Vitamin B6 deficiency	Low vitamin B6 levels
TF	rs3811647	Low iron levels anemia	Increased iron concentrations
SLC23A1	rs33972313	Vitamin C deficiency	Low levels of vitamin C
BCDIN3D	rs7138803	Diabetes	-
CB1-R	rs1049353	Renal fibrosis Metabolic disorders	-
GNPDA2	rs10938397	Obesity risk	-
FGF21	rs838133	Metabolic disorders Diabetes	Increased carbohydrate intake Decreased fat intake
KCTD15	rs29941	Diabetes	Higher carbohydrate intake
NEGR1	rs2815752	Diabetes	Higher carbohydrate intake
TMEM18	rs6548238	Obesity	-
MAP2K5	rs2241423	Diabetes	-
QPCTL	rs2287019	Diabetes	-
TNNI3K	rs1514175	Diabetes	-
GSK3B	rs334555	Bipolar disorder Brain disorders	Response to antidepressant pharmacotherapy
	rs11925868		
	rs11927974		
FKBP5	rs1360780	Depression Post-traumatic stress disorder	Glucocorticoid receptor sensitivity
OXTR	rs53576	Post-traumatic stress disorder	Regulation of mood, anxiety and social biology
AKT1	rs2494732	Psychosis	Regulation of dopamine levels in the prefrontal cortex
ANK3	rs10994336	Bipolar disorder	Sodium channel activity Increased excitatory signaling
	rs1938526		
CACNA1C	rs1006737	Mood instability Depressive and bipolar disorder	Altered brainstem volume Increased excitatory signaling
CHRNA3	Asp398Asn	Cigarettes smoking	Neurotransmission
CHRNA5	rs16969968	Pleasure response from smoking	Neurotransmission
OPRM1	Asn40Asp	Addictive behavior	-
CNR1	rs2023239	Addictive behavior	Normal reward signaling
FAAH	C 385A	Addictive behavior	Difficulty with withdrawal
GABRA2	rs279858	Sedation Amnesia Ataxia Anxiety Insomnia Alcohol addiction	Improved GABA production

Tab. I. *Continues.*

Gene	Polymorphism	Putative disease risks	Effect
1A HTR1A	C -1019G	Depressive disorder Bipolar disorder	Reduced serotonin signaling at post-synaptic sites
SLC6A4	rs1042173	Addiction-related disorders	-

encing the person's susceptibility to nutrition-induced obesity [3]. The major genetic variants influencing metabolic pathways involved in the increased risk of obesity and obesity-related disorders are located in the following genes: *ADIPOQ, FTO, LEPR, LEP, MC4R, INSIG2, PPARG, PCSK1, ADBR3, ADBR2, PPARγ, APOA1, GHRL, APOA5, FABP2, LIPC, MTNR1B, TCF7L2, CETP, GIPR, NPY, IRS1,* and *PCSK1* (Tab. I) [2, 56, 57]. Candidate genes involved in the regulation of food intake, lipid metabolism, or release of intestinal hormones have been investigated. For example, the *FABP2* (fatty-acid-binding protein 2) gene, expressed in the epithelial cells of the small intestine, is involved in fat absorption. Genetic variants in this locus may cause higher fat absorption and obesity [58]. Similarly, the *PPARG* (peroxisome proliferator-activated receptor-gamma) gene is expressed in the fat cells and plays a major role in adipocyte differentiation. In their study, Deeb et al. [59] demonstrated an association of the *PPARG* gene with insulin sensitivity and body mass index. So far, almost 500 genetic loci have been identified in association with obesity traits, like waist-to-hip ratio or body mass index [60].

The *FTO* genetic locus that is associated with fat mass and obesity is considered to have the strongest effect upon body weight. The *TMEM18* (transmembrane protein 18) gene is also known to regulate appetite, body weight, and obesity development. Similarly, decreased expression of the *MC4R* (melanocortin-4 receptor) gene results in a monogenic form of obesity [41, 47, 61, 62].

Genetic polymorphism interaction with physical activity

Research studies have revealed the significance of diet in combination with physical activity for maintaining a healthy body weight. Genetic polymorphisms associated with obesity might influence physical activity levels; conversely, physically active lifestyles might reduce obesity risk. For example, sixteen interventional and cross-sectional research studies performed on children and adults of European, East African, and African origin reported a significantly strong association of *FTO* intron 1 with physical activity [61, 62]. Additionally, a recent meta-analysis involving 111,421 individuals of European descent established a significant association between physical activity and genetic risk score for twelve obesity-linked polymorphisms [63, 64].

Similarly, another meta-analysis involving 19,268 children and 218,166 adults found higher leisure-time physical activity reduces *FTO* variants effects, whereas increased sedentary periods, like watching TV, enhance genetic predisposition to increased adiposity [65]. In the US, the Diabetes Prevention Program involving 869 individuals reported a strong association of *FTO* genetic variants with one-year lifestyle intervention processes related to physical activity, weight loss, and diet with reference to the subcutaneous fat area. They found an association of the minor allele of an *FTO* variant with more subcutaneous fat mass within the control group as compared to the lifestyle intervention group. Similarly, another recent study indicated that physical activity, along with a vegetarian diet, could reduce elevated body mass index due to the minor allele of a variant in the *FTO* gene (rs3751812). Other physical activity-related genes are influenced by dietary intake and are involved in muscle strength and structure [66-68].

Additional studies have described the protective effect of physical activity on obesity-linked genetic variants in the form of a combined genetic risk score. In their study, Li et al. [69] have shown that the genetic susceptibility to obesity in individuals with higher genetic risk scores could be reduced by high physical activity levels [29, 69].

Conclusion

Every human being possesses an exclusive nutritional blueprint inside his/her genes. Bioactive food components and nutrients affect the expression of such genes. Nutrigenomics is the branch of science that analyzes gene-nutrient interactions, allowing the development of personalized nutrition approaches to maintain good health and prevent disease. Nutrigenomics combines different branches of science like nutrition, bioinformatics, genomics, molecular biology, molecular medicine, and epidemiology. Studies have revealed a myriad of interconnections at various levels amongst nutrients and genes. More specifically, genes regulate the intake and metabolism of different nutrients, while nutrients positively or negatively influence the expression of different genes at the epigenetic, transcriptional, and translational level. Nutrigenetic testing may soon become a fundamental technique to plan individualized weight loss and to better understand gene-nutrient interactions.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; AKK, GB, KD, JK, KLH, LS, FF, SN, MP, PC, FB, PG: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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REVIEW

Metabolomics application for the design of an optimal diet

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Keywords

Metabolomics • Mediterranean diet • Precision nutrition • Food metabolome • NMR

Summary

Precision nutrition is an emerging branch of nutrition science that aims to use modern omics technologies (genomics, proteomics, and metabolomics) to assess an individual's response to specific foods or dietary patterns and thereby determine the most effective diet or lifestyle interventions to prevent or treat specific diseases. Metabolomics is vital to nearly every aspect of precision nutrition. It can be targeted or untargeted, and it has many applications. Indeed, it can be used to comprehensively characterize the thousands of chemicals in foods, identify food by-products in human biofluids or tissues, characterize nutrient deficiencies or excesses, monitor biochemical responses to dietary interventions, track long- or short-term dietary habits, and guide the development of nutritional therapies. Indeed, metabolomics can be coupled with genomics and proteomics to study and advance the field of precision nutrition. Integrating omics with epidemiological and clinical data will begin to define the beneficial effects of human food metabolites. In this review, we present the metabolome and its relationship to precision nutrition. Moreover, we describe the different techniques used in metabolomics and present how metabolomics has been applied to advance the field of precision nutrition by providing notable examples and cases.

Introduction

Metabolomics is the study of small metabolites – such as amino acids, nucleic acids, lipids, or carbohydrates – and complex secondary metabolites present in biological systems inside the cells or extracellular fluids. Metabolomics uses analytical techniques, such as liquid or gas chromatography (LC or GC), mass spectrometry (MS), liquid chromatography-mass spectrometry (LC-MS), Fourier transformed infra-red spectroscopy (FTIR), and nuclear magnetic resonance (NMR) spectroscopy to create metabolomic profiles that enable identification and relative quantification of metabolites at a given time [1]. Metabolomic profiles are simply the secondary metabolites produced under extra- and intracellular environmental conditions. Qualitative and quantitative metabolomics compares and identifies specific metabolites produced under specific stimuli and generates metabolomic profiles based on various statistical methods. Metabolomics is becoming increasingly popular in nutritional research and provides a wealth of biological data, dependent upon intake of a specific diet, dietary pattern, age, gender, lifestyle, and health status [2]. Metabolomic approaches are deployed in nutritional research for the identification of metabolites in human bodies in response to certain dietary food regimens [3, 4], and the resulting metabolomic profiles are used to design personalized dietary and lifestyle interventions to improve overall health.

Precision nutrition

Precision nutrition is one of the most promising branches of nutritional sciences, combining genomics, proteomics, and metabolomics to identify an individual's metabotype and response to their dietary intake and lifestyle pattern [5]. Based on an individual's metabotype, tailor-made dietary regimes and physical activity plans are suggested to prevent or cure specific pathophysiological condition [6]. In addition, metabolomics is used to characterize thousands of chemical constituents of foods, to monitor biochemical response to specific food intake, to recognise food by-products in human tissues or biofluids, to identify nutrient deficiencies, to track dietary habits, and to devise nutritional regimes based on all obtained data [7, 8].

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Metabolomics and Nutritional Science

Metabolomics is the branch of analytical chemistry that studies metabolites, which are small biological molecules (molecular weight under 1,500 Da) found in cells, tissues, and/or biological fluids. Metabolomics is different than other omics sciences because it uses a variety of sophisticated instrumentation to obtain detailed information about metabolites. In contrast, transcriptomics, proteomics, or genomics are single instrument-based techniques, and therefore metabolomics provides a better insight into biological data [9]. Over the past decade, three main spectroscopic techniques have emerged as well-known metabolomics pillars: nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS) [10]. NMR identifies and quantifies high-abundance molecules, whereas the other two techniques are good at quantifying and detecting low-abundance metabolite molecules [11]. Overall, these spectroscopic techniques are the backbone for the identification of organic compounds, such as amino acids, lipids, organic acids, and amines. The myriad of literature available on metabolomics studies using these sensitive analytical advanced techniques authenticates their sophistication and diversity [10-12].

Targeted and untargeted metabolomics

Generally, metabolomics approaches are classified as targeted and untargeted. As stated by its name, targeted metabolomics deals with the identification of selected metabolites via cross comparison with their known standards, which in turn facilitates in developing biomarkers or hypotheses testing [13]. On the other hand, untargeted metabolomics mainly focuses on the discovery of novel, yet unknown compounds [14]. Given the high demand and rapidly growing interest in the identification and quantification of biologically active compounds, targeted metabolomics has a broad spectrum of applications, mainly in the diet and nutrition sector [13, 15], for example to identify nutritional disorders or deficiencies [16] and biomarkers of food intake (BFIs) [17], to analyze food composition, to estimate dietary intake [18] and to provide appropriate recommendations for chronic disease management [19].

Metabolomic tools and techniques

A large variety of spectroscopic techniques are employed as conventional characterization platforms in metabolomics studies, including Fourier transformed infrared (FT-IR) spectroscopy [20], high-performance liquid chromatography (HPLC) [15], mass spectrometry (MS) [21], and nuclear magnetic resonance (NMR) spectroscopy [22]. Ultra-performance LC (UPLC) is an advancement of conventional HPLC that operates at higher pressure, offering 2-3 times enhanced spectral sensitivity over conventional HPLC, alongside short measurement times and small analyte quantity requirement [23]. Mass spectrometry is sensitive in the detection of negligible analyte concentrations, but it requires laborious preliminary separation steps using GC/LC tools. On the other hand, NMR is preferred over the other spectroscopic techniques due to its non-destructive nature, high reproducibility, sample preparation feasibility, and both qualitative and quantitative modes of sample identification [24]. Nonetheless, weak NMR signal sensitivity in case of multicomponent analyte analysis is its main limitation [25], but it can be reduced by using cryogenically cooled probes, microprobes, and/or the dynamic nuclear polarization approach [26]. Overall, the huge diversity of metabolite structures – in terms of concentration, polarity, size, and stability – prevents the collective analysis of all metabolites using only one or two analytical techniques. Therefore, sequential coupling of different techniques has been proven to be beneficial to improve NMR signal. Currently, the main limitation of these coupled techniques is their cost-effectiveness, even though they will probably become the most prevalent metabolomics approach in future [27].

Metabolomics and comprehensive food characterization

The aim of precision nutrition revolves around the basic understanding of food composition and its correlated health benefits. Conventionally, food composition is analyzed in terms of macronutrient and essential nutrient content, but also by exploiting national food company databases (such as USDA or Health Canada) [28]. The few reported essential nutrients, however, do not cover the full spectrum of food composition, which refers to the micronutrient profile of a food product. Generally, the average fruit or vegetable consists of a cocktail of over 15,000 different components, belonging to over 100 chemical classes in variable concentration, ranging from 10^{-12}M (vitamins) to 10^{-3}M (sugars) [28]. These micronutrients impart basic properties to the food, including health benefits, food aroma, flavor, and color, which are due to polyphenols, terpenes, and pigments [29]. Metabolomics helps elucidate micronutrients present in food, thus enhancing our knowledge of various food constituents.

MS-NMR coupled spectroscopic metabolomics studies on a wide variety of foods (such as milk, banana, wine, beer, rice, tomato) have identified a vast majority of previously unknown nutrient species [10]. Moreover, these studies helped in developing food-nutrients/metabolome databases, including Phenol-Explorer, PhytoHub, and FooDB (Tab. I) [10, 30]. The statistics of these food metabolome databases is as follows:

- Phenol-Explorer: 501 polyphenols from 459 food varieties;
- PhytoHub: > 1,800 phytochemicals from 356 food varieties;
- FooDB, > 71,000 chemicals in nearly 800 food varieties [31].

All these databases act as a guide for nutrition scientists to develop precision nutrition and to understand the nutritional dynamics required to maximize expected health benefits.

Tab. I. Metabolite databases and repositories related to the food metabolome.

Database/ Repository	Website	Types of metabolites	Number of metabolites or foods	References
Food Metabolome Repository	http://metabolites.in/foods/	Food metabolites identified using LC-MS	222 food items analysed via LC-MS	[43]
FsDatabase	http://www.kazusa.or.jp/komics/en/tool-en/218-fstool.html	Flavonoids	6,867	[43]
HMDB	www.hmdb.ca	Microbial transformed Endogenous, and exogenous/ xenobiotic compounds identified in humans	Over 40,000	[42]
Exopome Explorer 2	www.ecmdb.ca	Dietary and pollution biomarkers	908	[40, 44]
FooDB	www.foodb.ca	Food constituents and additives	28,000	[28]
Phenol-Explorer	www.phenol-explorer.eu	Polyphenols in the diet	502	[45]
PhytoHub	www.phytohub.eu	Phytochemi-cals and their metabolites in the diet	1,500	[46]

Food metabolome

The term 'food metabolome' refers to the collection of all the metabolites of food that are derived by ingestion, digestion, and absorption. The term is broadly coined as 'human-food metabolome,' because humans consume a maximal amount of food metabolites [32]. Food consumed by humans contains approximately 25,000 compounds, which get further metabolized after ingestion, creating a complicated and extensive array of molecules [32, 33]. Nonetheless, the great diversity in human food metabolites is the biggest challenge in characterizing them completely: it can only be done by accurately monitoring dietary intake and any health effects defined in epidemiological and clinical investigations.

The food metabolome as part of the human metabolome

Human metabolomes are highly complex and vary depending on several factors, such as diet, health status, gender, age, genetic makeup, and physiology of an individual [34]. This is because humans, unlike laboratory animals, are free-living omnivores, and are exposed to multiple environments associated with a tremendous variety of ingested foods. Hence, the human metabolome comprises four different categories: endogenous metabolome (chemicals linked with cellular metabolism), food metabolome (derived from foodstuff), xenobiotics linked with drugs, and xenobiotics linked with environmental chemicals. The exact composition of the human metabolome is hard to ascertain; at least 50,000 detectable compounds have been identified in the human metabolome to date [35]. The composition of the human metabolome also varies depending on the type of biofluid and/or body part to which it is sampled from. For instance, the chemical composition of oral or gastric compounds is identical to the chemicals extracted from ingested food or drugs, whereas food constituents found in urine and blood are entirely different from the parent compounds because they get further metabolized into secondary metabolites in the liver, kidneys, or intestines. Sometimes the parent compounds get extensively metabolized and thus turn into end products, which are similar to chemicals naturally produced by the body. In addition, the gut microbiota is a massive contributor to the composition of the human metabolome [36]. Typically, vitamins, certain amino acids, and fatty acids are specific microbial metabolites; however, there are other metabolites derived from biotransformation of both endogenous and food metabolomes by the gut microbiota. Gut microbiota-mediated metabolites include secondary bile acids, amino acid metabolites, short-chain fatty acids, and plant polyphenol metabolites [37].

Metabolomics and dietary biomarkers

One of the important preludes of precision nutrition is to have a detailed understanding of an individual's diet and overall dietary status. Traditionally, assessing the nutritional status of an individual was done using several means, such as nutritional assessments, such as surveys, dietary diaries, 24 h dietary recalls, and food frequency questionnaires; however, these methods present several limitations. These include, but are not limited to, deliberate deception in reporting dietary intake, recall bias, memory lapses, and difficulties in estimating portion sizes. These limitations can lead to incorrect or inconsistent data collection, which leads to ambiguity in identifying dietary biomarkers, thus highlighting the necessity to deploy analytical tools that can correctly measure an individual's dietary intake and facilitate corresponding BFI detection. In this regard, a major initiative was launched in 2013: the so-called Food Biomarker Alliance, also known as FoodBAll [38].

Food Biomarker Alliance (FoodBAll)

The aim of FoodBAll was to use metabolomics in BFI identification and to create an inventory of metabolite biomarkers in biological fluids produced after intaking

a specific food [38]. This inventory helps to elucidate the metabolites produced in the human body as a response to dietary intake as well as overall metabolism. Understanding the metabolism of foods with respect to its type, quantity, and metabolic rate is a key to precision nutrition: such detailed information helps nutritional scientists and dieticians to tailor personalised dietary regimes both for healthy individuals and for patients to improve their overall health and wellbeing.

The members of the FoodBAll consortium identified several BFIs for various classes of foods and developed protocols and definitions for their identification and validation [39]. The BFIs have now been listed in dedicated databases, such as Exposome-Explorer [40], MarkerDB [41], and Human Metabolome Database (HMDB) [42].

Dietary biomarker discovery using dietary patterns

Dietary pattern analysis aids scientists and dieticians in gaining a broader insight into an individual's dietary intake, food preferences, and eating habits. Dietary pattern analysis encompasses the quantities, proportions, variety, and combinations of consumed foods/beverages as well as consumption frequency.

Identification of dietary biomarkers involves the combination of dietary and metabolomic patterns, which are analysed by applying chemometrics coupled with multivariate strategies to develop models for food intake behavior and metabolic patterns [47]. Both supervised and unsupervised methods can be used to identify similarities and differences in detected metabolites. The data are then subjected to various cluster analyses – such as hierarchical clustering – to identify similar groups [48]. Supervised learning methods, such as partial least-squares discriminant analysis (PLS-DA) and partial least squares regression (PLSR), are used to identify food metabolites that act as biomarkers to predict diet-related metabolic patterns [49, 50].

Food metabolites have an influential effect on human health. A person's metabolomic profile reflects the overall metabolic state under a particular environmental/pathophysiological condition and changes with respect to changes in said condition. In addition, an individual's metabolomic profile is dependent upon overall genetic makeup, phenotypic expression of genes, and dietary intake [51]. Here, dietary intake and choice of food play a dual role: not only do they affect the type of metabolites being produced, but they also influence the gut microbial community and the way this microbiota will metabolize the diet [51, 52]. It has been widely accepted now that gut microbiota plays a pivotal role in maintaining overall gut health, and changes in the gut microbiota may give rise to metabolic disorders and initiate or aggravate non-communicable diseases such as obesity, diabetes, or hypertension [53].

The gut microbiota is comprised of 1,000 different species of approximately 10^{14} individual microbes, with a total biomass of 2 kg [54]. These microbes not only help in metabolism and absorption of micronutrients, but they modulate the host's immunity against pathogens as well [55]. Lactic acid bacteria and Bifidobacterial species residing in the human gut are essential for human health, as they synthesize vitamin K and several B vitamins, like thiamine, biotin, folates, cobalamin, pantothenic acid, nicotinic acid, pyridoxine, and riboflavin [56]. A cross talk exists between human gut microbiota, dietary intake, and the way it is metabolized: for instance, dietary intake influences the microbial community structure in the gut. In turn, the microbial community in the gut affects the way food components are metabolised and absorbed. The result of this cross talk determines the overall metabolomic state of an individual, which greatly influences their health and wellbeing [57]. Moreover, dietary changes affect the functionality of gut microbiota, thus increasing human dietary flexibility. In addition to dietary intake, the microbiota is affected by an individual's genetics, environment including psychology, bacteriophage action, and use of antibiotics or other treatments [58-61].

Untargeted metabolomics has been used to evaluate the physiology and metabolomic profile of gut microbiota in response to intake of dietary supplements (Table II). A recent study has reported the effect of seven dietary supplements on a consortium culture of bacteria containing *Blautia producta*, *Bifidobacterium longum*, *Anaerostipes caccae*, *Clostridium ramosum*, *Bacteroides thetaiotaomicron*, *Clostridium butyricum*, *Lactobacillus plantarum*, and *Escherichia coli*. GC-MS analysis of the dietary supplement's metabolism by the consortium (in comparison to placebo) detected 131 metabolites, which included organic acids, fatty acids, nucleic acids, amino acids (the predominant class of metabolites), phenolic compounds, steroids, sugars, alcohols, and inorganic nitrogenous compounds [54]. The study indicated a modulatory effect of dietary supplements on the microbial community and on gut metabolism, inhibiting or inducing specific metabolic pathways.

Moreover, metabolomics can help identifying food-induced shifts in metabolites, thus providing useful information about an individual's diet. For instance, an intervention study classified two dietary patterns, the New Nordic Diet (NND) and the Average Danish Diet (ADD), using untargeted metabolomics coupled with multivariate analysis, with a low misclassification error rate (19%) [62]. This reveals that untargeted metabolomics can be used as a powerful screening tool to estimate compliance to a certain dietary pattern [62]. A similar study was conducted to explore the effects of an isocaloric Mediterranean diet (MD) intervention on overall metabolic health, systemic metabolome, and gut microbiome in individuals having lifestyle risk factors for metabolic diseases. The results revealed that switching to the Mediterranean diet whilst retaining the overall caloric intake resulted in changes in the gut microbiome as well as the metabolites in urine, with a marked reduction in blood cholesterol levels and an improvement in overall health [63].

In another study, 1H NMR was used to analyse the urinary metabolome of 1,848 Americans, which revealed 46 metabolites that can help differentiate healthy and unhealthy individuals. These metabolites indicated the correlation of vitamin C, glucose, and fructose with

Tab. II. Targeted and untargeted metabolomic approaches for food metabolites biomarker identification.

Techniques	Purposes of the study	Study groups	Findings	References
Targeted Metabolomics				
Fluorescence spectroscopy	Comparative analysis of the effects of dietary levels of proline betaine on glycine betaine excretion, homocysteine, and betaine concentrations in plasma	8 healthy males Age: 18-50	Proline and betaine had little effect on plasma total homocysteine concentrations in healthy humans	[65]
Mass spectrometry	Blood metabolites that correlate red meat consumption to the onset of type 2 diabetes	790 males and 1,257 females, including 801 with type 2 diabetes Age: 35-64	Six biomarkers were linked to elevated red meat consumption and diabetes risk	[66]
Mass spectrometry	Analysis of demographics, dietary habits, and metabotypes	740 males and 760 females Age: 18-90	Two subgroups identified for postprandial insulin levels and fasting metabolic profile	[67]
Targeted mass spectrometry	Effect of Western dietary patterns on metabotypes	16 females and 21 males Age: 18-50	Western dietary pattern with high saturated fat intakes resulted in higher levels of short chain acylcarnitine and amino acids as dietary biomarkers	[68]
Untargeted metabolomics				
NMR	Identification of coffee consumption biomarkers	7 females and 1 male Age: 28-45	Identification of putative biomarker 2-furoylglycine	[69]
Mass spectrometry	Characterization of dietary walnut fingerprinting	275 subjects, both male and female Age: 55-80 (males) and 60-80 (females)	Identification of 18 markers of fatty acid metabolism and intermediate metabolites of the tryptophan/serotonin pathway	[70]
Mass spectrometry	Compliance tool development based on metabotyping strategies to compare Average Danish Diet vs New Nordic Diet for 6 months	79 females and 28 males Age: 18-65	Identification of 22 unique food markers for 7 food groups (chocolate, cabbage, beetroot, citrus, green beans, strawberry, and walnut)	[71]

biomarkers of citrus fruit consumption, such as 2-hydroxy-2-(4-methylcyclohex-3-en-1-yl) propoxy glucuronide, 4-hydroxyprolinebetaine, and proline betaine. In addition, these metabolites highlighted the association of calcium and sodium with citrate, and formate with hypertension, renal function, and adiposity [64].

These studies indicate the potentials of metabolomic approaches in revealing the metabolomic status of an individual in response to dietary intake and pathophysiological conditions, in detecting gut microbial changes, and in applying precision nutrition.

Metabolomics and customised diet design

The most impressive use of metabolomics data in nutrition sciences is perhaps the design of tailor-made diets, based on an individual's metabolomic profile, dietary preferences, gut microbiome, lifestyle, and pathophysiological status. In addition to that, the integration of the other omics technologies provides an excellent platform for prevention and management of metabolic disorders like diabetes, obesity, hyperlipidemia, and hypercholesterolemia [72]. For instance, one study reported the integrated use of metagenomics, metabolomics, and precision nutrition coupled with machine learning to devise a dietary regime to manage postprandial blood glucose levels. The algorithm was based on anthropometric data, physical activity, metabolomic-based blood parameters, self-reported dietary intake, and gut microbiome composition of 800 participants considered healthy or prediabetic [73]. A high interpersonal variability was observed in postprandial glycemic responses of participants, irrespective of the fact that they were given the same food. Based on these findings, DayTwo Inc., the first precision nutrition company, was established, with the aim to design custom diets for prevention and control of prediabetes [74].

Similarly, another study used LC-MS based metabolomic profiling on blood samples from 40 healthy adults with normal blood sugar levels to predict their risk of acquiring type 2 diabetes, resistance to insulin, and associated comorbidities. Based on their metabolomic profiling, subjects were given customised diets, nutritional supplements, physical activity, and lifestyle recommendations for 100 days. The follow-up metabolomic analysis after 100 days, showed a significant decline in the risk of developing type 2 diabetes and associated comorbidities [75].

These and similar studies indicate that precision nutrition guided by metabolomic profiling is a promising arena for further research. Thus far, most of the metabolomic studies had focused on identifying metabotypes and metabolomic markers associated with obesity, diabetes, and metabolic disorders. Further studies in this regard will enable scientists and dietitians to design customised diets, based on an individual's metabolomic status for achieving better health and controlling lifestyle-mediated diseases.

Conclusions

Metabolomic studies coupled with genomics, proteomics, and multivariate analysis, provide an excellent platform for new advancements in the field of precision nutrition. Not only does this approach generate a repertoire of novel human food metabolome biomarkers, but it will also greatly enhance the development of molecular nutritional epidemiology, thus contributing to a better prescription of dietary regimes and physical activity for managing a healthy lifestyle and preventing and curing lifestyle-mediated diseases. In addition, precision nutrition not only brings hope for patients suffering from various metabolic disorders, but also provides nutritionists with a tool for designing diets that match the nutritional requirements, metabolic function, and gut microbiota to achieve maximum benefits.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; SC, KD, MCM, MS, KLH, BA, VV, GM, FF, AI, MAP, LDG, EG, PC, SN, STC: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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Review

Physical activity for health

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Keywords

Physical activity • Med Diet • Health • Non-communicable diseases

Summary

Physical activity plays a substantial role in maintaining people's good health and mental wellbeing, but that is not all: not only it positively affects the individuals' mental and physical health, but a lack of physical exercise exerts a negative impact also on the overall economy of a nation. In addition, physical inactivity not only increases the risk of non-communicable diseases (NCD), but also contributes significantly to the increased morbidity and mortality in patients suffering from these diseases. On the contrary, physical activity reduces the risk of NCDs – such as cardiovascular diseases, type 2 diabetes, and cancer – in a dose-dependent manner; regular exercise is also associated with many health benefits and delayed mortality. However, understanding the role of physical activity in modern society and creating an awareness in the general population is one of the most important tasks of health and recreation promoters. Correspondingly, there is a dire need to enhance our knowledge, perception, and awareness of physical activity and its impacts on an individual's health, ultimately contributing to developing a healthy society. The current review will focus on the health benefits of the two most widely studied modifiable lifestyle risk factors, physical activity and diet, focusing particularly on the Mediterranean diet.

Introduction

The conventional Mediterranean diet (Med Diet) – which includes olive oil, fresh vegetables, fruits, legumes, nuts, fish, and red wine, and is characterized by minimal consumption of red/processed meats, fat-containing dairy products, and sweets – was proven to have several health benefits for humans, including a marked reduction in chronic diseases and improved health [1]. On the other hand, also physical activity (PA) has been proven a booster of healthy life. Accordingly, the synergistic effects of Med Diet and physical activity are apparent from several observational studies, as well as from personal experience of people belonging to different geographic area. Both Med Diet and physical activity have been reported to improve longevity and quality of life. For instance, several studies showed that Med Diet has an inverse relationship with total mortality [1-3]. Also, there are strong inverse associations between the consumption of foods following the principles of the Med Diet and cardiovascular mortality [3]. Correspondingly, synergizing physical activity with Med Diet reduces the risk for incidence and progression of cancer, mortality, and CVD. A recent study, which hypothesized the combined effect of Med Diet and physical activity on the reduction of total mortality, utilized data from the cohort of Seguimiento Universidad de Navarra (SUN), which had 19,464 participants who were followed up between December 1999 and February 2016 [4]. The study reported that the synergistic effects of both PA and Med Diet reduced the hazard ratio (HR) and respective mortality. Furthermore, Med Diet alone lowers the mortality rate up to 95%, with multivariable adjusted HR 0.66. This effect is further enhanced when combined with high or moderate physical activity: in this case, the HR is reduced to 0.36 [2].

The positive impact of PA on health has been known for centuries. For instance, the ancient philosopher Plato emphasized the importance of PA by stating that "Lack of activity destroys the good condition of every human being, while movement and methodical physical exercise save it and preserve it". In fact, this statement is as valid today as it was in ancient times. The modern lifestyle not only has led to decreased PA in doing the normal day-to-day chores, but also promoted human beings' dependency on machines: for instance, people do not need to walk to work or school, they depend on vehicles instead. This lifestyle has brought many comforts, of course, but at the expense of people's physical wellbeing: the reduced PA has raised great concerns about public health and wellbeing. In fact, decreased PA, also coupled with diet regimes rich in fast and fried foods, has increased the obesity rates to alarming levels more or less worldwide [5, 6]. In addition, this has resulted in an increased incidence of non-communicable diseases such as diabetes, cancer,

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cardiovascular problems, inflammatory diseases, thus leading to increased morbidity and mortality [3-6]: lack of PA has become the fourth leading cause of mortality [7]. Not only this is an alarming situation, but it also has a huge socioeconomic impact on the healthcare system, with nations spending millions of dollars as maintenance cost of physically inactive citizens and in PA promotion programs. Therefore, it is direly needed to make the public realize the importance of physical activity, alone or in combination with healthy dietary regimes like the Med Diet, so that the full potential of both can be reaped for shaping a healthy and active lifestyle.

Physical activity and health

Physical activity can be defined as 'any bodily movement generated by skeletal muscles at the expense of energy utilization': lifting, working out, playing, travelling, walking, cycling, dancing, gardening, housework are all examples of PA [7]. The compendium of PA is helpful in estimating the metabolic intensity of activity, as compared to a resting stage, in terms of metabolic equivalent tasks (METs) [8]. METs are used to categorize activities, for example sedentary or inactive (such as watching television, lying in supine position, desk work, light intensity or effortless behaviour grocery shopping, slow walk), moderate intensity (such as slow cycling, lawn mowing), and energetic activity (such as fast cycling, jogging, running) (Tab. I). Apart from PA levels, also the individual's genetics influence PA results and the onset of several NCDs, among which obesity and cardiovascular diseases [9-13]. Several studies have reported that physical inactivity leads to an enhanced risk of all-cause mortality, overall poor health, and lower life expectancy, with high rates of morbidity and mortality in patients with underlying chronic diseases such as hypertension, diabetes, CVD, chronic obstructive pulmonary disease (COPD), especially when the patient's BMI is above 30, indicating obesity [14-16]. These studies have also pointed out that a minimum increase in physical activity, such as adding an hour of walk weekly, could significantly improve the overall health and reduce the risk of mortality. Furthermore, vigorous or high intensity physical activity not only stimulates the body systems, but also exerts positive effects on overall health condition. For instance, strong aerobic or endurance activities (like swimming or fast running/walking 150 minutes per week) not only reduce high blood pressure and type 2 diabetes, but also improve the lipid profile and decrease the risk of cardiovascular diseases (Tab. II) [17-19]. Additionally, weight endurance and weight-supporting exercises such as skipping, jumping, weight training, and using playground equipment, lead to the development of a healthy bone mass, bone health, muscular power, and musculoskeletal fitness [20].

Effects of physical activity on cardiovascular diseases

Physical activity does wonders on heart health and circulation. This can be assessed by a simple fact: men are less prone to serious heart disease than women are, because generally they are more physically active. Physical activity, however, reduces 30-40% CVD risk in women [17, 24, 29, 31]. Regular exercise will attenuate chronic heart diseases by improving the cardiovascular system (the heart contraction and relaxation is refined with effective blood pumping and circulation) and by increasing lung capacity to facilitate oxygen intake and to improve dilation of blood vessels.
Additionally, altering blood lipid profiles by increasing ratio of protective high-density lipoprotein (HDL) to low density lipoprotein (LDL) and an enhanced usage of fat as fuel. This leads to a reduction of heart disease and stroke risk factors, such as high blood pressure and abnormal blood lipid profiles [32-34].

Effects of Physical activity on Type 2 diabetes

Type 2 diabetes is mostly seen in adults over 40 years, but also in children and young people having a seden-

Tab. I. Classification of physical activity levels in terms of METs and their corresponding risk of mortality in case of underlying chronic diseases.

Metabolic equivalents (METs)	Classification of activity	Examples of activity	Risk of mortality in case of underlying chronic diseases and High Body mass Index (BMI) > 30*
1.0-1.5	Sedentary or inactive behaviour	Lying in supine position, meditating, desk work, watching TV, listening to music without any physical activity	High (risk rate: 2.0-2.5 on a 4 scale)
1.5-2.9	Light-intensity activity	Slow walk, cooking, gardening, washing, arts and crafts, playing an instrument, slow walking, fishing, light yoga	High (risk rate: 2.0-2.5 on a 4 scale)
3.0-5.9	Moderate-intensity activity	Slow dancing, cycling at a speed of less than 10 mph, vigorous cleaning, mowing the lawn, painting of walls, dancing, exercise class	Moderate (risk rate: 1.0-1.5 on a 4 scale)
> 6	Vigorous-intensity activity	Weightlifting, laborious jobs (such as carrying heavy loads or farming), fast dancing, cycling at more than 10 mph, running more than 4 mph	Low (risk rate: < 1.0 on a 4 scale)

*Based on a study conducted by Myers et al., 2002 [14] and reviewed by Warbuton et al., 2006 [15].

Tab. II. Effects of physical activity on cardiovascular conditions.

Conditions	Subjects	Exercise/ physical activity	Results	Reference
Cardiovascular (CV) diseases and Cancer	10,224 men 3,120 women	Variables acquired from maxETT	Higher fitness level and decreased CV and cancer mortality were related to higher fitness levels	[17]
Cardiovascular diseases	4,276 men	Variables acquired from maxETT	Men with lower fitness levels had a higher risk rate (2.7) of dying from CV	[18]
Cardiovascular and all-cause mortality	1,960 men	Workout using bycycle ergometer	High fitness is linked to lower all-cause mortality and CV mortality	[19]
Chronic heart disease	12,138 men (MRFIT trial)	Self-informed physical activity during leisure time	63% reduction in fatal CHD observed because of moderate physical activity during leisure time as compared to lower leisure time PA	[21]
Cardiovascular (CV) diseases	12,516 men	Calories burned (kJ/week)	Burning more calories/week resulted in reduced risk of CVD in men	[22]
Cardiovascular (CV) diseases	10,269 men	> 4.5 METs Regular exercise	23% reduction CV and all-cause mortality with moderately vigorous PA, as self-reported by Harvard alumni	[23]
Risk of coronary events	72,488 women Nurses' Health Study	Total PA (walking, vigorous exercise)	Inverse relationship between total PA and coronary events: the higher the PA, the lower the risk of developing coronary events (even in women starting PA later in life)	[24]
Coronary heart disease	39,372 women	Total calories burnt by walking	Time spent on walking/week has an inverse effect on the risk rate of CHD	[25]
Coronary heart disease	9,758 men	Net energy expenditure during leisure-time	Lower risk of hard CHD events associated with high leisure time energy expenditure	[26]
Coronary heart disease	44,452 men	Various exercises such as walking, running, weight-training, rowing	Intense exercise, with a higher MET associated with reduced CHD risk	[27]
Cardiovascular (CV) diseases	6,213 men	Variables acquired from maxETT	In CVD patients, death risk in subjects having exercise capacity < 5 METs was approximately double than those who could exercise > 8 METs	[14]
Myocardial infarction	15,152 MI cases vs. 14,820 controls	Self-reported PA	Regular physical activity diminished the risk of myocardial infarction	[28]
All-cause premature death	9,777 men	Variables based on maxETT	Fit men had a lower risk rate of all cause and CVD death rate between follow up intervals than the unfit men	[29]
Diabetes mellitus and hypertension	2,478 men and women (18–30 yrs)	Walking duration on treadmill during ETT	Low fitness level associated with high risk of developing DM, HTN, and metabolic syndrome	[30]

tary lifestyle and obesity. Studies have shown that PA enhances blood glucose control and slows down the onset of type 2-diabetes [35] in both men and women [36]. Studies also show that, rather than sedentary activities, physical activity reduces the risk of type 2-diabetes in both men and women. High risk individuals with obesity and inheritee or impaired glucose tolerance, can reduce their risk of having type 2-diabetes risk by brisk exercises [37]. Physical activity reduces risk of diabetes by long-term and short-term improvements in insulin action

for better glucose control. In older men, exercise training for two months shows significant improvement in insulin sensitivity and fasting glycaemia [37].

Effects of physical activity on weight management and obesity

Obesity has a strong correlation with physical inactivity. It's a common observation that people having sedentary lifestyle have a lower metabolic rate and tend to gain weight over time and become obese while those having regular exercise, walking or other forms of physical activity have a higher metabolic rate and are lean. In addition, less physical activity means less energy expenditure, which results in weight gain over time and vice versa [38]. Obesity has turned into an epidemic nowadays, with nearly half of the world population being obese [39]. For instance, nearly three-fourths of the adult population in the USA are obese. Similar trends exist in other westernised countries and, according to the EU, countries estimate that in 2008 23% women and 20% men are overweight or obese in the European countries [40]. In addition, a gradual increase in obesity has been observed also in children and adolescents in Europe [41]. These increasing trends in obesity and being overweight are largely dependent upon the physical inactivity as well as on dietary habits. Westernised food regime, with fried and fat-rich food coupled with high sugar and salt intakes, less vegetables and fruits more red meat are the contributory factors of high BMI in half of the western population. Besides that, inadequate physical activity with more sedentary lifestyle is the major cause for increased obesity and overweight in Europe. Physical activity coupled with proper food intake has an inverse effect on weight gain and obesity. High physical activity leads to high energy expenditure and, correspondingly reduction in stored fats (adiposity) and lower BMI. In addition, this helps to lower diabetes and high blood pressure, and also improves lipid profile that ultimately reduces the risk of developing NCDs [42-49].

Effect of Physical activity on Cancer

Cancer has become a major concern because of the sedentary lifestyle of the European population. Cancer is a leading cause of death in Europe, existing health-care programs must be improved, and new initiatives must be developed to raise awareness of the role of PA in reducing cancer risk. Cancer is becoming the leading cause of death in Europe. Physical activity plays a significant influence in cancer risk reduction [50]: physically active men have a 30-40% lower risk of colon cancer, whereas physically active women have a 20-30% lower risk of breast cancer [50]. Physical activity lowers the risk of cancer through reducing long-term inflammation in the gut, which aids in the reduction of colon cancer, improves the immune system's ability to fight cancer, and improves hormone balance [44].

The effect of physical activity on various cancers is mentioned below:

- **Colon cancer:** Individuals who exercise regularly have a 40-50% lower risk of colon cancer than those who do not [51];
- **Breast cancer:** Women who are involved in vigorous physical activity can minimize up to 30-40% risk of breast cancer in both premenopausal and postmenopausal active women [52, 53, 54]. Also, if women increase physical activity after menopause, they are less prone to breast cancer [54, 55];
- **Bladder cancer:** People who participated in recreational activities had a 15% decreased risk of bladder cancer [56];
- **Lung cancer:** Physical activity can reduce the incidence of lung cancer in both smokers and non-smokers, according to meta-analysis research [57, 58];
- **Endometrial cancer:** Obesity is the leading cause of endometrial cancer, and it can be prevented with regular exercise. Endometrial cancer risk is reduced by 20% in highly active women [59];
- **Uterine cancer:** Uterine cancer risk was decreased in women who engaged in more physical activity. There is a lower risk of uterine cancer in more active women [60];
- **Oesophageal cancer:** Increased physical exercise lowers the risk of esophageal cancer by 21% [61];
- **Renal cell cancer:** Renal cell cancer risk is reduced by 12% in people who engage in vigorous physical activity [62];
- **Gastric cancer:** Individuals who were the most physically active had a 19% lower risk of stomach cancer than those who were the least active [63].

Effects of Physical activity on Musculoskeletal health

Regular exercise promotes bone density, healthy joints, strong muscles, tendons and ligaments, as well as optimum growth and development. Additionally, it will develop functional ability for elderly individuals to lift, carry, climb stairs, etc. and lower the risk of osteoporosis and hip fracture [44, 64]. Physical activity can have beneficial effects on several musculoskeletal diseases (Tab. III).

Effects of Physical activity on Psychological health

Everyone's mental state can indeed be enhanced by exercise, which can lessen the effects of depression by elevating mood and sensations, it [80, 81]. Additionally, it can improve awareness of stress and sleep efficiency. Physical activity helps young people's cognitive, learning, and judgmental abilities, and their academic performance [82]. It will strengthen older peoples' cognitive abilities, including short-term memory, planning, and decision-making [83]. Mental health can

Tab. III. Effects of physical activity on musculoskeletal diseases.

Diseases	Condition	Symptoms	Treatment	References
Fibromyalgia	Ailment that causes pain throughout the body. Broad, diffuse, non-inflammatory, treatment-resistant joint and muscle aches, lasting at least three months	Reduced muscle strength, rapid fatigue	Aerobic training	[65-67]
Osteoarthritis	A degenerative condition that progresses over time and frequently causes severe discomfort	Discomfort, stiffness, sensitivity, loss of flexibility bone spurs, a grating sensation, and swelling	Physical exercises or aerobic exercises training, Non-Steroidal Anti-inflammatory Drugs (NSAID)	[68-72]
Osteoporosis	A condition where the quantity and thickness of bone tissue declines	Fractures resulting from fragility, loss of height, gum recession, stooped posture, lower back pain	Weight bearing exercises, walking, sunlight exposure for vitamin D production, bone and muscle strengthening exercises	[73-75]
Rheumatoid Arthritis	A chronic disorder that affects the joints and results in pain, swelling, and stiffness	Morning stiffness, joint discomfort, tenderness, and edema	Regular low intensity exercise	[71-74]

be enhanced by exercise, as reported by many studies: physical activity involves moving one's body and using their muscles, for example for walking, running, dancing, swimming, practicing yoga, or working in the garden [84]. The length of exercise regimens appears to mitigate the association between physical activity and psychological health, as lengthier programs regularly report. Mild to moderate mental health disorders, including depression and anxiety, may be effectively managed by engaging in physical activity. Increased aerobic exercise or strength training has been proven to dramatically improve depressive symptoms, even though people with depression often engage in less physical activity than those who do not suffer from it. Moreover, regular exercise appears to have benefits comparable to those of meditation or relaxation for anxiety symptoms and panic disorder [85].

Current physical activity levels

Modern technologies are currently helping people in doing less physical labour; for example, using a private vehicle lessens the need for physical activity-based transportation, like walking or cycling. The introduction of television, computers, and other electronic leisure gradually made engaging in sedentary activities more appealing than exercising, particularly for young people. Accelerometers are the accepted methods of monitoring physical activity to evaluate people's transportation, household, and leisure activities. Low-income and middle-income countries are more active than high-income ones in terms of cycling and walking [86-88]. In the future, low- and middle-income countries will reduce their levels of physical activity because of technological changes [89].

The method used to estimate an activity's energy expenditure is called METs. According to MET hours, physical activity in the UK decreased by 20% between 1961 and 2005. Eurobarometer surveys are used to monitor the levels of sports and physical activity. The survey reveals that in Europe the physical activity falls with age and men are more enthusiastic than women. Correspondingly, well-educated individuals are more active than less educated ones. The Northern part of Europe is more active as compared to the Southern part, mostly engaging in activities like cycling, dancing, and gardening (particularly interesting to nations like the Netherlands and the Nordic region), while walking is more prevalent in Southern and Eastern Europe. According to the results of the overall survey, one in ten Europeans are unusually sedentary and unable to walk for 10 minutes each day [90].

The WHO public health recommendations for physical exercise are not being followed by about one third of Europeans [87, 90]. A according to the "Health Behaviour in School-aged Children" (HBSC) research, an average of over 45% of girls and 2/3 of boys are unable to meet the recommended 60 minutes per day of moderate activity, with girls being less physically active than boys, according to reports (Tab. IV) [91]. Furthermore, accelerometers showed that 11-year-olds exercise more than 15-year-olds.

Socioeconomic effects of physical activity as opposed to physical inactivity

Brain development, emotional and social health, job performance, and productivity are all enhanced by physical activity, resulting in the improvement of life skills like grit, self-control, punctuality, emotional regulation, decision-making, and goal-setting [94].

Physical activity-related risks typically involve the musculoskeletal system, such as straining a mus-

Tab. IV. Recommended PA for various age groups.

Age group	Recommended PA	Reference
Preschool children (3-5 years)	Physical activity throughout the day	[92]
Children and Adolescents (6-17 years)	Daily 60 minutes or more PA	[93]
Adults (18-64 years)	150 minutes moderate intensity PA weekly	[93]
Older adults (65 years and more)	At least 150 minutes of moderate-intensity aerobic PA weekly	[93]
Adults with chronic disabilities	150 minutes of moderate intense aerobic activity in a week	[92]
Pregnant and postpartum women	150 minutes of moderate intense aerobic activity (brisk walk)	[92]

cle or twisting a joint. These issues are brought on by performing too many activities without adequate warm-up or training. A person with heart disease runs a higher risk of having a heart attack if they participate in vigorous activity without warming up first. These hazards can be reduced simply by appropriately warming up before starting the more intensive exercises [95, 96]. To prepare the body for the increased demands on bones, muscles, the heart, and the lungs, muscle-strengthening exercises should be done gradually over time [97].

Mediterranean diet and physical activity

One of the most important factors that affect health globally is the lack of physical activity [97]: physical inactivity is in fact a major risk factor for the development of chronic diseases and early death, as demonstrated by a wealth of research on the benefits of PA on health. The aging process will eventually be negatively impacted if PA or exercise is replaced with inactivity or sedentary behaviour [98]. The two most widely studied modifiable lifestyle risk factors, diet and PA, increase the risk of developing lifestyle diseases like CVDs, obesity, type 2 diabetes, and several malignancies, as well as their mortality and morbidity. In clinical practice, diet and PA are usually advised for promoting general health, weight loss or maintenance, preventing chronic diseases, and improving quality of life [99], being regarded as intricate variables that may interact [100]. When compared to the MedDiet or PA alone, there is already data to suggest that greater adherence to both is linked to improved health biomarkers, a lower risk of disease, and a lower mortality rate [100, 102]. If the person avoids smoking and alcohol abuse, the Med Diet may lower the risk of non-communicable illnesses, improve health status, and lower total lifetime healthcare expenses [103]. When combined with PA, these benefits may be even greater. This interaction of effects implies that lowering either element would also lower the likelihood that the other factor would result in a particular outcome. The presence of interaction demonstrates that the effect of the two exposures is different from the mere sum or multiplication of their individual effects, depending on the nature of the link between exposures and the anticipated scale (additive or multiplicative) for the interaction [104].

Conclusion

It can be concluded that not only physical activity is beneficial for having a healthy life, but it is indeed essential to alleviate drastic consequences of various chronic non-communicable diseases. Recommended level of physical activity, coupled with a healthy diet such as Med Diet, can help to restore and maintain a healthy body and, correspondingly, an ecstatic life.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; KD, ZN, MCM, FF, PC, MAP, SN, PM, SX, MB, DB, STC, KLH: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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Review

Dietary supplements for obesity

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Keywords

Dietary supplements • Obesity • Weight loss • Nutritional disease

Summary

Obesity and associated complications including diabetes, cardiometabolic dysfunction, disability, malignancy and premature mortality are considered epidemic. Research on obesity is therefore of worldwide importance. The development of obesity is a multifactorial phenomenon with contributions from biological, behavioral, genetic and environmental factors. Obesity and its associated issues require various lifestyle modifications and treatment options such medication, exercise, diet, surgery, pharmacological therapy and dietary supplements. Dietary supplements are considered an attractive alternative to traditional therapy due to their low toxicity profile and their accessibility to the general population. Dietary supplements may include one or more dietary ingredients. In this narrative review, we analyze the effects on obesity and obesity-related issues of various natural components. For example, there are a myriad of supplements that have been used as dietary supplements for weight loss such as minerals, vitamins, amino acids, metabolites, herbs, and plant extracts. This narrative review aims to present the benefits and side-effects of several ingredients of dietary supplements for weight loss and treatment of obesity. In particular, the mechanism of action, results of clinical trials, and possible side effects will be presented for the following ingredients: β-Glucans, bitter orange, calcium, vitamin D, chitosan, chromium, cocoa, coleus forskohlii, conjugate linoleic acid, ephedra sinica, fucoxanthin, garcinia cambogia, glucomannan, green coffee, green tea, guar gum, raspberry, hoodia gordonii, irvingia gabonensis, phenylpropylamine, pyruvate, white kidney bean.

Introduction

Obesity is one of the most prevalent nutritional diseases in industrialized countries and is recognized as a significant public health issue. By definition, obesity is the excessive accumulation of adipose tissue. Excess body weight is classically determined on the basis of a person's BMI (body mass index): BMI = weight (kg)/height (m)^2. A BMI of 30 kg/m^2 is recognized as the obesity threshold, while a BMI comprised between 25 and 29.9 kg/m^2 is used to identify overweight people; BMIs over the obesity threshold are associated with higher morbidity and mortality rates and a wide range of symptoms. Excessive weight gain is considered a multifactorial phenomenon that involves variably interacting biological, behavioral, genetic, and environmental factors [1-4].

Obesity and associated complications like cardiometabolic dysfunction, diabetes, disability, malignancy, and premature death are considered epidemic in the western world [5-7]. Treatment of obesity is therefore of major significance for researchers around the world. In the United States, the obesity rate continues to increase, and worsened during the COVID-19 pandemic [8]. Obesity, overweight and associated issues call for preventive and treatments approaches. Indeed, several solutions have been proposed, such as following a healthy lifestyle, following eating plans for weight loss, and exercising. Moreover, interventional approaches such as surgery, pharmacological therapy and dietary supplements have also been developed [7, 9]. The U.S. Food and Drug Administration (FDA) has approved various traditional approaches including pharmacological, surgical, and endoscopic bariatric therapies that may promote weight loss of 5-35% [10]. Dietary supplements are considered an attractive alternative to traditional therapy due to their low toxicity and accessibility to the general population. Almost 33.9% of adults attempting weight loss, largely young adults, women, and lower socioeconomic groups, use dietary supplements [7, 10].

Dietary supplements are products intended to aid or supplement the diet and enhance nutritional status. They may include one or more ingredients such as minerals, vitamins, amino acids, metabolites, herbs, or extracts. Since they are generally taken orally, they are mostly available in the form of tablets, capsules, powders, or liquids. Dietary supplements are not suggested alone for treatment of a disease, but they should act synergistically with other treatments to facilitate healing or recovery. Though dietary ingredients may show certain effects in preclinical and clinical settings, the evidence may not be clinically significant in clinical trials [10-12]. Thus, clinical studies and meta-analyses should be performed to prove the effectiveness of a dietary supplement. Moreover, different factors must be considered in selecting dietary supplements, among which purity of the supplement, the patient's overall lifestyle (such as dietary habits and exercise), other health-associated conditions of

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the patient (such as concomitant diseases and nutritional status), accurate dosage, food–drug interactions, absorption profiles, and potential side effects are the most relevant [7].

The U.S. Dietary Supplement Health and Education Act of 1994 deregulated the dietary supplement industry. The Office of Dietary Supplements (ODS) at the National Institutes of Health (NIH) has strengthened and enhanced understanding and knowledge of dietary supplements through the evaluation of scientific information, supporting scientific research and public education [13]. A product integrity profile is required for research projects funded by the ODS, including a collaborative approach to documentation of the commercial product. The Obesity Society believes that it is useful to conduct a qualitative analysis of non-FDA approved therapies to provide valid scientific evidence for the guidance of members [10].

Researchers have shown keen interest in the molecular and theoretical mechanisms of bioactive ingredients and the weight-loss effects of commonly used dietary supplements [7]. Table I and following paragraphs report a non-exhaustive list of dietary supplements with evidence supporting their use in weight loss.

β-Glucans

Glucans are soluble polysaccharide fibers derived from D-glucose and classified by their α or β interchain linkage. β-Glucans consist of D-glucose monomers linked together by beta-glycosidic bonds and are arranged in linear β1-3,1-4-D-glucan structure. Glucans are mostly found in cell walls of cereal grain endosperm and are also a major structural component of mushroom cell walls. As β-glucans are indigestible and readily fermented by gut microbiota in the colon and small intestine, it has been suggested that they could have a prebiotic role; they do not have significant side effect [14, 15, 52]. Their weight-loss effect is attributed to their being soluble fibers that may increase satiety due to the total time they take to move through the gastrointestinal track, and to their ability to reduce absorption of glucose. Their weight-loss effects were discovered as secondary outcomes of clinical trials designed to evaluate the effects of β-glucans on health conditions like insulin resistance, high blood pressure and dyslipidemia [14, 15].

Bitter orange

Bitter orange, *Citrus aurantium* or Seville orange, has been used in various traditional South American and Chinese folk medicines for different health conditions [53]. Bitter orange extract has been used as a supplement to treat obesity and to enhance exercise performance. It contains phytochemicals such as octopamine, alkaloids and particularly synephrine. Several studies suggest there is a positive effect of bitter orange extract on weight loss [16, 53-55]. In a clinical trial by Kaats et al., a single dose of synephrine alone or synephrine and flavonoids combined both increased the basal metabolic rate in humans. Synephrine derived from *C. aurantium* is safe and no side effects were observed when up to 98 mg/day was taken for 60 days [16].

Tab. I. Non-exhaustive list of dietary supplements with evidence supporting their use in weight loss.

Ingredient	Mechanism of action	References
β-Glucans	Reduce appetite and glucose absorption	[14, 15]
Bitter orange	Increase metabolic rate	[16]
Calcium-vitamin D	Improve metabolism, regulate triglyceride storage and adipocyte lipid metabolism	[17]
Chitosan	Reduce fat absorption	[18]
Chromium	Increase insulin sensitivity	[19, 20]
Cocoa	Stimulate thermogenesis and lipid catabolism	[21]
Coleus forskohlii	Increase lipolysis	[22, 23]
Conjugated linoleic acid	Increase lipolysis	[24, 25]
Ephedra sinica	Increase metabolic rate	[26]
Fucoxanthin	Reduce lipogenesis and increases thermogenesis	[27-31]
Garcinia cambogia	Reduce lipogenesis	[32, 33]
Glucomannan	Reduce appetite and fat absorption	[34-36]
Green coffee	Increase lipolysis	[37-40]
Green tea	Reduce appetite and increases thermogenesis	[26, 41]
Guar gum	Reduce appetite	[42]
Hoodia gordonii	Reduce appetite.	[43-45]
Irvingia gabonensis	Reduce lipogenesis	[46, 47]
Raspberry	Reduce lipogenesis and increases lipolysis	[48]
Phenylpropylamine	Reduce appetite	[49]
Pyruvate	Reduce appetite and fatigue, increases glucose uptake my skeletal muscles	[50]
White kidney bean	Reduce glucose absorption	[51]

Calcium-vitamin D supplementation

Calcium is an essential nutrient/mineral often associated with healthy teeth and bones. It is also required for muscle, heart, and nerve function and for blood clotting. Vitamin D helps the body absorb calcium after conversion to calcitriol in the kidneys. Combined supplementation of calcium and vitamin D may improve metabolic health, regulate triglyceride storage and adipocyte lipid metabolism, and reduce body weight. Three different studies have reported significant weight loss over time in adults treated with calcium, without any stated side effect [17].

Chitosan

Chitosan is a natural marine polysaccharide fiber derived from insect exoskeletons and shells of crustaceans like lobster, shellfish etc. [10]. Although it does not occur naturally in human tissues, it appears to be biocompatible, nontoxic, biodegradable and nonimmunogenic [7]. It is used in wound dressings to stop or reduce bleeding, and orally to decrease absorption of lipids in the gastrointestinal tract and reduce body weight [18]. Its mechanism of action for weight loss may involve binding fat molecules in the intestine, preventing their absorption. Chitosan is an insoluble animal fiber that exerts a bile acid sequestration or resin effect, decreasing absorption of cholesterol. It is currently available over the counter for treatment of conditions like obesity, hypertension and hypercholesterolemia [56]. A meta-analysis by Ernst and Pittler showed a statistically significant loss of weight (2.38 kg) after chitosan supplementation for 28 days [57]. Five additional studies on the effects of chitosan supplementation on weight loss reported statistically significant changes between groups, while others reported a weight reduction (2.3 kg) after 6 weeks of supplementation [10, 58].
A Cochrane meta-analysis of 13 clinical trials on chitosan reported a statistically significant weighted mean difference in body weight (1.7 kg) between the chitosan supplement and placebo groups. However, when only higher quality trials were analyzed, the average weight loss fell to 0.6 kg, which was, however, still statistically significant [59].

Chromium

Chromium is considered an essential trace mineral, present in small amounts in different foods. It is also taken as a supplement. Chromium plays a significant role in the metabolism of amino acids, glucose, and lipids though its effects on insulin. It may directly increase the activity of serotonin and regulate its downstream impact on dopaminergic signaling on central insulin receptors. Chromium is considered to affect numerous pathways involving the central control of satiety, energy homeostasis and food intake by modulating these neurotransmitters [19, 20]. Chromium has been shown to reduce body weight while maintaining lean mass, thus it is favored by manufacturers of dietary supplements for weight loss [60].

Cocoa

The cocoa bean or cocoa seed is the dried and fermented seed from the fruit of *Theobroma cacao*. These seeds are roasted and ground to obtain cocoa. Cocoa beans contain substantial amounts of various bioactive compounds, such as methylxanthines and antioxidant polyphenols (theobromine and caffeine) that may help weight loss by converting white adipocytes into brown adipocytes and enhancing lipid catabolism and endothelial function, while reducing insulin resistance and oxidative stress [21].
A randomized controlled clinical trial proved that a combination of catechins and cocoa reduces food intake withouth any side effect in young adults [61], while a second blinded placebo-controlled study proved that cocoa by-products reduced body weight in adults [62].

Coleus forskohlii

Forskolin is a bioactive compound extracted from the root of *Coleus forskohlii,* a relative of the mint family. *Coleus forskohlii* is native to India and has been used in Ayurvedic medicine for centuries for the treatment of various health conditions, like respiratory disorders, heart disease and abdominal colic. Forskolin is a strong cAMP stimulator that activates hormone-sensitive lipase, causing release of fatty acids from adipose tissue. A few limited clinical trials have examined its effect on weight loss and found a more significant effect in males than females. A placebo-controlled, double-blind clinical trial involving 15 obese males treated with 500 mg/day forskolin extract (10%) for 12 weeks found a significant reduction in body fat in the forskolin group and an increase in lean body mass without any stated side effect, although the basal metabolic rate remained unchanged [22, 23, 63].

Conjugated linoleic acid

Conjugated linoleic acid is a natural essential omega-6 fatty acid derived from linoleic acid, found mostly in meat and dairy products. Animal studies have shown its many beneficial effects including immune enhancement, reduction of atherosclerosis biomarkers and altered body composition (lower fat mass, higher lean mass). In humans, dietary conjugated linoleic acid supplements may improve insulin sensitivity and lipid metabolism by decreasing plasma levels of triglycerides and low-density cholesterol [24, 64]. Conjugated linoleic acid proved to reduce hunger in adults, with no remarkable side effects [25].

Ephedra sinica

Ephedra sinica is a plant that occurs naturally in Asia, although it is cultivated in other parts of the world. For thousands of years, *Ephedra sinica* has been used in Chinese medicine. Ephedrine is the bioactive ingredient of *Ephedra sinica* associated with weight loss; it is mostly taken with caffeine. In clinical trials, ephedra and ephedrine demonstrate only short-term weight-loss effects. The proposed mechanism of action involves an increase in the metabolic rate and stimulation of fat burning. For these reasons, ephedrine is used in weight-loss supplements [26].

Fucoxanthin

Fucoxanthin is a marine carotenoid widespread in nature and mostly isolated from diatoms and seaweeds. Several preclinical trials have investigated a mechanism of action of fucoxanthin in the treatment of obesity and associated cardiometabolic alterations. Fucoxanthin decreases plasma and hepatic concentrations of triglycerides. It also reduces expression of acetyl-CoA carboxylase, thus decreasing malonyl-CoA formation, fatty acid synthase expression and saturated long-chain fatty acid synthesis [27-29].

Research studies have reported that fucoxanthin may downregulate low density lipoprotein receptor expression in the liver [28]. It also downregulates expression of the *C/EBP*α (CCAAT/enhancer-binding protein-alpha), *PPAR*-γ (peroxisome proliferator-activated receptor gamma) and *SREBP-1c* (sterol regulatory element-binding protein 1c) genes during intermediate to late adipocyte differentiation stages, whereas during the initial adipocyte differentiation stages, fucoxanthin enhances expression of the proteins *C/EBP*α, *PPAR*-γ, A-FABP (adipocyte fatty acid-binding protein), *SREBP-1c* (sterol-regulatory element binding protein-1C), glucose transporter 4 (GLUT4) and lipoprotein lipase [30]. Similarly fucoxanthin has been known to stimulate UCP-1 (uncoupling protein1) expression in white adipocytes, thereby increasing energy expenditure and thermogenesis [31].

In a study of 151 obese premenopausal women who were given supplements containing fucoxanthin from brown seaweed and pomegranate seed oil extracts at various doses for almost 16 weeks, the group receiving 300 mg pomegranate seed oil and 300 mg seaweed extract (2.4 mg fucoxanthin) had a statistically significant decrease in waist circumference, body weight and body fat, without any side effects. Moreover, the group that received more than 2.4mg fucoxanthin showed an increase in resting energy expenditure compared to the placebo [65].

Garcinia cambogia

Garcinia cambogia is a plant native to Asia, Polynesia, Africa, and Australia. It has various significant anti-inflammatory, antineoplastic, hypolipidemic and antidiabetic effects on the body, and its extracts have anorexigenic effects. *Garcinia* has also been analyzed for weight management. Its rind is high in hydroxycitric acid, believed to be a bioactive component that causes weight loss by inhibiting extra-mitochondrial citrate lyase or ATP-citralyase that influences the synthesis of cholesterol and fatty acids. As ATP-citralyase is the major enzyme involved in the synthesis and storage of fatty acid in cells, hydroxycitric acid may inhibit lipogenesis [10, 32, 33].

Glucomannan

Glucomannan is a water-soluble polysaccharide dietary fiber that is easily extracted in large amounts from softwoods, tubers, plant bulbs and roots. Most glucomannan is extracted from the tuber of konjac (*Amorphophallus konjac*), an Asian plant for use as an herbal remedy. Glucomannan high molecular weight polysaccharide is composed of β-(1-4)–linked D-mannose and D-glucose monomers and is considered a soluble fiber. This highly viscous dietary fiber can absorb 50 times its weight in water. Since human salivary and pancreatic amylase cannot break β-1,4 glycosidic bonds, glucomannan reaches the colon almost unchanged and is fermented by gut microbiota. Glucomannan is lately being analyzed for its beneficial effects on weight loss, blood glucose, and dyslipidemia, amongst other uses [34, 35]. Some clinical trials question its effectiveness in weight loss [35, 66]. Thus, new studies will be needed to confirm glucomannan positive action on weight reduction.

Various mechanisms of action could explain the effects of glucomannan on weight loss: it may cause satiety through greater mastication effort, by prolonging gastric emptying, and by shortening the time food remains in the small intestine. Fecal energy loss may be another mechanism of action, as soluble fiber decreases absorption of fats and protein. Sood et al. reported statistically significant weight loss (-0.79 kg) after almost 5.2 weeks in humans taking glucomannan [36].

Green coffee

Green coffee extract is obtained from unroasted green coffee beans and marketed in caffeinated and decaffeinated forms. The bioactive ingredients of green coffee extract include chlorogenic acid, a polyphenol of the phenolic acid subfamily [67]. Different mechanisms of action of green coffee extract on weight loss may be caused by a reduction in pancreatic lipase activity, a lipolytic effect on adipocytes, inhibition of hydroxymethylglutaryl-CoA (HMG-CoA) reductase, acyl-CoA- cholesterol acyltransferase (ACAT) and fatty acid synthase (FASN), an increase in β-oxidation and increased expression of PPAR-α in liver, a nutrient sensor that regulates genes important in peroxisomal and mitochondrial β-oxidation, and fatty acid transport. A meta-analysis

reported statistically significant weight loss after green coffee extract supplementation (180-200 mg/day) for a period of 4-12 weeks, with no side effects [37-40].

Green tea

Green tea is made from the steamed and pan-fried unfermented (unoxidized) leaves of *Camellia sinensis*. Green tea has caffeine that is thought to contribute to the suppression of appetite and stimulation of thermogenesis. Antioxidants in green tea like the catechin orepigallocatechin-3-gallate, inhibit norepinephrine breakdown that in turn causes an increase in calories burned [26, 41]. A randomized, double-blind, placebo-controlled clinical trial reported that green tea extract reduces body weight without side effects on more than 100 women [68].

Guar gum

Guar gum, a dietary fiber obtained from seeds of the plant *Cyamopsis tetragonolobus,* is used in various food products particularly as a thickener and emulsifier in baked items [10]. It consists of high molecular weight galactomannan polysaccharides in linear, β-1,4–linked D-mannopyranosyl chains with α-1,6 D-galactopyranosyl side chains [69]. Guar gum may cause weight loss by virtue of its bulking characteristics in the gut which delay gastric emptying [7, 70]. Guar gum supplements have therefore been used to reduce food intake and to increase satiety, but a meta-analysis did not sustain its effectiveness, and side effects were reported including diarrhea, flatulence, and cramps [42, 70].

Hoodia gordonii

Hoodia gordonii, also known as Bushman's hat, is a leafless succulent plant with medicinal properties occurring naturally in Namibia, Botswana, and South Africa. Researchers have studied *Hoodia gordonii* as a weight-loss adjuvant due to its appetite suppressing properties, although its use was questioned due to several adverse effects, among which were nausea and skin reactions [45]. Metabolites such as pregnane glycosides that contain 6-deoxy and 2,6-dideoxy sugars have been isolated from *Hoodia gordonii*. While the active compound causing the anorexigenic effect of *Hoodia gordonii* is not yet clear, P57AS3 (P57), an oxypregnane steroidal glycoside, is commonly considered to be the metabolite responsible for these effects [43-45].
Research into the effects of P57 in vivo have shown that intraventricular injection of purified P57 in rats decreases food intake and significantly increases hypothalamic ATP production, which may decrease the appetite response. According to an in-vitro study, P57 stimulates cholecystokinin secretion in human enteroendocrine cells, while cholecystokinin has been studied for insights into its appetite-suppressing effect through the vagus nerve. However, since oral administration of *Hoodia gordonii* is known to cause gastric breakdown of P57, the extract must be taken in high doses to obtain significant clinical effects [45, 69, 71], or easy to absorb derivatives developed.

Irvingia gabonensis

Irvingia gabonensis, also known as African wild mango, is a native to western and central Africa. Its seed is high in saturated fatty acids and its flesh is rich in polyphenols, specifically flavonoids. Oben et al. treated adipocyte cultures with *Irvingia gabonensis* extract and observed inhibition of PPAR-γ (peroxisome proliferator-activated receptor gamma) expression, a decrease in leptin protein levels and upregulation of adiponectin expression, thus inhibiting lipogenesis [46, 47]. Other randomized double-blind clinical trials analyzed the weight loss effects of *Irvingia gabonensis* extracts [72, 73]. Most of these studies were systematically reviewed by Onakpoya et al., who reported that administration of 200-3150 mg/day *Irvingia gabonensis* extract for 4-10 weeks could lead to statistically and clinically significant weight loss and decreased waist circumference compared to a placebo group. Side effects of the extract included headache and sleep difficulty [74].
Irvingia gabonensis emerged from these studies as a useful adjuvant dietary supplement in weight reduction management. However, many of these clinical studies were conducted on relatively small samples of black people from Africa. The research should be extended to larger and more diverse populations [72].

Raspberry

Raspberry ketones [4-(4-hydroxyphenyl)-2-butanone] are natural aromatic substances found, for example, in red raspberries and rhubarb. Extracted from raspberries, they are used as flavoring in the food industry. Several *in-vitro* studies on the effect of these ketones on adipocytes have reported increased fatty acid oxidation, reduced lipid accumulation and enhanced secretion of adiponectin [48]. Molecular studies have established that raspberry ketones downregulate expression of various genes associated with adipogenesis, such as C/EBPα, PPAR-γ, ACC1 (acetyl-CoA carboxylase1), SCD1 (steroyl-CoA desaturase1), A-FABP 2 (adipocyte fatty acid–binding protein 2) and FAS (fatty acid synthase), whereas it increased the mRNA levels of genes involved in the process of fatty acid oxidation, such as HSL (hormone-sensitive lipase), CPT (carnitine palmitoyl transferase) and ATGL (adipose triglyceride lipase) was observed [75]. Moreover, in-vivo studies on rodents fed a high-fat diet have shown that raspberry ketones prevented any increase in body weight or visceral adipose tissue. The mechanism of this effect presumably involves stimulation of brown and white adipose tissue and inhibition of pancreatic lipase activity [76].

These properties suggest that raspberry ketone supplementation could be an alternative weight-loss measure, but sufficient clinical evidence is lacking and certain teratogenic and cardiotoxic effects of raspberry ketones identified by in-silico research, indicate the need for further detailed study [77].

Phenylpropylamine

Phenylpropylamine (PPA) is a sympathomimetic agent that has structural similarity to ephedrine and amphetamine. It may act as a decongestant and appetite suppressant and induce significant weight loss. It is used as an over-the-counter supplement for weight loss. Researchers believe that PPA acts through the α-1 adrenergic receptor (α1-AR) [49]. In a double-blind placebo-controlled study, Schteingart administered a 1200 kcal diet and 75 mg/day PPA to 101 overweight subjects for 6 to 20 weeks and reported greater weight loss (2.59 kg) in the PPA-supplemented group than in the placebo group, without untoward side effects [78].

Pyruvate

Pyruvate is the simplest alpha-keto acid having a carboxylic and a ketone functional group. It is the end product of glycolysis but is also obtained from other sources. It breaks down glucose and produces energy and is also a key intermediate in several cell metabolic pathways. Pyruvate helps lipid metabolism by reversible conversion to phosphoenolpyruvate, increasing glucose uptake by skeletal muscle. It may therefore promote weight loss [50]. By virtue of these properties, pyruvate has been used to promote weight loss in subjects with obesity, to improve athletic performance, and in the treatment of cataracts, high cholesterol and cancer. A meta-analysis which analyzed six clinical trials concluded that pyruvate is effective in reducing body weight, and that adverse effects include gas, bloating and diarrhea [79]. Pyruvate should be administered with thiamine, a cofactor of pyruvate dehydrogenase, an essential enzyme that allows pyruvate to enter the Kreb's cycle [80].

White kidney bean

Extract of white kidney bean (*Phaseolus vulgaris* L.) contains alpha-amylase inhibitor (phaseolamin) that inhibits pancreatic amylase activity, lowering glycemia and calorie absorption by delaying or preventing complex carbohydrate digestion. It is therefore used as an over-the-counter dietary supplement for weight loss [51]. Several proof-of-concept clinical trials have established a dose-dependent reduction in glucose absorption after supplementation with white kidney bean. However, weight-loss effects were more prominent in some relatively small, short-term studies that used 1.5-6 g/day of white kidney bean extract. For instance, a clinical trial involving 60 subjects with obesity reported greater reductions in fat mass, body weight and thigh, hip and waist circumference in the group taking white kidney bean extract than in the placebo group, without any side effect [81, 82].

Conclusions

Obesity is a prevalent nutritional disease and a significant public health issue in industrialized countries. Due to their low toxicity profile, dietary supplements are considered an attractive alternative to traditional therapy, which usually comprise surgical and pharmacological treatments. Dietary supplements for weight loss or obesity managements may act through several mechanisms, among which are reduction of lipogenesis, appetite, and nutrient absorption, or increasing lipolysis and energy expenditure. Among the many ingredients presented in this study, chitosan and green tea showed promising results with limited side effects. Weight loss and absence of side effects were reported for both ingredients by meta-analysis. More clinical studies on chitosan and green tea, as well as other promising ingredients, will be needed to increase our knowledge on the efficacy and on the mode of actions of dietary supplements for weight loss and obesity management.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

Gabriele Bonetti, Karen L. Herbst: These authors contributed equally to this work.
MB: study conception, editing and critical revision of the manuscript; GB, KLH, Kevin D, Kristjana D, AKK, BA, VV, GM, AI: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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Review

Dietary supplements for lipedema

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Keywords

Weight loss in lipedema • Dietary supplements • Fat burning supplements • Fat burners

Summary

Lipedema is a chronic disease that mostly manifests in females as the abnormal distribution of subcutaneous adipose connective tissue, usually coupled with bruising, pain, and edema. Lipedema molecular pathophysiology is currently not clear, but several studies suggest that genetics and hormonal imbalance participate in lipedema pathogenesis. Women with lipedema present in some cases with elevated body mass index, and the appearance of obesity in addition to lipedema, where the obesity can cause serious health issues as in lipedema-free individuals with obesity, such as diabetes and cardiovascular disorders. Unlike obesity, lipedema tissue does not respond well to diet or physical exercise alone. Therefore, in this review we discuss the effect of various dietary supplements that, along with diet and physical exercise, cause fat burning and weight loss, and which could potentially be important in the treatment of lipedema. Indeed, an effective fat burner should convert stored fats into energy, mobilize and break down triglycerides in adipocytes, boost metabolism and inhibit lipogenesis. Common ingredients of fat burning supplements are green tea, caffeine, chromium, carnitine, and conjugated linoleic acid. The use of fat burners could act synergistically with a healthy diet and physical exercise for decreasing adipose tissue deposition in patients with lipedema and resolve related health issues. The effects of fat burners in human studies are sometimes contradictory, and further studies should test their effectiveness in treating lipedema.

Introduction

Lipedema is a chronic and progressive disease that is characterized by the abnormal distribution of subcutaneous adipose connective tissue causing disproportionate and painful limbs [1]. Usually, it develops in females during their time of puberty or other times of hormonal, shape and weight change, including menopause or childbirth. Lipedema is characterized by the symmetrical enhancement of nodular subcutaneous adipose connective tissue on the lower body and arms, while leaving the upper trunk, hands, and feet unaffected. Lipedema tissue often affects the lower abdomen, thighs, buttocks, and calves. In about 80% of cases, lipedema can also affect the arms, whereas the hands and feet are not affected [2]. Although lipedema greatly affects women's health around the world, it remains either undiagnosed or misdiagnosed as other similar diseases, such as obesity or lymphedema [3]. Polygenic susceptibility along with lymphatic, hormonal and microvascular disorders might be at least partially responsible for the development of lipedema. Moreover, lipedema can progress to the point where lymphedema develops. The major causes for the onset of lymphedema include genetic susceptibility, lymphatic vessel malformations, surgery and trauma [4]. Lipedema does not respond well to restrictive diets, contrary to usual forms of obesity. Thus, lipedema leads to a disproportionate increase in lower body tissue that stubbornly retains its shape (waist to ankles) after diets or bariatric surgery. Lipedema is not restricted to just subcutaneous adipose connective tissue, in fact, women with lipedema had significantly lower muscle strength and a non-significant, but clinically relevant lower exercise-endurance capacity than women with obesity [5]. Excess subcutaneous adipose connective tissue and lower muscle function results in later stages, patients with lipedema with an elevated body mass index, increasing the risk of developing severe obesity with metabolic complications, which in turn worsens the symptoms of lipedema [1, 6-8]. The Mediterranean diet and ketogenic diet have been proposed for lipedema treatment, showing weight loss in both cases but failure to reduce pain with either intervention, and failure to reduce percent fat after the Mediterranean diet. [9, 10] The ketogenic diet trial was small; hence no evidence-based diet has been recommended for the treatment of lipedema. It is suggested that hypocaloric nutrition should be accompanied by suitable dietary supplements and exercise to overcome diet resistance and to manage weight gain in lipedema [8].

If a patient with lipedema develops obesity, it can cause similar serious health issues as in individuals with obesity but without lipedema. For example, it could trigger insulin resistance [2] resulting in hyperglycemia leading to the development of type 2 diabetes, which could further damage organs of the body as well as reduce quality of life. Moreover, obesity increases the risk of developing high blood pressure, hypercholesterolemia and increased blood clotting tendency that leads to higher risk of stroke and heart attacks [11-14].

In order to define an effective treatment option for lipedema, knowing the physiological process of subcutaneous adipose connective tissue deposition and reduction of muscle function and energy utilization is of outmost importance. Excess protein, fat and carbohydrates that are consumed daily can be converted into stored or ectopic fat though the process of lipogenesis in adipose tissue and the liver, and normally stored in adipose tissue as triacylglycerol [15-17]. When consumption of macronutrients, especially fat and carbohydrates, is in excess, fat can also be stored ectopically in muscle, liver, and other depots in the body [18]. Fat must be released from adipocytes through complex enzymatic/hormonal pathways in order to be utilized for energy production. When adipocytes are stimulated, they release triacylglycerol into the bloodstream as free fatty acids (FFA) via lipolysis. Then, FFA are transported by the blood stream to energy requiring tissues, especially muscles, and finally enter mitochondria where they are utilized for energy production [15, 19].

Dietary supplements that reduce adipose tissue and increase muscle (lean mass) could be an effective ally in the management of lipedema. Reduction of adipose tissue and increase in lean mass could improve body image, reduce pain, and improve mobility for patients with lipedema. The use of specific dietary supplements could help in reducing body fat and increasing lean mass thus preventing the invasive practice of microcannular tumescent lipedema reduction surgery including suction lipectomy, which is currently the most effective treatment for lipedema [20].

Fat-burning supplements

Fat-burning supplements are a specific type of dietary supplement that stimulates the fat burning process by several mechanisms. They can boost energy expenditure, increase fat metabolism, increase weight loss, increase oxidation of fats during exercise, as well as make long-term adjustments in metabolic pathways that enhance fat metabolism. Indeed, a good fat burner supplement should [15, 21, 22]:

- stimulate conversion of stored fats into energy;
- stimulate mobilization and break down of stored triacylglycerol from adipocytes;
- increase metabolism to burn stored fats;
- inhibit adipocyte enlargement via lipogenesis.

The efficacy of fat-burning supplements is supported by several studies. Indeed, weight loss induced by supplementation and diet together can be significantly higher than weight loss from diet alone [23]. In a study by Falcone et al., addition of thermogenic supplements containing caffeine, conjugated linoleic acid (CLA), multi-vitamins, and protein, to a hypocaloric diet with high-protein content for a period of 3 weeks resulted in 97% additional reduction in body weight and 35% additional fat loss when compared with the same diet alone. Thus, dietary supplements can increase overall weight loss, including fat loss [23].

Classes of fat-burning supplements

As described by El-Zayat et al., fat burners may be divided into several classes based on their mechanism of action: energy enhancers, protein and amino acids supplements, adrenergic enhancers, and lean mass enhancers [15]. Moreover, they can be comprised of several ingredients, each of which has its own mechanism of action. The main ingredients of fat burning supplements are green tea, caffeine, chromium, carnitine, and CLA. They can also include herbal stimulants such as ephedrine, pyruvate, yohimbine, and chitosan. Fat-burning supplements stimulate weight loss through several molecular mechanisms, mainly boosting metabolism and suppressing appetite [22, 24-26].

Energy enhancers

Fat-burning energy enhancers are usually caffeine or catechins. Caffeine is found in coffee, soft drinks, tea, cola nuts, and cocoa, and it can act as an energy enhancer and an exercise performance booster. Caffeine has the ability to increase stored fat release and the rate of calorie burn [15, 27-29]. Caffeine stimulates fat loss at the level of adipocytes and myocytes, mainly acting synergistically with beta-adrenergic drugs and neurotransmitters. Indeed, beta-adrenergic receptors stimulate fat loss and increase calorie burning, while alpha2-adrenergic receptors have the opposite function [30, 31].

Catechins are often derived from green tea. They have anti-obesity effects and their effect in weight loss is sustained by several human studies [32, 33]. Indeed, they stimulate fat oxidation and energy expenditure, decreasing dietary fat-induced weight gain. Their action is probably due to an increase in sympathetic neuronal activity, which in turn activates hepatic fat oxidation [34].

Protein and amino acids supplements

Protein supplements are utilized to create and maintain a positive nitrogen balance during the day without severely elevating caloric intake. They include whey protein and casein [15]. Whey protein is reported to help in building muscles, increasing strength, controlling appetite, improving endurance, aiding in weight loss, and boosting energy levels [35]. Casein provides all the essential amino acids required for exercise-induced growth of tissue. Casein also forms a gel within the stomach that causes it to slowly digest so that the amino acids/peptides are steadily absorbed over a longer period of time [36].

Adrenergic enhancers

Estrogens are thought to play a key role in the development of lipedema. Aromatase, produced by adipocytes, is an enzyme that converts androgens to estrogen. When mice had the aromatase gene knocked out, body weight gain and obesity-related metabolic complications occurred in both genders. This suggests that an optimal estrogen to testosterone ratio is important, and with increased lipedema tissue, this ratio may be high [37]; thus, dietary supplements that modify hormonal levels could be an effective strategy for lipedema treatment. Testosterone is a key molecule in the pathophysiology of weight gain and obesity. Indeed, it increases lean mass and decreases fat mass, where low testosterone levels are associated with energy imbalance, insulin resistance and dyslipidemia [38]. Supplementation of molecules that act on adrenergic receptors might help in increasing metabolic rate and fat burning, and in accelerating weight loss in women with lipedema. Adrenergic enhancers comprise 7-Keto dehydroepiandrosterone (DHEA) and yohimbine. 7-Keto-DHEA causes long-term changes in body levels of epitestosterone, testosterone, estradiol as well as other steroid hormones. It stimulates thermogenesis, diverting store fats in ATP and heat production [39, 40]. Yohimbe derives from the bark of *Pausinystalia yohimbe*. It is an alpha-2 receptor antagonist, and it accelerates weight loss, increasing testosterone levels, blood flow, thermogenesis, and fatty acid oxidation [15, 41-43].

Lean mass enhancers

The wide class of lean mass enhancers comprise several molecules that stimulate lean mass production through different molecular mechanisms. A non-exhaustive list, comprising their mechanism of action, is reported below in Table I [15]. Further studies will be needed to confirm their effects in humans, considering that a systematic review by Pittler & Ernst doubted the efficacy of several molecules, such as chitosan and pyruvate, in reducing body weight [41].

Fat-burning foods

Certain foods can help in the fat burning process by enhancing metabolism and suppressing appetite. Indeed, food consumption normally increases body weight, but certain foods stimulate lipolysis if coupled with regular exercise and sufficient water intake [15]. Fat-burning foods comprise good fats, medium chain triglycerides, and meat and dairy proteins. Good fats are lipids from several sources, among which are avocados, nuts, fish and vegetable oils [15]. These foods are rich in beta-sitosterol, oleic acids and omega-3 polyunsaturated fatty acids, and they accelerate fat burning and decrease triglycerides and LDL cholesterol levels [52, 53]. Medium chain triglycerides are found in cow butter, palm oil and coconut oil. They are easily digested and absorbed and are directly used for energy. Thus, foods rich in medium chain triglycerides suppress appetite and stimulate body fat loss [54]. Finally, meat and dairy proteins require a complex digestion and absorption processes, which burns energy. They stimulate satiety and use energy for their conversion and storage as fats, therefore meat and dairy they are excellent fat-burning foods [15, 55].

Conclusion

Lipedema is a chronic disease that results in the abnormal distribution of subcutaneous adipose tissue but also the loss of function of muscle. In some cases, obesity arises in later lipedema stages, leading to serious health issues. Lipedema adipose tissue is usually not responsive to diet and exercise, thus invasive techniques such as suction lipectomy are usually required. The use of fat-burning supplements and lean mass enhancers could improve the process of weight loss and but also increase muscle mass and possibly muscle function. Indeed, these supplements are known to increase fat mass loss boosting energy expenditure, increasing fat metabolism, and impairing fat absorption, which may in turn improve muscle function indirectly. Therefore, the use of dietary supplements could be a valid alternative to invasive techniques for the reduction of adipose tissue and related issues in lipedema patients. Human studies are needed to confirm their effectiveness in lipedema and to select the most effective dietary supplements.

Tab. I. Sources and mechanism of action of lean body enhancers.

Molecule	Source	Mechanism of action	References
Chitosan	Crustaceans	Reduces fat absorption	[40,41]
L-Carnitine	Chemical catalyst that is synthesized by human kidneys, brain, and liver	Participates in fatty acid transport into mitochondria during the breakdown of fats	[44,45]
Chromium	Trace mineral found in meat, grain, nuts	Reduces insulin resistance	[41,46]
Ephedrine	The plant *Ephedra sinica*	Stimulates sympathetic neuronal action	[47]
Synephrine	Citrus fruits	Stimulates thermogenesis	[48,49]
Pyruvate	Intermediate of glycolysis	Reduces appetite and fatigue, increases energy levels and muscle glycogen stores	[24,41]
Conjugated Linoleic Acid	Meat & dairy products	Transports dietary fats to cells for lipolysis	[50,51]

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

Gabriele Bonetti, Karen L. Herbst: These authors contributed equally to this work. MB: study conception, editing and critical revision of the manuscript; GB, KLH, KD, AKK, Serena M, Silvia M, MRC, Sandro M, MR, Marina C, Michela C, TB, FB, PG: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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REVIEW

Dietary supplements in neurological diseases and brain aging

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Keywords

Neurological diseases • Neurodegenerative diseases • Dietary supplements • Mediterranean diet • Ayurvedic herbs

Summary

A healthy diet shapes a healthy mind. Diet quality has a strong association with brain health. Diet influences the onset and consequences of neurological diseases, and dietary factors may influence mental health at individual and population level. The link between unhealthy diet, impaired cognitive function and neurodegenerative diseases indicates that adopting a healthy diet would ultimately afford prevention and management of neurological diseases and brain aging. Neurodegenerative diseases are of multifactorial origin and result in progressive loss of neuronal function in the brain, leading to cognitive impairment and motoneuron disorders. The so-called Mediterranean diet (MedDiet) with its healthy ingredients rich in antioxidant, anti-inflammatory, immune, neuroprotective, antidepressant, antistress and senolytic activity plays an essential role in the prevention and management of neurological diseases and inhibits cognitive decline in neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases. The MedDiet also modulates the gut-brain axis by promoting a diversity of gut microbiota. In view of the importance of diet in neurological diseases management, this review focuses on the dietary components, natural compounds and medicinal plants that have proven beneficial in neurological diseases and for brain health. Among them, polyphenols, omega-3 fatty acids, B vitamins and several ayurvedic herbs have promising beneficial effects.

Introduction

Neurodegenerative diseases (NDs) involve a progressive loss of neuronal activity, resulting in impairment of cognitive function. They have genetic and epigenetic etiology and are increasing at an alarming rate. For instance, 17.2 million people worldwide are suffering from NDs such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), Huntington's disease (HD) and dementia [1, 2]. As the symptoms appear only when neurological degeneration has reached an advanced stage, the prevention of NDs and the search for new therapeutic agents is a challenge. Although the mechanisms of NDs are multifactorial and complex, they share common pathways, such as oxidative stress, inflammation, mitochondrial dysfunction and intracellular Ca^{2+} overload. In addition, cross talk between these multiple pathways often makes therapeutic intervention less effective.

The brain is highly sensitive to oxidative stress and increased reactive oxygen species produced during neuroinflammatory processes. As the antioxidant defence system has low activity in the brain, increased oxidative stress results in NDs and aging [3]. Genetic and epigenetic factors greatly influence the onset and development of these disorders, while nutrition and metabolism play a key role in the manifestation of epigenetic modifications of DNA in the central nervous system [4-5]. Bioactive ingredients in food and gut microbiota can greatly influence DNA methylation in the adult central nervous system, indicating a role of diet and dietary components in NDs [6]. The so-called Mediterranean diet (MedDiet) is currently regarded as the healthiest diet in the world. It includes daily intake of whole grains, vegetables, fruit, legumes, white meats, fish, nuts, olives and olive oil. Rich in antioxidants, fibre, vitamins, minerals, phytosterols, probiotics, omega-3, and omega-6 fatty acids, it promotes human health and wellbeing. The MedDiet has been associated with improvements in overall health, prevention of cancer, maintenance of a healthy cardiovascular system and metabolism, and with preventing, alleviating and slowing neurological disorders (Fig. 1).

Clinical studies on efficacy of MedDiet in major neurodegenerative disorders

Several studies have evaluated the efficacy of the MedDiet in prevention and management of neurodegenerative disorders (Tab. I). These studies inferred that following the MedDiet not only decreases the incidence of NDs but also improves overall cognitive function and hampers the onset and progress of decline caused by NDs and cerebral aging.

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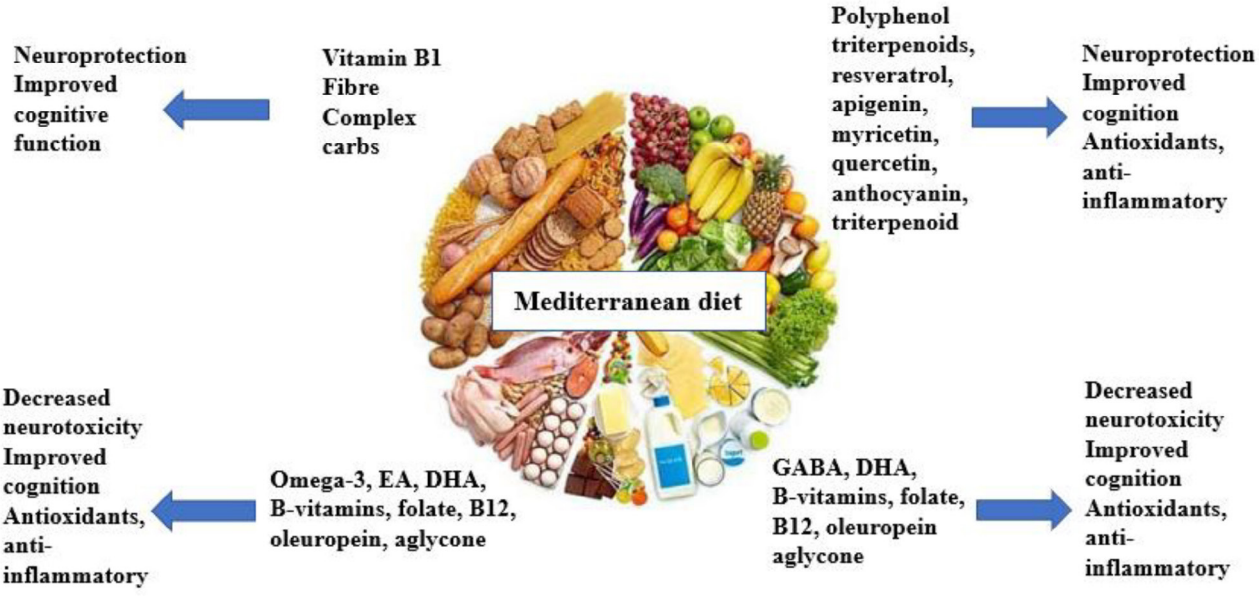
Fig. 1. Effects of dietary components of Mediterranean diet in neurodegenerative diseases and cerebral aging.



Animal studies and clinical trials have shown that the MedDiet has anti-inflammatory, antioxidant and free radical-scavenging properties that alleviate or mitigate neurotoxicity and neurodegeneration (Tab. II).

MedDiet and depression

Increasing evidence suggests that depression, the foremost global cause of disability, is a subtle neurological disorder [22, 23]. Besides other therapies, diet may be useful for improving overall mental health and relieving stress and anxiety. The MedDiet, rich in vitamins, minerals, antioxidants, healthy fats and proteins, reduces the risk of depression [24]. Research-based evidence suggests that dietary measures can be an adjunctive treatment for mental disorders. For instance, a healthy diet has been tested clinically in two different trials for its effects on symptoms and remission rates of depression, showing promising results [25, 26]. As many as 37 studies have reported a reduction in symptoms of depression in groups of persons on diets rich in polyphenols [27]. An observational study also revealed that following the MedDiet was crucial for reducing depressive outcomes in overweight patients with metabolic syndrome [28]. Vicinanza et al. (2020) reported a positive impact of the MedDiet on mental health in elderly patients with multimorbidity [29]. They also observed that the diet prevented symptoms of depression in these patients, promoting healthy aging. Yet another promising clinical study named PREDI-DEP is underway to assess the MedDiet supplemented with extra virgin olive oil or nuts for precluding relapse of unipolar depression [30]. Diet plays an important role in shaping behaviour and modulating mood (Tab. III). For instance, omega-3 essential fatty acid supplements alleviated symptoms of bipolar disorder in 30 patients [31].

Effect of natural compounds and medicinal plants on neurological disorders

Several medicinal plants and natural compounds have been deployed to prevent or alleviate neurological diseases and symptoms in vivo and in clinical trials. Here we discuss important natural compounds that can be obtained from dietary sources or nutritional supplements and that mediate various aspects of practical utility in the management of neurodegenerative disorders.

N-acetylcysteine (NAC) in neurological disorders

N-acetylcysteine (NAC) is a mucolytic thiol known for its ability to alleviate stress and mediate the impacts of toxicity, infections and inflammatory conditions by supporting the body's antioxidant and nitric oxide systems [37]. It crosses blood brain barrier (BBB) and is a precursor of l-cysteine and reduced glutathione GSH, as well as a source of sulfhydryl groups in cells. It scavenges free radicals and interacts with reactive oxygen species (ROS) [38]. It has a multifaceted mode of action, acting as a drug, a xenobiotic and a cytoprotectant. The effects of NAC on various neurological and neurodegenerative diseases are summarized in Table IV.

Effects of phospholipids on neurological conditions

The brain and nervous system have a more diverse lipid composition than the rest of the body, showing a pre-

Tab. I. Clinical studies demonstrating effect of MedDiet on ND progression.

Neurological conditions	Description	Study type	Participants	Duration	Findings	Reference
Alzheimer's disease	Formation of widespread extracellular amyloid plaques and intraneuronal neurofibril tangles in the brain (Reitz and Mayeux, 2014), a major cause of dementia	Follow-up study	70 subjects with normal cognitive function, age 30-60 years	3	Not following MedDiet was correlated with progressive AD abnormalities	[7]
		Cohort studies	1393 normal and 482 with mild cognitive impairment, age 76.7-77.5 years	4.5	Following MedDiet reduced risk of cognitive impairment and AD	[8]
Dementia	Loss of cognitive function due to brain aging or neurodegenerative diseases	Longitudinal	1865 (41%M) patients with dementia, mean age 73 years	1.4	10% decrease in dementia on MedDiet. Cereals shown to have positive impact on mental performance	[9]
		Cross sectional	52 subjects with normal cognitive function	-	Low intake of rice and higher intake of milk and soybean reduced risk of dementia	[10]
Huntington's disease	a rare, hereditary condition that causes progressive neurodegeneration	Prospective	211 patients with expanded CAG repeats	3.4	Diet and high energy intake may delay onset	[11]
Parkinson's disease (PD)	Neuronal degeneration, dopaminergic loss. PD symptoms include tremors, motoneuron changes, cognitive decline, dementia and loss of muscle strength (Gratwicke et al., 2015)	Population-based cohort	1731 (41% male) PD-free individuals, age 65 and over	-	MedDiet lowered probability of prodromal PD in elderly people	[12]
Amyotrophic lateral sclerosis (ALS)	Degeneration of brainstem and spinal cord motoneurons resulting in progressive muscle atrophy, paralysis and respiratory failure (Oh et al., 2015)	Cross sectional baseline analysis	302 patients with a history of ALS symptoms of 18 months or less	-	Better function associated with antioxidants and with carotenes in fruit and vegetables	[13]
Multiple sclerosis	Demyelination of nerve fibres and myelin sheaths, affecting the optic nerves, brain and spinal cord	Survey	396	-	MedDiet reduces risk of relapses	[14]

dominance of phospholipids [45]. Phospholipids occur in varying concentrations in the brain, e.g. 31 nmol/mg phosphatidylcholine, 54 nmol/mg phosphatidylethanolamine, 8 nmol/mg phosphatidylserine and 5 nmol/mg of phosphatidylinositol [46]. Sphingomyelin levels in the hippocampus and prefrontal cortex are similar to those of phosphatidylethanolamine in adult male rats [47]. Phospholipids also occur in the membranes of organelles such as mitochondria, endoplasmic reticulum, Golgi apparatus, peroxisomes and lysosomes, which illustrates their importance in cells. Studies have revealed that phospholipid-enriched diets can modulate cognitive processes [48] and phospholipid supplementation has been shown to increase cognitive function in a polyunsaturated fatty acid-deficient mice model and to improve memory in piglets on permanent supplements [49].

Phosphatidylserine

Phosphatidylserine (PS) is an acidic phospholipid and a natural component of brain neuronal membranes and other biological membranes. It plays a pivotal role in normal neuronal function by determining neuronal membrane surface potential and the local ionic environment [50]. Phosphatidylserine is a brain-specific nutrient [51] and activates protein kinase C (PKC) in neural membranes. It is thought to decrease in the brain with aging, leading to cognitive decline and impairment as well as lower PKC

Tab. II. Antioxidant and anti-inflammatory nutrients of the Mediterranean diet used in animal and human studies.

MedDiet component	Nutrient	Study design	Study population	Proposed antioxidant activity	References
Extra virgin olive oil	Total polyphenol fraction of olive oil and hydroxytyrosol.	In vitro	Endothelial cells and murine myoblasts	Redox potential enhanced by increasing glutathione levels and free radical scavenging	[15,16]
	Hydroxytyrosol and tyrosol	Randomized	Male Wistar rats	Hydroxytyrosol and tyrosol activate GSH, reduce lipid peroxidation, restore glutathione balance in liver	[17]
	Extra virgin olive oil, oleuropein aglycone	Randomized	TgCRND8 mice	Inflammation and neurotoxicity reduced by induction of autophagy and recovery of lysosome system	[18]
Fish and dairy	B-vitamin folate (vitamin B9) and vitamin B12	Transverse	ALS patients	Less inflammatory damage and oxidation, improvement in myocytic atrophy	[19]
Citrus and green tea	Phytochemicals, triterpenoids, resveratrol	Randomized clinical	SOD1 (G93A) mice	Increased SIRT and AMPK resulting in enhanced survival of motor neurons Resveratrol treatment reduces activation of NF-kB pathway in LPS-activated microglia and stabilizes autophagic flux	[20]
Diet enriched with oily fish, seafood, dairy, nuts, vegetables, fruit and eggs	Docosahexaenoic acid (DHA)	Transverse	BV-2 murine microglial cells	Unsaturated fatty acid-based decrease in toxic effects of 7-ketocholesterol	[21]

Tab. III. Effect of dietary components and regimes on mood and psychological disorders.

Dietary components/ regimes	Effect on mood	Study type	Participants	Reference
Vitamin D	Improved mood	Double-blind placebo-controlled	44 healthy volunteers	[22]
Vitamins, minerals and essential fatty acids	Reduction in antisocial behaviour	Double-blind, placebo-controlled	231 young adult prisoners	[32]
Tryptophan depletion	Worsening of mood in seasonal affective disorder/winter type (SAD)	Randomized, balanced, double-blind crossover	11 SAD patients with recurrent episodes of winter depression	[33]
Folic acid therapy	Improved intellectual function	-	16 patients with impaired intellectual function	[34, 35]
Folic acid deficiency	Increased depression, impaired cognitive function, impaired abstract thinking	-	260 healthy subjects 60 to 94 years old	[36]
Omega 3 fatty acids	Improved short-term course of illness in bipolar disorder	Placebo controlled	30 patients with bipolar disorder	[23]
Traditional vs western diet	Traditional diet reduced odds in bipolar disorder	Epidemiological cohort study	23 women with bipolar disorder and 691 normal subjects	[31]

Tab. IV. Mechanism of action of NAC in different neurological disorders.

Disease	Mechanism	References
Unverricht–Lundbor type SCD, tardive dyskinesia, myoclonus epilepsy	Antioxidant effect by scavenging free-radicals and enhancing glutathione	[39]
Multiple sclerosis	Scavenges free-radicals and inhibits TNF toxicity	[40]
Amyotrophic lateral sclerosis	Enhances glutathione peroxidase and free-radical scavenging	[41]
Parkinson's disease	Enhances glutathione and free-radical scavenging	[42]
Huntington's disease	Scavenges free radicals and prevents mitochondrial dysfunction	[40]
Alzheimer's disease	Boosts glutathione levels	[43]
Focal cerebral ischemia	Enhances glutathione levels, improves microcirculation and tissue oxygenation, inhibits NOS and regenerates endothelium-derived relaxing factor	[44]

Tab. V. Effect of phosphatidylserine on neurological conditions.

Neurological conditions	Subjects	Nutrients	Findings	References
Alzheimer's disease	Aged patients with AD and dementia	Soy lecithin-derived phosphatidylserine plus phosphatidic acid	Improved cognition, mood, and memory	[58]
Attention deficit hyperactivity disorder (ADHD)	Children with ADHD	Phosphatidylserine	Improved short-term auditory memory and ADHD symptoms	[59]
Premenstrual syndrome (PMS)	40 women age 18-45 years diagnosed with PMS	400 mg PS + 400 mg PA per day or a matching placebo	Significant reduction in PMS symptoms	[60]
Cognitive impairment	Elderly persons with impaired memory	100 mg/day phosphatidylserine enriched with docosahexaenoic acid (PS-DHA)	May improve or maintain cognitive status	[61]
Cognitive function improvement	Elderly persons with impaired memory without dementia	Phosphatidylserine enriched with docosahexaenoic acid (PS-DHA)	May improve cognitive performance	[62]
Acute cognitive effects	Healthy young volunteers	Ginkgo biloba extract with soy-derived PS	Significantly improved memory task speed and improved secondary memory	[63]
Cognition and cortical activity after mental stress	Healthy subjects doing cognitive tasks under induced stress in a test–re-test design	Phosphatidylserine supplementation	Continued supplementation significantly was connected with a more relaxed state compared to the controls	[64]
Age-related cognitive function	130 elderly persons with cognitive impairment	PS derived from soybean 300 mg/day	Safely improved cognitive function	[65]
	494 elderly persons with cognitive impairment	300 mg/day PS supplements	Improved cognitive function in 6 months	[66]

activity [52]. Phosphatidylserine functions equally well in adults, children and the elderly. For instance, in young healthy males it mitigates stress-induced activation of the hypothalamus-pituitary-adrenal axis [53]. Phosphatidylserine-omega 3 supplementation reduces attention deficit hyperactivity disorder (ADHD) symptoms in children [54]. This indicates that PS may prove beneficial in correcting disrupted neural function under various conditions. It also modulates several important enzymes and proteins, such as synapsin I, that maintain neural function [55]. Table V lists some of the studies depicting the role of PS in neurological conditions.

Phosphatidylcholine

Phosphatidylcholine (PC) is the major phospholipid component of cell membranes, lecithin, organ meats, nuts and spinach. Phosphatidylcholine supplements derived from egg yolk are well-absorbed in the gut and their levels can vary in different regions of the brain under different circumstances. For instance, PC and phosphatidylethanolamine levels increase in the whole brain of a stress-induced mouse model [56], while phosphatidylethanolamine and sphingomyelin levels decrease in the prefrontal cortex, and sphingomyelin in the hippocampus [47]. Likewise, an age-induced reduction in PC and phosphatidylethanolamine levels was detected by HPLC in the hippocampus and frontal cortex of elderly persons (89-92 years) [57].

Effects of Gamma-aminobutyric acid on brain and behaviour

Gamma-aminobutyric acid (GABA) is a non-protein amino acid found in high concentrations in different

Tab. VI. GABA and prevention of neurological disorders.

Neurological conditions	Sources of GABA	Subjects	Effect on neurological conditions	Reference
Alzheimer's disease	Naturally produced by cerebral cortex	Thirty-eight AD risk participants, 14 with normal cognitive function, 11 with cognitive decline, 13 with impaired cognitive function	In high-AD risk participants GABA levels were associated with the dorsomedial-dorsoanterolateral prefrontal cortex	[71]
Menopausal depression, insomnia and autonomic disorder	GABA-enriched rice germ	Twenty menopausal patients	Improvement in sleep, somnipathy and depression	[72]
Depression	GABA-rich Monascus-fermented product	Depression animal model	Prevented depression	[73]
Sleep quality	GABA powder from lactic acid bacteria fermentation	32 Japanese volunteers	Prevented sleep disorders	[74]
Sleep latency and non-REM sleep	GABA (90.8%) and l-theanine (99.3%)	Pentobarbital-induced sleep in ICR mice	Decreased sleep latency and enhanced sleep duration	[75]
Stress	GABA from natural fermentation with lactic acid bacteria	8 stressed volunteers	Increased relaxation, reduced anxiety and raised immunity	[76]
Cognitive function	GABA-enriched product fermented with kimchi-derived lactic acid bacteria	50 mice	Improved long-term memory loss and increased neuronal proliferation	[77]
	GABA-enriched fermented *Laminaria japonica* product	40 elderly persons	Prevented cognitive impairment in the elderly	[78]

parts of the brain [67]. Foods such as germinated brown rice, soybean, green tea, cabbage, yogurt, kimchi and pickles are excellent sources of GABA. GABA is the main inhibitory neurotransmitter in the human cerebral cortex [68]. As a food supplement it is used to alleviate anxiety and improve sleep quality. Several studies have reported that GABA crosses the blood-brain barrier, albeit in small amounts [69]. GABA is a known antihypertensive, anti-inflammatory, antidiabetic, antimicrobial, antiallergic, hepatoprotective, renal protective and intestine protective agent [70], and it demonstrated effects on several neurological disorders (Tab. VI).

Melatonin in neurodegeneration

Melatonin, a neurohormone secreted by the epiphysis cerebri and extra pineal structures, has several important functions (chronobiotic, normothermal, immune-modulating, antioxidant, oncostatic, cryoprotective and anxiolytic) in the body [79]. Melatonin affects the gastrointestinal tract, cardiovascular system, reproductive system and metabolism, and regulates body weight. Acting as a chronobiotic, melatonin modifies the phase and amplitude of biological rhythms. It acts as a cytoprotective molecule in neurodegenerative disorders and aging by reversing inflammatory damage. It also prevents neurodegeneration in experimental models of Alzheimer's and Parkinson's disease. Melatonin supplementation has been recommended for the treatment of insomnia [80]. Table VII lists the effects of melatonin supplementation on various neurological conditions.

Omega-3 fatty acids

Omega-3 fatty acids are essential for a variety of physiological functions involved in neuroinflammation, neurotransmission and neurogenesis and therefore play a major role in brain development, performance and aging. The importance of omega-3 fatty acids is indicated by the fact that a deficiency leads to many neurological conditions such as depression, ADHD, schizophrenia, bipolar disorder, dementia and autism (Tab. VIII). Eicosapentaenoic (EA) and docosahexaenoic (DA) acid modulate inflammatory processes and maintain mental health, while a deficiency results in mental disorders (Tab. VIII). They also directly affect neuronal membrane fluidity and receptor function. Although omega-3 supplementation and enriched foods have long been studied for their vital role in neurological homeostasis, randomized clinical trials investigating their therapeutic potential have yielded inconclusive results, limiting their use in psychiatry. High-quality clinical trials are urgently needed to evaluate the effectiveness of omega-3 fatty acids in inhibiting and treating NDs.

Neurotropic B vitamins

Neurotropic B vitamins have crucial roles in the nervous system, not only as coenzymes. Their importance is in-

Tab. VII. Effects of melatonin supplementation on various neurological conditions.

Clinical condition	Melatonin dose	Findings	References
Parkinson's disease	0.25 and 1.25 mg/kg i.v.	Striking improvement in symptoms	[81]
Amyotrophic lateral sclerosis	60 mg/day oral for 13 months	Neuroprotective effects	[82]
	300 mg/day rectal for 2 years in 31 sporadic patients	Reduced oxidative damage	[83]
Muscular dystrophy	70 mg/day for 9 months	Mitigated hyperoxidative state of erythrocytes	[84]
Multiple sclerosis	50-300 mg/day oral for 4 years	Improved overall symptoms of progressive MS with long-term use	[85]
Migraine	3 mg/day for 4 months	Lower duration, frequency, and intensity of pain	[86]

Tab. VIII. Neurological implications of omega-3 fatty acids.

Neurological condition/function	Subjects	Study type	Supplements/ doses	Findings	Reference
Anxiety and inflammation	68 medical students under low-stress such as exams	Placebo-controlled, double-blind 12-week RCT	n-3 (2.5 g/day, 2085 mg eicosapentaenoic acid and 348 mg docosahexaenoic acid) or placebo	14% decrease in lipopolysaccharide-stimulated interleukin 6 production and 20% reduction in anxiety symptoms; lowered n-6:n-3 ratio and anxiety	[87]
Dementia	5386 patients without dementia	Prospective evaluation of incidence of dementia	Fatty-acid-rich fish	Fish intake decreased dementia	[88]
Cognitive function	867 elderly persons	Observational epidemiological	Oily fish containing long-chain PUFA	Fish consumption was positively associated with delayed unadjusted recall in CVLT	[89]
Parkinson's disease	31 patients with major depression	Double-blind, placebo-controlled	Fish oil (containing omega-3 fatty acids) or mineral oil capsules for 3 months	Omega-3 enriched fish oil improved depression	[90]
Alzheimer's disease and vascular dementia	49 controls, 25 AD and 15 VD	Cross-sectional	Excess intake of n-6 polyunsaturated fatty acids	AD and VD associated with higher intake of n-6 animal fats	[91]

dicated by the fact that their deficiency leads to various NDs such as depression, beriberi, Wernicke's encephalopathy, seizures, subacute combined degeneration of the spinal cord and peripheral neuropathy [92, 93]. Synergistic interaction of vitamins B1, B6 and B12 has been reported to improve neuropathic pain, motor control and nociception (Tab. IX) [94].

S-adenosyl methionine (SAMe)

S-adenosyl methionine (SAMe) is a major methyl donor that influences central nervous system function via cell transmethylation pathways, including but not limited to DNA methylation. It is a strong antidepressant with impacts in mouse models of amyotrophic lateral sclerosis, epilepsy and Alzheimer's disease [100]. SAMe supplementation alters brain bioenergetics and is an effective treatment for depression (Tab. X) [101, 102].

Tryptophan

Essential amino acid tryptophan (TRP) is involved in various physiological processes including immunity, neuronal function and gut homeostasis. Its metabolism in humans takes place via the kynurenine and serotonin pathways and produces niacin, serotonin and melatonin. In addition, to endogenous TRP, the gut microbiota also produces specific TRP metabolites that indirectly influence host physiology. An alteration in TRP metabolites results in neurological and psychiatric disorders. Tryptophan supplementation has been used to treat a number neuropsychological disorders in various clinical trials (Tab. XI) and has been found to improve serotonin and tryptophan deficiency, thus alleviating the severity of symptoms in depression, schizophrenia and bipolar disorder.

Magnesium

Magnesium is an important mineral for homeostasis in the human body. It plays an essential role in neuroprotection, neuromuscular conduction and nerve transmission. It is a mineral of intense interest due to its capacity to protect the nervous system against ecotoxicity, thus im-

Tab. IX. Sources and neurological implications of vitamins B1, B6 and B12.

Vitamin	Sources	Coenzyme for	Deficiency symptoms	Implications in nervous system	Reference
B1 (thiamine)	Fish, beans, lentils, cereals, yogurt, sunflower seeds, cereals	Pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase, transketolase	Beriberi, polyneuritis	Energy supply to nerve cells for synthesis of nucleic acids, neurotransmitters, and myelin	[95, 96, 97]
B6 (pyridoxine)	Salmon, tuna, beef liver, chicken, leafy greens, orange, banana, papaya, cantaloupe	Cystathionine-beta-synthase/lyase, serine-hydroxymethyl transferase, aromatic L-amino acid decarboxylase	Cognitive impairment, depression, premature aging of neurons	Metabolism of DNA/RNA, amino acids and neurotransmitters	[98]
B12 (cobalamin)	Dairy products, fish, poultry, eggs, meat	Methionine synthase, methylmalonyl CoA mutase	Cognitive impairment, impaired neurotransmitter production, polyneuritis, subacute combined spinal cord sclerosis	Metabolism of nucleic acids, fatty acids, amino acids, neurotransmitters, myelin	[99]

Tab. X. Neurological implications of S-adenosyl methionine (SAMe).

Neurological conditions	Study Design	Subjects	Dose	Findings	Reference
Abstinence from smoking	Three-arm, randomized, blind, placebo-controlled, dose-ranging clinical trial	120 adults	Oral SAMe 800 or 1600 mg/day or matched placebo for 8 weeks	SAMe holds little promise for the treatment of tobacco dependence	[103]
Parkinson's disease	Open label clinical trial	13 patients with depression	800 to 3600 mg/day for 10 weeks	SAMe is a well-tolerated, safe and effective alternative to antidepressants	[104]
Depression	Double blind randomised controlled trial	49 patients with depression	800 mg/day SAMe monotherapy versus placebo	Depression improved	[105]

Tab. XI. Clinical trials evaluating efficacy of tryptophan in neuropsychological disorders.

Neurological conditions	Study Design	Treatment	Findings	Reference
Depression	Human pilot clinical trial	Tryptophan	Replenished serotonin deficiency	[106]
Schizophrenia	Open baseline-controlled trial	Tryptophan	Improved impaired serotonin synthesis	[107]
Bipolar disorder	Clinical trial	L-tryptophan	Alleviated tryptophan deficiency	[108]

proving many neurological disorders. Table XII shows some selective studies of magnesium in NDs.

Polyphenols

Polyphenols are important nutrients abundant in spices and foods. They have antioxidant, anti-inflammatory and senolytic activities; they inhibit oxytosis, modulate the gut microbiome and promote protein aggregation and stability. They also maintain GSH levels and neurotrophic signalling pathways [113]. They show promise for preventing neurodegenerative diseases such as dementia, PD, HD, ALS, stroke, TBI, diabetes, cardiovascular diseases, liver disease and cancers. In addition, polyphenols control symptoms of depression. For instance, the antioxidant potential of polyphenols could possibly improve depression symptoms in women [114]. However, the effect of polyphenols in disease prevention and treatment depends on adequate dietary consumption. Fresh fruit and vegetables contain plenty of polyphenols that offer a variety of neurological benefits (Tab. XII). Polyphenol supplements, such as Pycnogenol® (a procyanidin) obtained from French maritime pine bark by Horphag Research (Geneva, Switzerland), have shown promising antioxidant and anti-inflammatory properties in various in vitro, animal and/or human models [115], besides improving endothelial function and showing beneficial effects in ADHD [116]. Another important commercially available polyphenol is silymarin, extract-

Tab. XII. Neurological implications of magnesium.

Neurological conditions	Study Design/type	Subjects	Treatment/ supplementation/ assessment	Findings	Reference
Risk of dementia	Prospective cohort Hisayama Study, Japan	1081 Japanese without dementia, age > 60 years, 17-year follow-up	Dietary intake of potassium, calcium, magnesium	Higher self-reported dietary potassium, calcium and magnesium intake reduces risk of dementia	[109]
Alzheimer's disease	Comprehensive geriatric assessment	101 geriatric patients with slight to moderate cognitive impairment	Assessment of Mg levels in blood samples	Mg ion levels were directly related to cognitive function	[110]
Parkinson's disease	Case control study	249 patients with PD for < 6 years and 368 controls	Dietary intake	Higher intake of magnesium, iron and zinc associated with lower risk of PD	[111]
Cerebral ischemia	Intravenous magnesium efficacy in stroke (IMAGES) trial	2589	A bolus dose of 16 mmol of MgSO4 was infused over 15 min and then a maintenance dose of 65 mmol MgSO4 was given over 24 h	Highly beneficial in early-treated patients	[112]

Tab. XIII. Dietary sources and neurological benefits of polyphenols.

Class of polyphenols	Biologically active compound	Dietary source	Neurological benefits	References
Non flavonoids	Curcumin	Turmeric	Significant reduction in severity of depression and improved cognitive function in elderly AD patients	[121, 122, 123, 124]
	Resveratrol	Grapes	Improved cognitive function, reduced oxidative stress and neuroinflammation, and neuroprotection in AD patients	[125, 126]
Anthocyanins	Cyanidin petunidin	Berries strawberries tea	Improved cognitive function and reduced risk of dementia	[127]
Flavones	Apigenin kaempferol myricetin quercetin	Apple skin, broccoli, fruit peel, lettuce, olives and onions	Quercetin reduces risk of AD and regulates microglial activity, neuroinflammation, oxidative stress and neural injury	[128, 129]
Flavonones	Fisetin hesperitin	Citrus fruit and peel	Fisetin slows loss of cognitive function and maintains brain health in dementia models	[113]

ed from milk thistle and sold with various brand names. It is commonly known as flavonolignans and is a mixture of eight stereoisomers: taxofolin, silybin A and B, isosilybin A and B, silychristin, isosilychristin and silydianin [117].

Silymarin shows neuroprotective mechanisms in AD, PD and cerebral ischemia including mediation of antioxidant mechanisms, regulation of kinases in cell signalling pathways, anti-inflammatory properties, neurotropic effects, modulation of neurotransmitters and inhibition of apoptosis [118, 119]. Silymarin also controls production of amyloid-β by inhibiting β-amyloid precursor protein and cholinesterase activity, thus inhibiting the onset of AD [120]. Its low cost, bioavailability and safety make silymarin a natural drug of choice for neuroprotection and hepatoprotection [119, 120].

Ayurvedic herbs in the treatment of neurodegenerative diseases

Ayurvedic medicine has been practised in the Asian subcontinent since ancient times. Many herbs and medicinal plants have been explored for their antioxidant, anti-inflammatory, antidiabetic, anticancer and cytoprotective properties. Medicinal plants such as *Withania somnifera* (ashwagandha), *Baccopa monnieri*, *Acorus calamus* and *Hypericum perforatum* have been shown to prevent or alleviate neurological diseases and symptoms (Tab. XIV).

Bacopa monnieri

Bacopa monnieri is a traditional Indian ayurvedic medicinal plant belonging to the family *Scrophulariaceae*. This memory enhancer, known as Brahmi, has been used

Tab. XIV. Neuroprotective properties and therapeutic potential of selected medicinal plants.

Medicinal plants	Active ingredients	Neuroprotective properties	Therapeutic potential	Reference
Bacopa monnieri (L.) Wettest (folk name: brahmi)	Bacopasides III–V, bacosides A and B, bacosaponins A, B and C	Antioxidant, antistress, anti-inflammatory, anti-microbial and smooth muscle relaxant. Improves memory	Neuroprotection in AD and bipolar disorder, improves intelligence and memory	[130, 133, 134]
Withania somnifera (L.) Dunal (folk name: ashwaganda)	Ashwagandhine, withanolides, withasomniferin, withasomniferols and withanone	Memory enhancer and anti-stress agent with effects on locomotor function and neural growth	Inhibits oxidative stress, improves cholinergic function and mitochondrial respiration in rotenone-induced Parkinsonism in *Drosophila melanogaster*	[131]
Acorus calamus (folk name: sweet flag, sway or muskrat root)	145 compounds α-asarone, β-asarone, eugenol, isoeugenol, 44 sesquiterpenes including lactones, monoterpenes (C-10), triterpenoid saponins	Antioxidant, anti-depressant, anti-inflammatory, anticonvulsant, neuroprotective, antianxiety, cytoprotective, immunomodulatory	Neuroprotection and anti-inflammatory agent in AD and PD	[132, 135]
Hypericum perforatum (Folk name: St John's wort)	Quercetin, hyperoside, quercitrin, rutin, hypericin, kaempferol, hyperforin	Antidepressive, antioxidant, neuroprotective	Restoration and improvement of microglial viability, inhibits amyloid-β toxicity in AD and brain malondialdehyde in PD	[47]

traditionally for more than 3000 years to treat various neurological disorders, to enhance digestion and to improve learning, cognitive function and concentration. It helps restore cognitive deficit and enhances mental and brain function. This nootropic plant promotes repair of damaged neurons, neuronal synthesis and synaptic activity. Recent studies show that it contains surplus bioactive phytochemical compounds with synergistic properties that are useful in the management of ND [130].

Withania somnifera

Withania somnifera or ashwagandha is another traditional Indian medicinal plant that promotes long life, youthful vigour and good intellectual powers. It is used traditionally in the treatment of neurodegenerative diseases, general frailty, nervous exhaustion and insomnia. It has anti-inflammatory, anti-tumour, antioxidant, immunomodulatory and anti-neuropsychiatric effects [131].

Acorus calamus

Acorus calamus or vacha is a traditional Indian ayurvedic medicinal plant. Its rhizomes are used to treat insomnia, melancholy, memory loss, hysteria, depression and mental disorders. Almost all parts of the plant have proven beneficial in the treatment of neurological, gastrointestinal, kidney, respiratory, liver, and metabolic disorders. Its action is anticonvulsant, anti-depressant, anti-hypersensitive, anti-inflammatory, cardioprotective, immunomodulatory and anti-obesity [132].

Hypericum perforatum

Hypericum perforatum or St. John's wort is a perennial plant. It is used in traditional medicine to treat external and internal disorders such as minor burns, anxiety and mild to moderate depression. It is also a herbal remedy for neurological disorders such as mental aliments, hypersensitivity, neuralgia, spinal convulsion, hydrophobia, spastic paralysis, spinal irritation, coxalgia and menopausal neurosis [47].

Conclusion

Oxidative stress and neuroinflammation are key factors in the onset and progression of neurodegenerative diseases. A diet rich in biologically active compounds with antioxidative and anti-inflammatory properties affords significant neuroprotection. Many recent studies have attempted to evaluate and recommend the Mediterranean diet and its ingredients with neuroprotective, oxidative stress mitigating and anti-inflammatory properties that impede the progression, delay the onset and reduce the severity of neurodegeneration in neurological disorders such as AD, PD, HD, MS, ALS and natural age-related brain aging. Several natural compounds, minerals and medicinal plants have been tested in clinical trials and animal studies and some, such as PLs, GABA, NAC, omega-3 fatty acids, magnesium, curcumin, resveratrol, *Hypericum perforatum, Acorous calamus* and *Bacopa monnieri,* have proven beneficial, safe and economical in the treatment of NDs. However, their effects are dose-dependent and must be administered in a precise manner. These potentially beneficial dietary components need to be evaluated in large clinical trials to assess their wider application across patients of different ethnic origin.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; KD, MCM, Paola C, PM, Pietro C: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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Review

Dietary supplements in retinal diseases, glaucoma, and other ocular conditions

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Keywords

Ocular diseases • Retinal diseases • Glaucoma • Med Diet • Antioxidants

Summary

Environmental pollution, inadequate eating habits and unhealthy lifestyles have led to a tremendous increase in ocular diseases worldwide. Given the costly treatments that are currently available for the most common and threatening eye diseases (such as cataract, dry eye disorder, or diabetic retinopathy), curing these diseases or preventing refractive errors by taking nutraceuticals and natural compounds that are present in our daily diet is a very valuable intervention. The eyes are the most important part of our visual system and require micronutrients such as vitamins, carotenoids, trace metals, and omega-3 fatty acids in order to function properly and to protect themselves against light-induced and age-mediated degenerative disorders. The Mediterranean Diet (MedDiet) has been in the limelight since the 1980s because of the several health benefits it provides, including eye health. MedDiet is characterized by the consumption of small amounts of red meat, while emphasizing the intake of fish, eggs, nuts, legumes, citrus fruits, green vegetables, olives and their derivatives, especially olive oil, and dairy products in a proportionate manner, in order to achieve the maximum health benefits. The antioxidant, anti-inflammatory, and neuroprotective properties of these foods – both when used as an ingredient in the dietary regime or as a source of nutritional supplements – have shown promising results in the management of chronic degenerative ocular diseases, both in animal models and in human subjects. In this chapter, we will focus on the importance of MedDiet and natural compounds for the visual system and its role in slowing down age-related ocular degeneration.

Introduction

Eye health and vision have extensive and overwhelming effects on the overall quality of life, health, education, sustainable development, and even on the economy. Lacking the access to high quality treatment regimens and even to affordable eye care has rendered many people blind or visually impaired. In 2020 an estimated 596 million people in the world had distance vision impairment, out of which 90% of cases could be treated using high-cost treatment regimen [1, 2]. Out of these, 43 million suffered from complete or partial blindness. On the other hand, 510 million people could not improve near vision impairments because they could not afford reading glasses, showing the extremely poor socioeconomic conditions in which they live. These patients included all age groups, mostly affecting children and the elderly. In addition, eye diseases were more prevalent in women, ethnic minorities, and people living in rural areas. One of the major factors observed in these patients was that they were malnourished, due to the inadequate food supply because of overall poor socioeconomic conditions.
Diet, being a key lifestyle factor, can exert long-term effects even on ocular health and play a vital role in preventing visual dysfunction, which can lead to permanent visual impairment or blindness and it is estimated to affect 1 billion people worldwide by 2050 [1]. Consequently, the focus of the current chapter is how the Mediterranean dietary regime and the intake of supplements can prove to be beneficial for ocular health.

The visual system and vision impairment

Vision is the foremost among the human senses. It manifests through the coordinate function of the intricate visual pathways within the brain, interconnecting with eyes and their associated adnexal tissues. The cornea and lens of the eyes focus light onto the retinal photoreceptors, which then transduce light stimuli into neuronal impulses that lead to the development of a three-dimensional image by the brain. Clear vision requires structural and physiological integrity of all the components of the vision system (eyes, brain, and their pathways) and any abnormality results in visual dysfunction. Commonly, visual function is measured by distance visual acuity.

Common eye conditions that lead to visual impairment

Many factors contribute to the development of visual dysfunction, such as genetic mutations, environment, lifestyle, age, and malnutrition [3-6]. The global trend in the population growth index indicates an increase in adult population with visual impairment and dysfunctions, caused by many ocular disorders: it could be because of refractive errors, dry eye disease (DED) [2, 7], diabetic

retinopathy (DR) [8], ocular surface dysfunction (OSD), glaucoma, cataracts [2, 7], and age-related macular degeneration (AMD) [9]. Among children, on the other hand, the main causes of blindness and impaired vision are cerebral visual impairment, uncorrected refractive error, congenital ocular anomalies, cataract, retinopathy of prematurity, and corneal scarring. In addition, many conditions – such as allergic conjunctivitis, blepharitis, and dry eyes – can cause itching, pain and discharge in many people that do not suffer from visual dysfunction (Tab. I).

The effect of visual impairment on health and well being

Vision impairment not only affects the normal day-to-day routine of an individual, but it also has social, psychological, and cognitive implications that affect the overall quality of life. Visually impaired people often experience reduced educational and employment prospects and are often forced to take up low-paid jobs [16]. Moreover, these people are often targeted with negative attitudes such as bullying, social exclusion, loneliness, violence, and sexual assault [17].

Good vision not only is closely associated with an improved quality of life, but it also exerts profound effects on the overall health of an individual. On the contrary, poor vision or visual impairment can predispose a person to several physiological and psychological complications, such as cardiovascular diseases, cancer, dementia, and depression [18]. Although the causal relationship of vision impairment with health conditions mentioned above is complex and difficult to assess precisely, it has been broadly categorized into three kinds:

- direct effects of vision impairment on the individual's health and wellbeing, such as injuries, and indirect effects caused by difficulties (or even impossibility) to access health care due to social isolation and limitations in mobility and physical activity;
- other factors that are not specifically related to vision, like poor diet, poverty, smoking, sun exposure, ageing, etc;
- effects of systemic health conditions on visual impairment such as dementia, diabetes, cancer, and ocular metastases.

Hence, vision impairment negatively affects the patients' mobility and mental health, even exacerbating the risk of dementia, it increases the likelihood of injuries

Tab. I. Most prevalent eye conditions causing vision impairment.

Ocular condition	Description	Epidemiology	Treatment	References
Cataract	Opacities in lens obstruct or scatter the incoming light	It is the most important cause of blindness worldwide (17.8 million in 2020) and the second leading cause of moderate or severe vision impairment (83.2 million). Cataract results from age-related changes, smoking, UV damage, dehydration crisis, diabetes, galactosemia, and steroid use	Surgery with intraocular lens (IOL) implantation	[2]
Refractive error	Blurred vision because light is not sharply focused on the retina, due to a mismatch between the eye axial length and the refractive power of the cornea and/or lens. Hypermetropia (long sight) and myopia (short sight)	It is the second main cause of blindness (3.7 million) and the main cause of vision impairment (157.5 million)	Refractive error can be corrected using spectacles, contact lenses, IOL implantation during cataract surgery or laser refractive surgery	[10, 11]
Glaucoma	Progressive damage of the optic nerve in one or both eyes, resulting in irreversible blindness	The third main cause of blindness (3.6 million) and the fourth cause of moderate or severe vision impairment (4.1 million). The risk of developing glaucoma increases with age and is increasing globally with population ageing	Medication, trabeculoplasty, iridotomy, or surgery	[2, 12]
AMD	Degeneration of the macula, resulting in loss of clear vision. It has both "dry" and "wet" forms	The fourth prominent cause of blindness globally (1.9 million) and the third in moderate to severe vision impairment (6.2 million). AMD is the primary cause of vision loss in high-income countries	Long term anti-VEGF intravitreal injections	[13-15]
DR	Damage to the small blood vessels in the retina leads to leakage of plasma fluid and blood, which may damage central vision ("diabetic macular oedema, DMO")	The fifth cause of blindness (1.1 million) and vision impairment (3.8 million)	Retinal laser; intravitreal injection of anti-VEGF or steroid; retinal surgery	[8]

like falls and road traffic crashes, thus increasing also the patients' need for social care, ultimately leading to higher mortality rates [2, 19].

Diet and eye health

Several population-based studies have revealed the importance of diet, specifically of micronutrients, in the management of age-related ocular diseases. These studies have led researchers to focus their studies on a wide variety of nutrients in the management of eye problems, such as retinal conditions (light mediative oxidative damage), refractive errors, age-related macular degeneration, cataract, glaucoma, and dry eye [1].

Nutrient deficiencies led to manifestations of ocular diseases in the adults and the effects of malnutrition on the development of newborns' visual system have suggested that nutrition plays an important role not only in the proper development of the visual system, but also in enhancing or suppressing the various ocular manifestations leading to visual impairment and blindness. For instance, studies have demonstrated that taking vitamin A in recommended doses has beneficial effects on retinitis pigmentosa, while vitamin E is detrimental to the same condition [20]. In addition, lutein and docosahexaenoic acid (DHA) supplementation, along with vitamin A, was found to delay retinal degeneration and visual decline. Similarly, several studies have reported a link between vitamin A deficiency and night blindness and xerophthalmia [21]. On the other hand, vitamin B deficiency due to poor intake or absorption results in nutritional amblyopia, which causes blurred vision and reduced visual acuity [22]. Furthermore, carotenoids have been useful in improving the vision throughout life, as well as in reducing retinal and lens degeneration – leading to AMD and cataract in the elderly population [23].

MedDiet and eye health

A healthy lifestyle, indicated by healthy eating habits and plenty of physical activity, offers a valuable intervention to fight against the tremendously increasing risk of eye disorders, such as myopia progression, dry eye disorders, diabetic retinopathy, cataracts, glaucoma, or AMD. Providing certain micronutrients such as minerals, Omega 3 fatty acids, vitamins, and carotenoids in recommended daily doses ensures an overall good health and exerts positive effects on eye health and on the visual system. However, including these micronutrients in the diet as supplements might raise concerns in the underdeveloped countries, where most of the population lives below poverty line and are thus unable to afford nutritional supplements. In this regard, diet regimes that are affordable by a wide range of socioeconomic groups are a method of choice to introduce beneficial nutrients in the body.

The MedDiet in this regard has received considerable attention in the past few decades, mainly because people dwelling in the Mediterranean region tend to have lower incidence of cardiovascular diseases and other chronic disorders [24]. Furthermore, adherence to the MedDiet is linked with increased antioxidant activity, reduced incidence of several diseases, reduced inflammation, and eventually reduced mortality [25]. A number of studies has shown that adherence to the MedDiet is also associated with reduced frailty [26]. In addition, people following this diet regime experience fewer visual disorders as well as a higher lifespan. This can be because this diet regime favors an enhanced consumption of whole meal bread, cereals, olive oil, fruits, legumes, and vegetables, and a low consumption of saturated fat, avoiding or reducing red meat consumption and taking with chicken and fish in moderate proportions, and an adequate intake of cheese and yogurt. Besides that, regular physical activity also helps enhance the beneficial effects of the MedDiet.

A recent retrospective clinical trial involving 7756 participants, with a mean age of 71 years, showed in 56.5% of cases the beneficial effects of following the MedDiet on AMD. The results indicated that closely following the MedDiet reduced the risk of progression to late AMD and to large drusen. This association was greater for geographic atrophy (GA) than neovascular AMD. In addition to that, fish intake contributed to a great extent in association [27].

In another prospective study, the researchers investigated the association between following the MedDiet and the incidence of advanced AMD in 4446 European people, aged 55 and older: it was observed that the likelihood of developing AMD in subjects that followed the diet was a 41% lower as compared to those who did not [28].

These findings establish the important role of a nutrient-rich MedDiet – containing whole grains, olive oil, fruits, vegetables, legumes, and fish – in AMD prevention. Although these studies could not prove a direct cause and effect relationship between MedDiet and AMD, it still highlighted that the reduced risk of developing AMD was due to the overall diet and not to the individual components (Tab. II).

Mediterranean diet– gut microbiota – eye axis

Gut dysbiosis has been recently observed to increase the predisposition to various ocular diseases, such as dry eye, uveitis, macular degeneration, and glaucoma [31]. This indicates the existence of a gut-eye axis (or gut-retina axis), mediated by the gut microbiota [32]. It is a well-known fact that alterations in the gut microbiota result in many intestinal and extra-intestinal diseases, including non-alcoholic fatty liver disease (NAFLD) [33], metabolic and inflammatory diseases, obesity, and cancer [34]. An increased intestinal permeability (termed as "leaky gut") allows leakage of bacterial endotoxin lipopolysaccharides (LPS) and pathogen-associated molecular pattern molecules (PAMPs), which activate pattern recognition receptors (PRRs), resulting in low-grade inflammation in several tissues. This crosstalk can extend to dendritic cells, perivascular macrophages, and retinal pigment epithelium (RPE) cells, and might end up in ocular inflammation. In addition, the gut microbial metab-

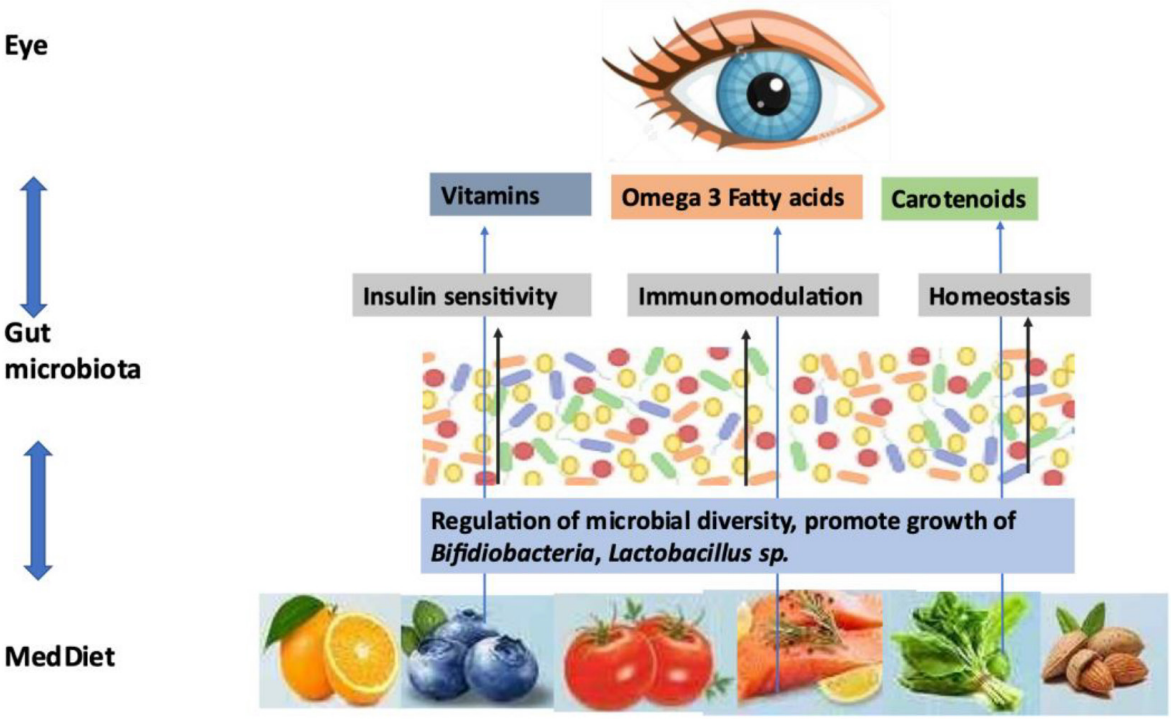
Tab. II. Dietary patterns as interventions to age-related eye diseases.

Diet pattern	Ocular diseases				
	Cataract	Refractive error	Glaucoma	AMD	Diabetic retinopathy
MedDiet	A small study on 500 African male subjects indicated a lower risk of developing cataract upon adherence to the MedDiet [25] Lack of evidence due to scarcity of clinical trials	Not assessed	Reduced risk of developing glaucoma [29]	Decreased risk of late AMD	Prevents diabetes and therefore diabetic retinopathy and lowers the chances of retinal degeneration during DR
Western diet	Not assessed	Not assessed	Not assessed	Increased risk of AMD	Not assessed
Prudent diet	Not assessed	Not assessed.	Not assessed	Lower incidence of AMD in some studies. However, results are variable [30]	Not assessed

olites might regulate retina-specific immune cells [35]. A recent study has shown that obesity-associated gut microbiota is the major role player in pathological angiogenesis of choroidal neovascularization (CNV) in retinal tissue [35], reinstating the existence of a gut-retina axis. Gut microbiota undergoes significant changes in respect to age and dietary patterns and greatly influences the overall health. MedDiet has been shown to alter the gut microbiota in many ways, such as increasing the microbial diversity, enhancing the *Bifidiobacteria*, and *Lactobacillus* sp population while reducing the proteobacteria [36]. Studies have shown that people following MedDiet had a better gut microbiota composition as compared to those following a Western diet regime [37]. As this diet regime avoids high caloric fatty foods and promotes fresh leafy vegetables and fruits, it results in higher proportion of short-chain fatty acids (SCFAs) and fiber-degrading bacteria in the feces [38]. In addition, some studies have reported an association between gut microbiome and ocular surface microbiome with ocular surface diseases. These studies have been recently reviewed by [39], and they suggest a convincing link between gut microbiota-eye health and gut microbiota-ocular surface microbiota-eye health. Whatever the case, the MedDiet and dietary intake of probiotics have been known to modulate a healthy gut (Fig. 1.).

As the MedDiet modulates gut microbiota, which in turn influences the onset of ocular diseases, it seems to exist a

Fig. 1. MedDiet- Gut Microbiota-Eye Axis.



MedDiet-gut microbiota-eye axis. Further studies in this area would be beneficial for the development of diet-mediated management of various ocular diseases.

Natural molecules

Food contains many essential vitamins, micronutrients, and biologically active compounds that can play a pivotal role in maintaining eye health. Some selected natural molecules and their effect on ocular diseases is presented in Table III.

Melatonin

Melatonin is a hormone secreted by the pineal gland and regulated by the hypothalamic suprachiasmatic nucleus (SCN) [40]. It plays an important role in circadian rhythms, as shown by its enhanced secretion at night and vice versa [41]. In addition to that melatonin has antioxidant, immunomodulation, and neuroprotective properties [42, 43]. Melatonin binds to two G-protein coupled receptors, MT1 and MT2, which are widely distributed in different tissues and it works in both a receptor-dependent manner and receptor-independent one [44]. Melatonin has low affinity towards another receptor, MT3, acting as an enzyme with different kinetic and ligand association and different pharmacological characteristics as compared to MT1 and MT2 [45]. In the past two decades, much attention has been paid to the relationship between melatonin and eye in connection with SCN, circadian rhythm, and ocular diseases [46]. Many researchers have reported melatonin production in various ocular tissues following the circadian rhythms, such as lachrymal gland, crystalline lens, retina, ciliary body, and iris [47]. Furthermore, studies have shown the presence of melatonin receptors in choroid, sclera, cornea, and retina, indicating an important role of melatonin in visual system [48]. Several studies have elucidated a link between melatonin and various ocular conditions, with a focus on melatonin as part of the treatment regime for glaucoma and for inflammatory and age-related diseases [49, 50]. Melatonin has been proven to be a strong neuroprotective agent when applied exogenously to control intra ocular pressure and preventing intraocular hypertension related retinal injury and glaucoma in animal models and clinical trials [49, 51, 52]. Furthermore, melatonin is a strong antioxidant, anti-inflammatory, and immunomodulation agent showing a therapeutic potential in AMD, diabetic retinopathy, and uveitis [53, 54]. In addition, melatonin protects the retina from oxidative stress by regulating the secretion of vascular endothelial growth factor (VEGF) in the retina [55]. Although it has been proven beneficial in the management of several ocular diseases, the causality between melatonin and ocular diseases is yet to be unidentified, testifying the dire need of more prospective clinical studies.

Taurine

Taurine is the most abundant amino acid in the retina of mammals, with taurine concentration reaching 50 mmol/g tissue higher than in any other ocular structure or in the brain [56]. The retinal pigment epithelium (RPE) and Müller cells provide taurine to the retina, which then collects and transfer it to photoreceptor cells [57]. Photoreceptors require an adequate amount of taurine from extracellular environment and its transport is dependent upon osmoregulation by high and low-affinity Na+ and Cl^--dependent taurine transporters present in the retina [58]. Although the function of taurine in the retina is still uncertain, a lack of taurine in nutrition has resulted in reduced plasma taurine concentration and atypical electroretinograms in children and adolescents [56].
Taurine depletion has been observed to increase light-induced photoreceptor degeneration in rats, indicating the role of taurine in retinal survival and photoreceptor protection [59]. Accordingly, the administration of taurine supplement might prove beneficial in preventing and counteracting retinal degeneration and light-induced pathologies.
Being a strong antioxidant, taurine exhibits cytoprotective effects by reacting and counteracting the neutrophil oxidant, hypochlorous acid. This reaction lead to the production of taurine chloramine, which inhibits the inflammatory pathway [60]. Moreover, taurine reduces the mitochondrial production of superoxide, prevents mitochondrial ROS induced oxidative stress, and protects the antioxidant enzymes [56]. The protective role of taurine in photoreceptor degeneration, retinal ganglion cell loss,

Tab. III. The effects of selected nutritional supplements on various ocular manifestations and their food sources.

Natural compounds	Food sources	Benefits for the eyes	References
Vitamin A	Almonds, carrots, tuna	Beneficial in retinitis pigmentosa	[31]
Melatonin	Fish, milk, eggs, seeds, pistachio	Neuroprotectant, IOP regulation	[34]
Taurine	Sea food, turkey, seaweed	Prevents retinal degeneration and light-induced damages	[52]
Omega-3 fatty acids	Fishes (mackerel, sardine, salmon)	Prevent inflammatory damage, reduce IOP, beneficial in dry eye disease and AMD	[66]
B-Vitamins	Milk, cheese, eggs, fishes, leafy vegetables, chicken	Antioxidant, neuroprotective, decrease cataract incidence	[60]
Palmitoylethanolamide (PEA)	Egg yolk, peanuts	Anti-inflammatory, retino-protectant in glaucoma and diabetic retinopathy	[78]
Saffron	Flower of *Crocus sativus*	Anti-inflammatory, anti-oxidant, protection against light damage to photoreceptors	[90]

and other retinal morphological injuries in in vitro and in vivo disease models shows that it plays an essential role in retinal function preservation, so it can be administered as a supplement or as part of a taurine-rich diet to prevent retinal degeneration in various ocular diseases [61]. Taurine has been observed to hamper the progression of retinal diseases, highlighting its promising candidature in preventing or treating retinal diseases.

Spearmint

Even when not consumed in large quantities, spearmint has plenty of vitamins and minerals that are beneficial for the body. The spearmint extract – rich in fiber, vitamin A, iron, manganese, and folate – along with forskolin, homotaurine, and B vitamins, has been recently observed as beneficial in counteracting the retinal dysfunction in retinal ganglion cell (RGC) death caused by optic nerve crush [60]. As the spearmint extract is rich in polyphenols, it contains flavonoids that exhibit neuroprotective effects on RGC loss by reducing inflammation and oxidative stress [62, 63]. Further research is required in order to determine the beneficial effects of spearmint extract in ocular diseases; however, as it is a regular dietary ingredient of MedDiet, it holds significant potential in managing various ocular and other pathological conditions.

B vitamins

The B-vitamins, comprising eight water-soluble vitamins, perform an essential role in cell function, acting as coenzyme in various metabolic reactions. They are specifically involved in energy production, DNA/RNA synthesis/repair, synthesis of numerous neurochemicals, genomic and non-genomic methylation, and cellular signaling, thus maintaining vital functions in the brain. B vitamins (like B6, B12, and folic acid) are beneficial in eye health, as these prevent AMD by reducing homocysteine levels in blood.

Deficiency of B vitamins results in reduced vision or formation of blind spots because the optic nerve is compromised. Several observational studies have reported the protective role of a high blood level of B vitamins, such as riboflavin, thiamin, and niacin, against the development of lens opacities [64]. Riboflavin and niacin also have protective effects against oxidative stress by acting as coenzymes of oxidative enzymes. Administering these two B vitamins in undernourished Chinese population resulted in decreasing the incidence of cataract [65]. An epidemiological observation study, involving 5442 female health professionals with preexisting cardiovascular diseases, has shown that vitamin B12 deficiency in women is linked with an increased risk of AMD, which are twice as much as those having normal B12 levels [64]. The randomized, double-masked, placebo-controlled trial data indicated that subjects with daily supplementation of 50mg B6, 1mg B12, and 2.5mg folate supplements for two years had 35% to 40% chances to be less prone to AMD. However, in another study, supplementation of vitamin B6, B12, and folate exceeding the recommended dietary doses resulted in an increased risk of causing cataract in U.S. physicians in a timespan of over 7 years [66]. Consistently with these findings, a high dietary folate intake in participants using multivitamins is associated with an increased risk of posterior subcapsular cataract (PSC) in Age-Related Eye Disease Study (AREDS) patients [67]. Another study reported a two-fold increase in cataracts in the PSC region with high folate intake [68].

Omega-3 fatty acids

Evidence suggests that the essential omega-3 fatty acids, obtained from a healthy diet or supplementation, provide benefits to the visual system. Omega-3 fatty acids can be obtained from a variety of food sources: for instance, the short-chain omega-3 called alpha-linoleic acid (ALA) is abundant in flaxseed and chia seeds, while marine-based foods are rich in long-chain omega-3 fatty acids, like docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). The absorption of long-chain omega-3 fatty acids in the cellular membranes is based on their competition with the long-chain omega-6 fatty acid called arachidonic acid (AA) [69], which is a pro-inflammatory agent. As a result, increasing omega-3 fatty acid intake regulates a systemic inflammation via production of anti-inflammatory metabolites. As the optimal dietary ratio of omega-6 to omega-3 fatty acid should be 4:1, a diet rich in omega-6 fatty acids – such as western fried foods – results in an imbalance of the two, thus leading to an appropriate absorption of omega-3 fatty acids in the cellular membranes [70]. This results in deficiency of omega-3 fatty acids in approximately 80% of the adult population of developed countries [71]. Balanced diets, having plentiful long-chain omega-3 fatty acids, have been demonstrated to be beneficial in several chronic ocular conditions, including dry eye disease and AMD [72]. Moreover, long-chain omega-3 fatty acids are beneficial in reducing ocular surface inflammation in dry eye disease, improving tear-lipid profile [73]. Studies have shown that women with a low dietary intake of omega-3 fatty acids tend to have higher chances to develop dry eye disease [74]. However, clinical trials conducted to assess the potential benefits of omega-3 fatty acids in dry eye disease resulted in contradictory findings. DHA, specifically, is implicated in structural and functional maintenance of retina [75].

In addition, diets rich in polyunsaturated fatty acids enhance the retinal cellular response in AMD animal models to oxidative, ischemic, and inflammatory damage [76]. Although epidemiological studies have shown the potentials of dietary long-chain omega-3 intake in lowering the risk of early-stage AMD [77] and of progression to late-stage visual impairment [78], the omega-3 supplements have not shown the same efficacy as whole foods [79]. On the contrary, a double-masked placebo-controlled randomized clinical trial conducted in Australia concluded that oral omega-3 supplementation for 3 months significantly reduced IOP in normotensive adults [80]. However, the synergistic interactions of different nutrients in the whole food is essential for har-

vesting their full benefits and this should be given due consideration during clinical recommendations.

Palmitoylethanolamide (PEA)

Palmitoylethanolamide (PEA) is a pleiotropic naturally occurring endogenous N-acetylethanolamine cell-protective lipid found in several foods and in many living organisms. Numerous studies have reported its anti-inflammatory and neuroprotective characteristics; however, its beneficial effects are dose-dependent and mediated via receptors such as PPAR-α, PPAR-γ, PPAR-δ, GPR, and TRPV1 [81]. In the past 50 years, many clinical trials for various ocular diseases (such as glaucoma, diabetic retinopathy, uveitis, and pathological conditions involving inflammation) have tested PEA effectiveness. This molecule is available both as via dietary sources rich in PEA and as via supplement. Several PEA supplementary products (like Normast, PeaVera, and Visimast) are administered to glaucoma and neuroinflammation patients in Italy for nutritional support. PEA holds promise in treating many retinopathies and has been evaluated in many double-blind placebo-controlled studies to be safe, effective, and tolerable up to 1.8 g/day. Moreover, as PEA downregulates proinflammatory genes, it has been beneficial as an anti-inflammatory and retino-protectant compound in glaucoma and diabetic retinopathy [82].

Saffron

AMD is a retinal neurodegenerative disease characterized in its early stage by large soft drusen and hypo-hyperpigmentation of the retinal pigment epithelium (RPE). Late AMD, the potentially blinding stage of disease, includes geographic atrophy of the RPE ("dry" age-related macular degeneration), or subretinal neovascular membranes ("wet" age-related macular degeneration) [83].

AMD is generally considered a multifactorial disease, whose development and progression are the results of a complex interaction between genetic and environmental risk factors. Both oxidative stress [84] and chronic inflammation [85] seem to play a significant role in the pathogenesis of AMD together with many risk factors [86-89]. The final outcome of all neurodegenerative retinal diseases is the death of photoreceptors, consequently visual functions progressively deteriorate up to complete blindness. Recently, new strategies able to mitigate photoreceptor death using natural products have been explored. Among the others, Maccarone et al. (2008) provided data showing that l'Aquila saffron is protective against light damage to photoreceptors, in a rat model [90]. A proof-of-principle clinical trial in AMD patients confirmed the potentiality of saffron treatment in neurodegenerative diseases and its consistency in time [91,92] and in patients carrying genetic mutations [93].

Saffron is a well-known spice, used widely in different cuisines. It has also long been used in traditional medical practice [93,94]. Egyptian healers used it to treat gastrointestinal ailments; in Roman times it was used to promote wound healing and relieve upper respiratory complaints. In more recent times it has been used as an anti-inflammatory, anticonvulsive and anti-tumor agent; it has been investigated in the treatment of cognitive defects. Because of this long history of medical use associated to a variety of different clinical applications it is essential to make an accurate analysis of relevant data specifically in the field of retinal diseases. Saffron is the commercial name of the dried red stigmas of *Crocus sativus* flowers. It is produced in many areas all over the world, but the bulbs may present different genetical characteristics and the cultivar and the drying strategies are quite different; this makes each single production to be unique and this could explain the variety of effects and some discrepancies in the literature. Chemically, saffron is known to contain more than 150 volatile and aroma-yielding compounds and many non-volatile biologically active components, including carotenoids (zeaxanthin and crocetin) and various alpha- and beta-carotenes. Its golden yellow-orange colour comes from alpha-crocin, a water soluble biogentobiose (sugar) ester of crocetin. Its flavor arises from the glycoside picrocrocin, a molecule containing safranal and a carbohydrate. Its most potent anti-oxidant ingredients appear to be crocin, and crocetin, a carotenoid dicarboxylic acid which forms the core of crocin. Several actions of crocin on mammalian tissues have been reported including anti-apoptotic activity and increased oxygen diffusivity [90, 95]. Kanakis et al. (2007) showed that metabolites of saffron bind directly to DNA and induce its partial conformation to beta-DNA, thereby protecting the cell from damage [96]. Saffron has been shown to have anti-inflammatory actions, including for example the inhibition of tissue necrosis factor [97]. Based on these observation it comes clear that the saffron extract does not act as a simple antioxidant. The peculiar characteristics of saffron components support the hypothesis of an involvement of very different ways of action going from antioxidant activity to direct control of gene expression as it is also suggested by microarray experiments [98].

Conclusion

Eyes are an integral part of our lives, well-being, development, and happiness. It has been proven now that a healthy diet helps maintain a healthy vision system, avoiding many ocular problems. Scientific evidence suggests that micronutrients, vitamins, and natural compounds with antioxidant and anti-inflammatory properties play a pivotal role in alleviating and protecting age-related ocular degeneration. Therefore, with aging we require an adequate supply of good fats, controlled carbohydrates, fibres, and proteins, along with micronutrients and vitamins to maintain our health. In addition, consuming a good amount of probiotics helps regulating the gut microbiota, which in turn mediates the ocular surface microbiota and prevents many ocular diseases. The Mediterranean diet, which is a blend of all these ingredients, holds significant potential as a dietary regime

for better eye care and therapy and should be given due consideration.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; MCM, ZN, KD, GP, BF: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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REVIEW

Dietary supplements in lymphedema

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Keywords

Lymphedema • Dietary supplement • Hydroxytyrosol • Nutrition • GARLIVE®

Summary

Lymphedema is a chronic inflammatory disorder resulting from ineffective fluid uptake by the lymphatic system, and the effects are principally felt in the lower limbs. The condition is said to be primary when caused by genetic mutations and secondary when caused by injuries, infections, or surgery. Lymphedema, a worldwide pathology, does not have an effective therapy so far. Leukotriene B4 has recently been identified as a key molecule in lymphedema pathogenesis. Surgical, nonsurgical, and pharmacological treatments have been proposed; however, they do not cure the disease and only ameliorate the symptoms. Nutrition and nutritional status are extremely important in lymphedema physiopathology. Obesity is a comorbidity that exacerbates the risk for secondary lymphedema and constitutes a negative prognostic factor. Indeed, anti-inflammatory foods and their effects on the inflammatory state and on oxidative stress are now being investigated for their possible therapeutic role in lymphedema. Although no special diet has so far been proven to be very effective, specific dietary tips could help in alleviating the edematous state of patients with lymphedema. A few supplements have been tested for lymphedema treatment. Among them, GARLIVE® containing hydroxytyrosol, hesperidin, spermidine and vitamin A, exhibited promising effects in the animal model. Hydroxytyrosol, a polyphenol from olives, showed anti-inflammatory effects and reduced leukotriene B4 synthesis, thus holding promise as a potential natural candidate for lymphedema treatment.

The lymphatic system

Our body maintains health and prevents diseases thanks to the coordinated action of different organs and organ systems, among which the lymphatic system is of utmost importance. The lymphatic system is composed of three main components: lymph, i.e. the interstitial fluid, originated by extravasation of fluid and proteins from blood capillaries; lymphatic vessels, which drain back lymph from the entire body into the bloodstream; and lymphocytes, cells of the immune system that concentrate in lymph nodes and check lymph content, including for the presence of microbes and viruses. The lymphatic vessels originate in the peripheral tissues and convey the lymph to the venous circulation [1, 2]. These vessels also transport antigens and immune cells to the draining lymph nodes, which foster immune response or tolerance [3].
Apart from participating in immunity, the lymphatic system is involved in many other physiological processes, among which circulation and metabolism are the most important [2]. The lymphatic system collects the lymph that is filtered from the arterial side of the capillary bed and also transports dietary fats, hormones, and waste substances [4, 5].
Always considered of secondary importance compared with the blood vascular system, growing evidence supports the primary role of the lymphatic vascular system. Blood vessels and heart develop earlier than the lymphatic vessels in embryos, and this first vascular system also has lymphatic functions crucial for defense mechanisms. Indeed, macrophages develop before erythrocytes, and in vertebrates, the early blood vessels express the lymphendothelial receptor VEGFR-3 [6].
Lymphatic vessels transport the lymph from the periphery to the venous circulation. Similar to blood vessels, they can have different dimensions, from the small lymphatic capillaries (or initial lymphatics) to the large collecting vessels (lymph ducts or collecting lymphatic vessels) [1]. The lymphatic vessels possess unidirectional valves and are composed mainly of lymphatic endothelial cells (LECs) and lymphatic muscular cells (LMCs) [1]. Disfunction in any of these components can lead to different pathologies, among which the most prevalent is lymphedema [5].

Lymphedema

A chronic inflammatory disorder resulting from fluid accumulation, lymphedema affects 3 million people in the United States and 140-250 million people globally. Lymphedema is considered primary when it is caused

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by genetic variants and secondary when it develops after surgery, infections, or injuries. Primary lymphedema has a prevalence of 1/100.000, whereas secondary lymphedema has a prevalence of 1/1000. The disorder mainly affects the lower limbs, but it can also affect the upper limbs, genitalia, and face [7-10]. Lymphedema is more frequently diagnosed in women than in men, because of: 1) hormonal differences, that lead to more severe manifestations in females, and possibly 2) the fact that women consult clinicians more readily than men [11]. The pathophysiology of lymphedema and the molecular pathways underling the disease remain unclear although several studies have advanced our knowledge in this field. It is known that inflammation is an essential component of lymphedema and that it results in lymphatic damage, pain, fibrosis, and adipose tissue deposition [12-14]. Several genes, such as *FOXC2* [15, 16], have been found to be involved in primary lymphedema development or in secondary lymphedema predisposition; nevertheless, more research is needed to define new diagnostic and therapeutic targets [9]. Moreover, lymphedema treatments are currently unsatisfactory. Lymphedema, despite the etiology, is still virtually incurable, and the current therapeutics merely provide symptomatic relief by reducing swelling and preventing inflammation without offering a definitive cure for the disease [17].

Primary lymphedema

Lymphedema is classified as primary when it results from genetic mutations. Primary lymphedema is rare, and it is usually caused by malformations of lymphatic vessels and/or malfunction of lymphatic drainage [18]. More than 20 genes have been associated with various forms of primary lymphedema, but their mutations can explain only a portion of all primary lymphedema cases and a high degree of genetic heterogeneity is found among patients with lymphedema [19]. Apart from Milroy disease, many forms of primary lymphedema are usually sporadic. The disease can present at birth (congenital) or evolve during childhood, puberty, or even adult life, and may be diagnosed through genetic screening methods [18, 20].

Secondary lymphedema

Secondary lymphedema evolves after birth and results from lymphatic damage by different agents, such as infections, surgery, or traumas [18]. Particularly, lymphatic filariasis results from infections caused by nematodes belonging to the *Filariodidea* family, and it is the most common form of secondary lymphedema worldwide. The infection is mainly found in sub-Saharan Africa and India and affects more than 120 million people globally [18]. Moreover, herpesvirus infection can rarely cause lymphedema [21]. Secondary lymphedema typically results from malignancy-related therapeutic interventions. Surgical procedures usually lead to dissection or excision of lymph nodes. Furthermore, radiation therapy can damage dermal lymphatic vessels and cause nodal fibrosis [18, 22]. Finally, obstruction of lymphatic vessels by adipose tissue and reduced physical activity, which is typical of morbid obesity, can also cause secondary lymphedema [18, 23].

Pathophysiology of lymphedema

Lymphedema is a chronic inflammatory disorder. Fluid accumulation stimulates the activation of inflammatory cells, which in turn modify the extracellular matrix, decreasing lymphatic function [24]. Several inflammatory cells participate in its etiopathogenesis, mainly T cells and macrophages. CD4+ cells, specifically Th2 differentiation, are essential in controlling fibrosis and inflammation and in the development of lymphatic dysfunction [24, 25]. M1 inflammatory macrophages can be activated by Th1 and Th17 cellsand release nitric oxide and VEGF-C, which reduce the contraction of lymphatic vessels and increase lymphangiogenesis, respectively. On the other hand, M2 anti-inflammatory differentiation of macrophages seems to be beneficial in the initial stages of lymphedema and regulates lymphangiogenesis and tissue remodeling [24-26].

Following inflammation, lymphedema results in fibrosis and adipose tissue deposition. Fibrosis impedes the functioning of lymphatic vessels and exacerbates the symptoms of lymphedema and reduces lymph drainage. Fibrosis is positively regulated by Th1 and Th2 cells, which release TGF-β, IL-4 and IL-13, whereas macrophages negatively regulate the process. Adipose tissue is influenced by IL-6 dysregulation and Th2 cells, a negative regulator of adipose tissue deposition, whereas lymph stasis and leakage typical of lymphedema sustain adipose tissue proliferation [24, 25].

Leukotriene B4

Several studies have reported that leukotriene B4 (LTB4) is involved in the pathogenesis of lymphedema [21]. Leukotrienes are synthetized in leukocytes from arachidonic acid by the action of different enzymes, among which 5-lipoxygenase is prominent. Leukotrienes bind cognate G protein coupled receptors and elicit an inflammatory response. LTB4 signal is transduced by the receptors LTB4R and LTB4R2 [27]. LTB4 controls CD8+ and CD4+ cells as well as the recruitment of neutrophils and macrophages in the lymphedematous tissues. These inflammatory cells, especially neutrophils, produce more LTB4 upon activation, thus recruiting even more leukocytes to the inflammatory site [27, 28]. LTB4 stimulates Th17 differentiation and acts as a molecular link between adaptive and innate immunity in lymphedema [29]. LTB4 modulates VEGFR-3 and Notch signaling, both of which are important in lymphangiogenesis, and mediate pruritus caused by localized lymphedema. LTB4 has been proven to diminish the function of lym-

phatic endothelial cells, worsening the progression of lymphedema [25, 30].

Treatments

No absolute cure is available for lymphedema. The therapeutic approaches are divided mainly into nonsurgical (lymphatic drainage, compression therapy, electromedical devices, and specific exercising) and surgical (lymphatico–lymphatic bypass, used only in selective cases) methods. Other than specific treatments, skin hygiene and mild-to-moderate physical activity are of utmost importance [31, 32]. Some pharmacological treatments have been proposed for chronic edema pathologies, all with unsatisfactory results. Particularly, diuretics, analgesics, antibiotics, antifungals, and benzopyrones are being or have been used.

- Diuretics, used only in the initial stages of lymphedema, are usually ineffective, and their long-term use can result in fluid and electrolyte imbalance.
- Analgesics are used in cases of severe edema when the size and weight of the limb causes unbearable pain.
- Antibiotics are used in the presence of cellulitis, a common complication that results from lymphatic disfunction and local immunodeficiency.
- Antifungals are used for treating athlete's foot and fungal nail infections, typical complications of lymphedema in the lower limbs.
- Benzopyrones are considered the most effective pharmacological treatment because they stimulate proteolysis by macrophages, thus reducing excess proteins, swelling, fibrosis, and chronic inflammation and controlling microbial infections [31, 33, 34].

The lack of effective lymphedema treatments calls for research on new active molecules.

Proposed supplements and hydroxytyrosol

Apart from pharmacological therapies, a few studies have proposed the use of dietary supplements for the treatment of lymphedema. A combination of caloric restriction and synbiotic supplementation reduced the edema in survivors of breast cancer-related lymphedema, mainly via the antioxidant properties [35, 36]. Moreover, selenium supplementation proved to be effective in the treatment of secondary lymphedema and reduced the production of reactive oxygen species [37]. Finally, a new promising molecule has been recently proposed for the treatment of lymphedema in the form of supplements, namely hydroxytyrosol (HT). HT is a biophenol extracted from olive oil and leaves, exhibiting anti-inflammatory, antioxidant, and antimicrobial properties. HT has no side effects at any concentration; thus, it was proposed for prolonged consumption in the form of a nutraceutical. HT has been established to be an efficient inhibitor of LTB4 synthesis [8, 25, 38]. LTB4 inhibitors have already been tested for lymphedema treatment. Ketoprofen, a nonsteroidal anti-inflammatory drug, inhibits 5-lipoxygenase activity and regulates LTB4 synthesis. Ketoprofen alleviates inflammation and induces prolymphangiogenic factors, thereby reversing edema and re-establishing the lymphatic function [39, 40]. HT consumption has recently been correlated with an improvement in the quality of life in patients with lymphedema, supporting its therapeutic value [41].

Supplementation of HT in an animal model of lymphedema

A dietary supplement containing HT, GARLIVE®, was tested in a mouse tail model of lymphedema [7]. Other than HT, GARLIVE® contains the anti-inflammatory molecules spermidine, hesperidin, and vitamin A. Spermidine is derived from rice seeds, and it inhibits the production of proinflammatory mediators, such as TNF-α, IL-1β, NO, and PGE2 [42]. Hesperidin is extracted from citrus fruits and has been tested for lymphedema treatment, with promising results [43]. Vitamin A is usually correlated with low levels of inflammation. Moreover, retinoic acid, a metabolite of vitamin A, can interact with the receptor encoded by the *RORC* gene. Deleterious mutations in this gene have recently been reported in patients with lymphedema [44]. GARLIVE® supplementation resulted in reduced tail swelling after surgical intervention. The treated group presented a decreased tail volume, the peak of the swelling was reached faster, and the swelling remained for less time [7]. Considering the molecular effects of the used molecules, their possible synergistic effects, and the promising results in the animal model, further clinical studies should be conducted to establish the use of GARLIVE® in patients with lymphedema.

Lymphedema and nutrition

Diet plays an essential role in lymphedema progression. Particularly, the classical Western eating habits, based on carbohydrates and refined foods, favor systemic low-grade chronic cellular inflammation, which in turn stimulates edema. Proinflammatory and anti-inflammatory foods are now being actively investigated for their possible roles in lymphedema. Oxidative stress, another important aspect of lymphedema pathophysiology, could be reduced via nutrition and using supplements containing several natural antioxidant substances. Polyphenols seem to target the molecular pathways that form the basis of lymphedema. Polyphenols exert anti-inflammatory and antiedematous actions, and they elicit an effect directly on the lymphangion [32]. High body mass index has been correlated with lymphedema onset, and weight loss achieved via caloric restricted and healthy dietary patterns has been proven to reduce breast-cancer related secondary lymphedema [45]. Moreover, weight loss has been shown to improve lymphedema-related

symptoms and also offers other benefits such as improved body image and insulin control [32]. In the scope of dietary control, the intake of medium-chain fatty acids has been established to be correlated with a reduction in the volume of the limbs. Medium-chain fatty acids are hydrolyzed by pancreatic lipase and then absorbed in the duodenum. These fatty acids are not esterified or absorbed by the lymphatics but directly enter the portal system, thereby reducing accumulation and pressure in the lymphatic ducts [46]. However, extremely low-fat diets could require specific vitamin supplements; thus, the specific diet should be controlled by the clinician. Restricted fluid intake has also been proposed as a possible lymphedema intervention, but it has not been demonstrated to be beneficial in peripheral lymphedema. Thus, no special diet has so far been proven to be of high therapeutic value for patients with lymphedema [31]. Nevertheless, specific dietary tips could help in reducing the edematous state. Fibers present in fruits and vegetables lead to the formation of short-chain fatty acids in the bowel, which exhibit an anti-inflammatory activity [32, 47]. Other anti-inflammatory dietary molecules, such as omega-3, and several spices (turmeric, garlic, and curry leaves) may reduce inflammation and edema [32, 48]. On the contrary, foods such as salt, caffeine, omega 6 or 9, alcohol, and sweets exert the opposite effect [32, 49]. Thus, they should be avoided by patients with lymphedema. Finally, foods can control the physiologic hormonal response, which in turn influences inflammation and edema [32]. Apart from nutrition, specific physical exercises have been shown to be extremely important for improving the quality of life and ameliorating the symptoms of patients with lymphedema. In patients with secondary lymphedema, yoga practice, for instance, has been shown to be highly effective [50].

Lymphedema and obesity

Lymphedema may be a comorbidity of obesity. Several studies have correlated obesity with lymphedema onset, course, and prognosis [32, 51]. Patients with obesity also exhibit other conditions leading to limb edema, such as reduced mobility, concomitant metabolic syndrome, excess of fluid-retentive adipose tissue, hypertension, and hormonal alterations [32]. Obesity exacerbates the risk for secondary lymphedema by up to three times. Moreover, even without other conditions, people with a body max index of > 60 have an insufficient lymphatic flow. Adipose tissue physically compresses lymphatic vessels and triggers local inflammation. Obesity has been proven to strongly influence the treatments for both primary and secondary lymphedema, thus representing a negative prognostic factor [32, 52]. Several studies also suggested that a high-fat diet can deregulate gene expression in lymphatic endothelial cells, which implies a genetic link between obesity and lymphedema. In a recent study on 71 patients with lymphedema, 20% were obese, and several mutations in genes related to lymphedema pathogenesis were also detected in patients with obesity. These findings suggest the presence of a genetic association between lymphedema and obesity [41, 53, 54].

Conclusion

Lymphedema is a worldwide-diffused disease, still without an effective therapy. Surgical, non-surgical, and pharmacological treatments have been proposed, but they only ameliorate the symptoms. Several supplements have been proposed for lymphedema treatment, with GARLIVE® having promising effects in the animal model. New research and clinical studies will be needed to find the best treatment for lymphedema patients.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; GB, KD, Serena M, Silvia M, Sandro M, MR, MC: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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Review

Dietary supplements for polycystic ovary syndrome

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Keywords

Polycystic ovary syndrome • Curcumin • Vitamin D • Inositol • CoQ10

Summary

Polycystic ovary syndrome (PCOS) is one of the most prevalent female endocrine reproductive disorders, affecting between 4 to 18% of the women in their reproductive age. It is generally characterized by several clinical aspects, among which anovulation, inflammation and infertility. Moreover, PCOS has several health implications, including increased metabolic, reproductive, and psychological risks. Previously, metformin and to some extent thiazolidinediones were considered as drug of choice for PCOS management, but they had several side-effects, and controversial results were obtained about their efficiency, especially in non-insulin-resistant non-obese patients. Thus, alternative treatment options are now being studied for PCOS, including different natural molecules and complementary medicines (CM) for the improvement of their health, wellbeing and fertility. Recently, treatment of PCOS patients with different natural molecules, coming from nutritional supplements and herbal medicines, has attained satisfactory results with the absence of any side effects. In this review, four natural molecules, curcumin, vitamin D, inositol and CoQ10 are discussed for their therapeutic ability. These molecules proved to decrease insulin sensitivity and inflammation, to improve the restoration of ovarian function, and they could restore hormonal balance and regulate the menstrual cycle, all of which are the main features and major concerns for women suffering from PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is a very prevalent heterogeneous female endocrine reproductive disorder affecting 4-18% of women during their reproductive years. It is generally characterized by anovulation and overproduction of ovarian androgens. PCOS has a wide range of health implications, including increased risk of metabolic (obesity, cardiovascular disease, type 2 diabetes), reproductive (miscarriage, infertility, neonatal and pregnancy complications) and psychological disorders (stress, depression and anxiety) [1]. It is generally agreed that besides thorough examination with medical history, the reproductive expectations of the patient should also be considered, before any therapy is decided. Tailored or personalized therapeutic approaches involving weight loss, diet and lifestyle can significantly improve ovarian function and avoid the risks associated with PCOS [2]. A hormone-based approach has traditionally used metformin or oral contraceptives, personalizing according to specific needs and clinical situations. Although oral contraceptives, like estro-progestin compounds, are effective, they cannot be recommended to patients who wish to restore ovulation in order to achieve pregnancy [3]. Metformin and to some extent thiazolidinediones have been considered the drugs of choice for management of PCOS in women desiring motherhood. Although metformin has improved the condition of obese insulin-resistant women with PCOS, there have been controversial results regarding its efficacy among non-insulin-resistant, non-obese patients [4]. Furthermore, metformin causes recurrent gastrointestinal side-effects like vomiting, nausea, diarrhea, metabolic complications and abdominal bloating, while thiazolidinediones may induce fluid retention, weight gain, myocardial infarction, bladder cancer and coronary artery disease [1].

Women suffering from PCOS have been seeking alternative treatment options including natural molecules and complementary medicines [1]. A number of natural molecules have recently shown promise and an absence of side-effects in PCOS patients.

Natural molecules and sources

Studies have found that ingestible complementary medicines like nutritional supplements and herbal medicines have some positive effects in PCOS [5]. Women with PCOS show significantly higher oxidative stress and homocysteine plasma levels. Certain nutritional supplements have been found effective in these patients and in reducing the risk of PCOS in other populations [6]. The endocrine effects of the natural compounds used as supplements or medicine may improve hormonal balance and menstrual regularity [1, 7]. In this review we focus on the beneficial effects of the following natural compounds.

Curcumin

Curcumin is a dietary polyphenol derived from *Curcuma longa* (turmeric). It is a popular spice in many Asian

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countries. It acts as an antioxidant that increases glutathione peroxidase superoxide dismutase and catalase activities, as well as being a free radical scavenger and regulator of the Keap1-Nrf2/ARE signalling pathway that upregulates detoxification and antioxidant genes. Curcumin is also known to induce ovulation and enhance the biochemical profile of patients with PCOS [8, 9].

Polycystic ovary syndrome is a prevalent cause of infertility in women [10]. Some key features of PCOS are insulin resistance and hyperandrogenism. Increased insulin levels cause an increase in luteinizing hormone (LH) levels by activation of cytochrome P450c17. Curcumin may decrease cytochrome P450c17 activity, improving hyperandrogenism among patients with PCOS [11].

The androgen receptor signaling pathway has also been recognized as a possible mechanism of anovulation in women with PCOS. Curcumin substantially downregulates expression of ovarian androgen receptor proteins. Inflammatory factors, too, have a significant role in the pathogenesis of PCOS. Curcumin has anti-inflammatory action through reducing tumor necrosis factor-α and IL-6. It is also known to decrease LH, reduce insulin resistance, induce ovulation in PCOS patients, improve body weight and correct lipid abnormalities [12-14].

A meta-analysis by Chien *et al.* evaluated the effect of curcumin in PCOS patients, finding an improvement in glycemic control through regulation of fasting insulin, fasting glucose, HOMA-IR index and the quantitative insulin-sensitivity check index. However clinical use of curcumin is limited due to intrinsic and extrinsic factors often associated with herbal medicines. Intrinsic factors include variations in organ specificity, in chemical and pharmacological constituents, in activity between different Curcuma species and diurnal variations; extrinsic factors include storage, environmental, cultivation, manufacturing and substitution variables [8, 11].

Vitamin D

There is evidence that vitamin D deficiency could be a major cause of insulin resistance and metabolic syndrome in women suffering from PCOS [15]. A higher prevalence of vitamin D deficiency (67-85%) is observed in these women. Positive associations have also been reported between vitamin D deficiency and other diseases that co-exist with PCOS, such as insulin resistance, type 2 diabetes, cardiovascular disease and metabolic syndrome. This is sustained by the fact that vitamin D receptors control over 3% of the human genome, including genes critical for glucose metabolism [16]. Polymorphisms associated with vitamin D are linked to insulin resistance and vitamin D deficiency in PCOS. More precisely, vitamin D receptor variants as in the *DHCR7* and *Cdx2* genes are known to be associated with insulin sensitivity and insulin resistance, while variants in vitamin D receptor α-I are linked to testosterone levels in women with PCOS [17]. Hence vitamin D modulates glucose-insulin homeostasis through particular receptors in pancreatic β cells and skeletal muscle, leading to direct activation of human insulin receptor gene transcription and of peroxisome proliferator activator receptor δ, as well as stimulation of insulin receptor expression and increase in insulin-mediated glucose transport *in vitro* [18]. The endorsement of vitamin D supplementation in women with PCOS is based on the role of vitamin D in glucose metabolism: it enhances insulin synthesis and release, increased expression of insulin receptors and suppression of pro-inflammatory cytokines. Vitamin D may play a significant role in the development and onset of most clinical features of PCOS [19, 20].

A research study by Daniela Menichini and showed that vitamin D supplementation may help to restore physiological serum levels of 25(OH)D in women with vitamin D deficiency. Actually, since 67-85% of women with PCOS have inadequate vitamin D levels, vitamin D supplementation is recommended for all of them [21]. Randomized controlled trials have suggested that regular low dose supplementation of vitamin D (< 4000 IU/d) in PCOS patients or consumption of vitamin D as a co-supplement improves fasting glucose concentrations and insulin sensitivity as well as HOMA-IR index [22]. Other studies suggest that better results are achieved by higher doses of vitamin D (≥ 4000 IU/d) for at least 12 weeks. With these doses, significant improvements in insulin sensitivity, glucose levels, hormonal function and hyperlipidemia have been reported [19].

Vitamin D appears to improve reproductive and metabolic impairment in PCOS through its impact on insulin resistance. As far as reproduction is concerned, insulin resistance improves hyperandrogenism by insulin-mediated stimulation of ovarian androgen production and associated reduction in sex hormone-binding globulin [19, 23]. Vitamin D is also significantly involved in facilitating successful pregnancy. Many studies have reported a correlation between vitamin D deficiency and male as well as female infertility, which can be diagnosed with genetic tests [24-26], and that vitamin D improves the outcomes of assisted reproductive techniques. Likewise, vitamin D supplementation with myoinositol and melatonin increases pregnancy rate. The association between vitamin D status and pregnancy rate varies in different ethnic groups ($p < 0.01$). In the white non-Hispanic population, pregnancy rate decreases with decreasing vitamin D levels, whereas among Asians the reverse relationship is observed. Considering the age, quality and number of embryos transferred, the pregnancy rate in non-Hispanic whites was four times greater in vitamin D-sufficient than in vitamin D-deficient patients [27, 28].

Similarly, because the physiological effects of vitamin D act simultaneously or in cooperation with progesterone, vitamin D is considered to be a steroid hormone with progesterone-like activity [16]. Calcitriol or bioactive vitamin D plays a significant role in promoting endometrial receptivity. It also supports implantation and the progress of pregnancy via pathways similar to those of progesterone, creating substantial synergy of action. Thus the significance of vitamin D is clear from luteal phase onwards [27, 29]. Vitamin D also regulates

follicular development by influencing anti-müllerian hormone signals, ovarian sensitivity to follicle-stimulating hormone (FSH) and progesterone production in the granulosa cells of the ovaries [30].

Inositol

Inositol (cyclohexane-1,2,3,4,5,6-hexol) is a carbocyclic sugar/polyol that accumulates in brain, kidney, liver and other mammalian tissues. It regulates cell signal transduction in response to different neurotransmitters, growth factors and hormones. Inositol may be transformed into nine stereoisomers through epimerization of its hydroxyl groups. The most clinically important isoforms of inositol are myoinositol and D-chiro-inositol (DCI). Dietary intake of myoinositol is sufficient to achieve concentrations that could improve different endocrine disorders, including insulin resistance and diabetes, in which myoinositol plays a crucial role [31]. The two isoforms work as second messengers of insulin but with different activities. Myoinositol is primarily involved in cell uptake of glucose [32]. It may therefore be regarded as a semi-essential compound, deficient in different pathological and physiological conditions [31]. Myoinositol is an insulin sensitizer commonly used to treat PCOS due to its effectiveness in reducing the reproductive and metabolic disorders that are the key features of the syndrome. Myoinositol also plays a critical role in cytogenesis and cell morphogenesis, as well as being involved in cell membrane formation, cell growth and lipid synthesis. It is crucial for signaling pathways that operate throughout cell life to modulate various physiological processes, such as oocyte maturation, gamete development, fertilization and early embryo development [33].

Myoinositol is well established as a nutraceutical that improves insulin sensitivity and hormonal and reproductive functions [34]. Myoinositol supplementation decreases the prevalence of gestational diabetes among women at risk due to a family history of diabetes. Similarly, a recently published study reported a dramatic reduction in the prevalence of gestational diabetes among overweight pregnant women (33.6-14%) [35].

Myoinositol plays crucial roles in female as well as male reproduction. Various studies and the FDA have confirmed and approved this stereoisomer as very safe [30]. Restoration of ovarian function has been reported among women with PCOS on insulin-sensitizing myoinositol supplements, as hyperinsulinemia causes PCOS-related ovarian dysfunction. In the same way positive effects of myoinositol supplementation were found among post-menopausal women with other hyperinsulinemic disorders like metabolic syndrome [36].

Myoinositol plays a significant role in many cell pathways as it regulates hormones like insulin, FSH and thyroid-stimulating hormone and acts as a second messenger [37]. Several reviews of the literature suggest that myoinositol signaling is associated with production of anti-müllerian hormone in human granulosa cells [11]. Other studies have established that myoinositol treatment of women with PCOS decreases insulin resistance and androgen levels, inducing ovulation and regularizing the menstrual cycle [33].

Role of inositol in assisted reproductive technologies

Although assisted reproductive technologies face several bioethical issues [38], women with PCOS are the subject of several ongoing research studies. Wdowiak examined oral myoinositol activity in PCOS patients undergoing intracytoplasmic sperm injection [39]. A significant difference in the pregnancy rate was observed between women with PCOS treated with myoinositol (34.62%) and untreated PCOS controls (only 20%). Follicular fluid concentrations of superoxide dismutase (SOD) also increased significantly only in the myoinositol-treated group [32].

According to three different research studies on IVF patients, myoinositol showed beneficial effects on oocytes. The effects were essential in neutralizing various endocrine and metabolic abnormalities related to PCOS. Myoinositol is known to be effective in restoring spontaneous ovarian activity, and therefore promoting fertility among PCOS patients. For example, several clinical trials have investigated the effect of oral administration of 2 g myoinositol twice a day, a dose now considered standard for PCOS treatment [40].

Another clinical IVF trial on 133 women with PCOS and 137 non-PCOS controls with conserved ovarian reserves administered oral myoinositol daily for three months during ovarian stimulation and in the preconception period. The results showed a significantly higher number of total mature oocytes in the myoinositol-treated group than in non-PCOS controls. According to the 'take home baby' index, the miscarriage and pregnancy rates for the embryo transfers were similar, suggesting that myoinositol improves oocyte quality, which in turn improves IVF outcomes [32, 41].

A clinical study by Emekçi Özay *et al.* reported a higher pregnancy rate (18.6%) in myoinositol-treated patients than controls (12.2%). Similarly, several meta-analyses and systematic reviews and eight randomized controlled trials comprising 812 subjects have established that oral supplementation of inositol during controlled ovarian stimulation and assisted reproductive technologies can reduce the quantity of gonadotropins used, as well as the period of controlled ovarian stimulation in women with and without PCOS undergoing in vitro fertilization [42, 43].

Myoinositol combined with D-chiro-inositol

Although myoinositol is the most abundant inositol isoform, another stereoisomer with great therapeutic qualities is D-chiro-inositol (DCI) [44]. Women with PCOS

have shown significant reproductive improvement when they received supplements of inositol with certain other molecules [45]. Because of the ability of both isomers to lower insulin resistance and their positive affect on metabolism, the combination myoinositol-DCI is considered to be specifically beneficial for PCOS patients. Both act as second messengers of insulin and mediate various activities of insulin [44]. The latest research studies report that this combination with a high proportion of DCI improves the pregnancy rate with respect to the physiological rate, without any difference in embryo quality or oocyte maturation. D-chiro-inositol affects oocyte quality by acting directly on the ovum or by adjusting follicular fluid composition. Oocyte quality can be further improved at genomic level and by modifying the follicular microenvironment; the latter mostly influences oocyte maturity or cytoplasmic quality [45].

Thus the combination of myoinositol and DCI improves oocyte quality by decreasing testosterone levels and increasing insulin sensitivity. Thus this combination with a high fraction of DCI may also improve oocyte cytoplasmic quality with respect to physiological concentrations [45].

Myoinositol is converted into inositol-phosphoglycan (IPG) that acts as an insulin second messenger (myoinositol-IPG) involved in uptake of glucose by cells. On the other hand, IPG derived from DCI (DCI-IPG), which also acts as an insulin second messenger, plays a significant role in glycogen synthesis. Besides, myoinositol-IPG increases ovarian uptake of glucose and plays a crucial role in FSH signaling, while DCI-IPG is involved in the production of insulin-mediated androgen. Furthermore, myoinositol and DCI can decrease plasma concentrations of testosterone and LH, and the LH/FSH ratio, thus counteracting the effects of hyperandrogenism and reducing hirsutism and acne. These specific actions of myoinositol and DCI collectively justify the use of these two stereoisomers to treat women with PCOS [44, 46].

The myoinositol/DCI ratio is 100:1 in healthy females and 0.2:1 in those with PCOS. Myoinositol and DCI are found in a specific ratio in different tissues. Unfer *et al.* suggested that as ovaries do not show insulin resistance, hyperinsulinemia causes overstimulation of epimerase activity in women with PCOS, causing poor synthesis of DCI and associated myoinositol deficiency [47]. In order to define the ideal dose of myoinositol/DCI, the myoinositol/DCI ratio in plasma was used as a standard to understand the systemic physiological balance between these two inositol stereoisomers in humans [47].

Bevilacqua *et al*. studied a mouse PCOS model, finding a pattern similar to that in humans. They treated mice with 420 mg/kg myoinositol-DCI in 2 ml drinking water per day. When myoinositol/DCI was administered in a 40:1 ratio to PCOS mice, recovery of a physiological ovarian phenotype and normal reproductive function was faster than with other formulations of myoinositol/DCI or with water alone. The 40:1 myoinositol/DCI formulation was the most effective in restoring fertility, normal uterine structure and function, and regular follicle and ovarian structure [48].

Human preclinical and clinical studies sustain the results of Bevilacqua *et al*., showing that a myoinositol/DCI ratio of 40:1 is the most effective in restoring ovulation in women with PCOS. The activity of DCI proved most beneficial at a particular ratio with myoinositol, while increase in DCI concentrations could cause loss of its beneficial reproductive effects [44, 48].

Myoinositol combined with melatonin

Recent research has established that plasma concentrations of myoinositol and melatonin are predictors of oocyte quality: higher levels of these two molecules are positively correlated with good oocyte quality [33, 49]. Many clinical trials show that myoinositol supplements, alone or in combination with melatonin, improve oocyte quality and IVF outcomes in patients with PCOS as well as normal women. Chiu *et al.* showed a direct association between follicular fluid concentrations of myoinositol and oocyte quality; thus high myoinositol concentrations are a marker of high quality oocytes [50]. In the same way, myoinositol or melatonin supplementation affects oocyte and embryo quality. Particularly, in cases of ovarian stimulation, myoinositol supplementation reduces the number of FSH units (IU) administered which increases the chances of pregnancy. Moreover, the effects of gonadotropins on ovulation, follicular growth and luteinization are linked to differences in FSH and LH receptor concentrations [32, 51, 52].

Myoinositol combined with alpha lipoic acid

Insulin sensitizers are currently being used to treat obese and lean women with PCOS. Different isoforms of inositol without the side-effects of metformin are being compared with the latter for insulin-sensitivity-enhancing activity. Alpha lipoic acid (ALA) is a naturally occurring antioxidant derived from octanoic acid, considered effective and safe [53, 54]. Alpha lipoic acid exhibits anti-inflammatory activity, inhibiting NF-kB translocation to the nucleus and reducing release of proinflammatory cytokines. The combination of inositol derivatives and ALA is a therapeutic option to improve insulin sensitivity, ovulatory rhythm and hyperandrogenism. The combined action of myoinositol and ALA can therefore improve insulin resistance and chronic low grade inflammation in PCOS patients [55, 56].

In myoinositol-resistant patients, treatment with myoinositol and ALA leads to significant improvement in PCOS. This combination re-establishes ovulation, with substantial changes in metabolic and hormonal parameters, thus significantly increasing the chances of pregnancy in women with fertility issues. The bioavailability of myoinositol increases significantly when administered with ALA rather than alone [40].

Inositol and high-mobility-group-box-1 (HMGB1)

The best treatment option for PCOS should be determined on the basis of pathophysiology. High-mobility group box 1 (HMGB1) is a highly conserved non-histone chromatin-associated protein that plays a vital role in chromatin remodelling at nuclear level, as well as acting as a pro-inflammatory mediator in the extracellular environment. Plasma concentrations of HMGB1 are reported to be elevated in PCOS patients due to reduced ovarian expression of cystic-fibrosis-transmembrane-conductance-regulator, and are associated with inflammation and insulin resistance, both features of PCOS. Women with PCOS have considerably elevated circulating levels of inflammatory markers like hs-CRP (high sensitive c-reactive protein), IL-6 (interleukin -6) and TNF-α (tumor necrosis factor-α). Chronic low grade inflammation may contribute to different ovarian alterations as well as insulin resistance [34, 57-59].

PCOS patients have higher plasma concentrations of HMGB1, insulin and insulin-like growth factor I, and a higher HOMA-IR index and triglyceride/high-density lipoprotein-cholesterol ratio than controls. In adolescent girls with PCOS, combined inositol treatment significantly reduced concentrations of HMGB1 and insulin to levels almost similar to those of controls. In obese adults with PCOS, combined treatment with myoinositol-ALA lowered serum concentrations of insulin. Inositol treatment therefore had positive biochemical and clinical effects [34].

Coenzyme Q

Coenzyme Q10 (CoQ10) is a lipid-soluble antioxidant naturally synthesized by human cells and required for cell growth and maintenance. It is a mobile electron carrier essential for adenosine triphosphate synthesis by oxidative phosphorylation. CoQ10 acts as an intracellular antioxidant and effectively prevents the oxidation of lipids, proteins and DNA. Numerous randomized controlled trials have established significant beneficial effects of CoQ10 supplementation as an adjuvant in different clinical conditions [60].

The inflammatory state common in PCOS patients is demonstrated by higher plasma concentrations of inflammatory cytokines such as TNF-α, interleukin-6 and C-reactive protein [61]. Release of cytokines and chemokines promotes macrophage recruitment and adhesion molecule production. Enhanced circulation of adhesion molecules like vascular cell adhesion molecule-1, E-selectin and intercellular adhesion molecule-1 are prominent signs of impaired endothelial function [62]. In women with PCOS, increased plasma levels of these three biomarkers of endothelial dysfunction have been reported [7, 8]. Farsi *et al.* reported that CoQ10 supplementation for 12 weeks had major effects on plasma concentrations of high-sensitivity C-reactive protein and TNF-α in patients with non-alcoholic fatty liver disease [63]. Likewise Shiva Taghizadeh *et al*. found that CoQ10 supplementation (200 mg/day) for eight weeks had many beneficial effects and significantly reduced serum concentrations of the above three inflammatory and endothelial dysfunction markers in overweight patients with PCOS compared to placebo-treated controls [60].

Although the precise mechanism by which CoQ10 reduces inflammatory factors is unclear, there is evidence that CoQ10 can downregulate expression of genes encoding inflammatory cytokines in patients with PCOS or with diabetic nephropathy. Furthermore Schmelzer *et al*. showed that the anti-inflammatory effects of CoQ10 may be caused by lower expression of nuclear factor-$\varkappa$B-dependent genes [64]. This factor may be activated by reactive oxygen species and may increase pro-inflammatory cytokine expression. Antioxidants like CoQ10 could inhibit this NF-$\varkappa$B-activating cascade [65] and CoQ10 also activates the PPAR (peroxisome proliferator-activated receptor)-mediated anti-inflammatory response, that acts as PPAR agonist. Other mechanisms including attenuation of miR-146a, modulation of interleukin-1 receptor-associated kinases, reduced secretion of macrophage inflammatory protein-1 alpha and RANTES (regulated upon activation normal T-cell expressed and secreted) by CoQ10 are proposed. Further long-period studies are required to confirm the effects of CoQ10 supplementation on inflammatory factors in PCOS [60, 66, 67].

Conclusion

Polycystic ovary syndrome is one of the most prevalent endocrine disorders affecting women of reproductive age and is generally characterized by anovulation, overproduction of ovarian androgens, insulin insensitivity, inflammation, infertility and other reproductive complications. Metformin and to some extent thiazolidinediones have been considered drugs of choice for PCOS management but they have side-effects and their efficacy in non-insulin-resistant non-obese patients is controversial. In this review we discussed the therapeutic effects of four natural molecules. Curcumin has anti-inflammatory effects, improves hyperandrogenism, lowers LH, reduces insulin resistance and induces ovulation in women with PCOS. Vitamin D plays a crucial role in glucose metabolism, improving insulin sensitivity, decreasing insulin resistance and suppressing pro-inflammatory cytokines. Inositol lowers insulin resistance, improves reproductive function, improves oocyte quality and restores ovulation in patients with PCOS. CoQ10 exerts anti-inflammatory effects by downregulating expression of genes encoding inflammatory cytokines in PCOS. Hence these substances should be considered for treatment of PCOS. Additional research could provide further evidence of the beneficial effects of using these natural molecules in the treatment of PCOS. More natural molecules should be explored and analysed for their therapeutic activity in this syndrome.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; AKK, Kevin D, Kristjana D, LS: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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REVIEW

Dietary supplements for intestinal inflammation

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Keywords

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Summary

Intestinal inflammation leads to various chronic diseases, collectively known as inflammatory bowel disease (IBD). IBD mainly affects the large intestine, but it can also affect the gastrointestinal tract as a whole. Its major symptoms are pain, diarrhea, and weight loss, and it is usually associated with deficiencies of both macro- and micronutrients. Unluckily, after some time the body develops resistance against the already available drugs: thus, many patients fail to maintain remission, which is achieved in less than 50% of cases. Diet is a major determinant of gut inflammation. An unbalanced diet can affect the gut microbiota and cause dysbiosis, which is related to a dysregulated host immune response. The Mediterranean Diet its renowned for its anti-inflammatory effects and for preventing dysbiosis. In order to improve management and treatment of intestinal inflammatory diseases, it should become common practice to integrate the patient's diet with dietary supplements with anti-inflammatory effects (probiotics, butyrate, phosphatidylcholine, lactoferrin, palmitoylethanolamide, silymarin, and omega 3), which maintain the stability of the intestinal microbial cohort and strengthen the mucosal barrier, thus preventing or soothing IBD symptoms. Dietary supplements may help fight the high costs, the adverse side effects, and the recurrent relapses typical of drug use.

Introduction

Inflammation is an innate immunity mechanism, triggered by the complex biological response of the gut as well as of other tissues of the body toward harmful stimuli, like pathogenic bacteria [1]. The inflammatory bowel disease (IBD) consists of two heterogeneous chronic immune disorders, namely Crohn's disease (CD), affecting the whole gastrointestinal tract, and ulcerative colitis (UC), mainly affecting the large intestine [2]. With the advancement of technology and better understanding of the gastrointestinal pathophysiology, mucosal immunology and microbiology, research studies are now focused on the effects of diet on IBD pathogenesis [3]. To some extent, the increasing IBD incidence has been linked to the highly processed diet (Western diet), characterized by higher concentrations of fats and refined sugars. IBD patients present deficiencies both in macronutrients and in certain micronutrients. The major symptoms of IBD include abdominal pain, diarrhea, and sometimes weight loss [2].

Regardless of the availability of many novel drugs for IBD treatment, clinical remission is still achieved in less than 50% of patients, and even the initially responsive IBD patients eventually developed non-responsiveness, leading to further progression of disease as well as worsened quality of life. According to researchers, the role of diet in the clinical management of IBD patients is underestimated. Diet is considered as one of the major determining factor of gut microbiota, and an imbalanced diet could lead to dysbiosis and to many other effects on host homeostasis [4]. Gut dysbiosis is a major contributor to host immune response in various IMIDs (immune-mediated inflammatory diseases) [5]. Moreover, dysbiosis is known to directly or indirectly induce and sustain intestinal inflammation and to promote adipose tissue development. A diet with higher fat content increases intestinal permeability, thus leading to an excessive bacterial influx, which in turn causes the lack of tolerance for microbiota-derived antigens as well as gut inflammation among IBD patients [4].

The Mediterranean diet, comprised of olive oil, vegetables, fruits, whole grains, yogurt, and red wine, is famously associated with inflammation reduction and showed positive effects in IBD patients [4]. Particularly, extra-virgin olive oil (EVOO) is renowned for its nutritional characteristics as well as health benefits, specifically against inflammation and gastrointestinal (GI) tract diseases [6]. Experimental evidence suggested that the Mediterranean diet could have a preventive role in the onset of dysbiosis by favoring the existence of bacterial species with specific anti-inflammatory properties [4].

Lately, the use of various supplementations containing probiotics, butyrate, phosphatidylcholine, lactoferrin, palmitoylethanolamide (PEA), silymarin, and omega 3 has been strongly suggested to patients suffering from inflammatory diseases [2]. There are strong rationales to suspect the significant therapeutic potential of these dietary supplements in inflammatory bowel disease, which is a chronic intestinal disease with no medical cure [5]. The high price, the adverse side effects, and the recurrent relapses linked with the drugs usually prescribed have endorsed the need for alternative treatment options for many IBD patients [2]. We will now discuss each of the above-mentioned supplements in detail.

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Probiotics

Probiotics are live microorganisms that are added to fermented foods and are beneficial for health because they improve the overall stability of the intestinal microbial cohort. Probiotics possess immunomodulating properties that influence the intestinal microbial cohort and reduce the activity of intestinal pathobionts, like *Clostridium perfringens* and *Klebsiella pneumonia* [7].

Particular commensal species, known as novel probiotics or next-generation probiotics, restore intestinal health by inhibiting inflammation and restoring the epithelial barrier. These goals could be achieved either by *de novo* administration of specific microbial species to the gut as probiotic/bacterio-therapeutic preparations or by growth acceleration of particular species within the gut through dietary means, or by combining these methods. The main intestinal phyla are the Lactobacilli – that cause a significant reduction in the number of toxin-producing intestinal bacteria as well as increase in longevity – and Bifidobacteria – that protect the intestinal health thanks to the substances they secrete or their extracellular structures. *Eubacterium hallii*, *Faecalibacterium prausnitzii*, and *Roseburia intestinalis* metabolize the dietary fibers and provide energy for enterocytes, also providing anti-inflammatory effects within the gut. *Akkermansia muciniphila* exerts a beneficial effect on metabolic syndrome and strengthens the gut mucosal barrier. Recently, Bacteroides species have been reported to release immunomodulatory molecules with further beneficial effects [8].

Clinical studies provide evidence supporting the beneficial effects of probiotics in the prevention as well as in the treatment of numerous gastrointestinal diseases in both adult and pediatric patients. Therefore, evidence-based probiotic formulations could be given to prevent or decrease the intensity of intestinal inflammations modulated by pathogenic bacteria. Probiotic supplementation could also be used to treat inflammation-associated complications of inflammatory bowel diseases. *In vivo* studies have also reported the effectiveness of probiotics in the prevention or reduction of inflammatory responses linked with colitis [7, 8].

The probable mechanisms of action of probiotic supplementation have been studied under various experimental conditions, both *in vivo* and *in vitro*. The efficacy of probiotics depends upon several factors, including the agents used, total dose and dosing pattern, their metabolism, the metabolites they produce, the molecules expressed on their surface, the characteristics of the host and luminal microbial environment. [8]

In a recent research study Kim et al. supported probiotic supplementation in inflammatory bowel disease by exhibiting that LA1 (*Lactobacillus acidophilus*) significantly affects endoplasmic reticulum stress and suppresses the activation of NF-$\varkappa$B (which leads to IBD pathogenesis), and could therefore be used as a potential immunomodulator in IBD treatment [9].

Probiotics are also employed to treat celiac disease, a prevalent systemic disorder affecting the small intestine and caused by an abnormal immunity response to the ingestion of gluten: since probiotics can regulate the composition as well as the functions of the microbiota, their supplementation might delay or even prevent the disease onset. Probiotics modulate immune response, toxin receptors degradation, nutrients competition, adhesion sites blockage, and the production of inhibitory substances against pathogens [10, 11]. Lindfors et al. [12] have highlighted that certain probiotics, like *Bifidobacterium lactis* or *Lactobacillus fermentum*, play a protective role against gliadin toxic effects in cultures of intestinal cells (Caco-2 cells from the human colon), causing a dose-dependent inhibition of the amplified intestinal epithelial permeability induced by the stimulation of gliadin and the production of IL-10 by T- regulatory cells [11, 12].

Certainly, the major symptoms of the condition are caused by the inflammation activation via cytokines cascade induction through the NF-κB pathway. Other strains of Bifidobacteria improve the composition of gut bacteria in celiac disease and reduce the inflammation, as evidently reported by Laparra et al. [13]. Another research study by Laparra et al. shows that Bifidobacteria and gliadin-digested fragments cause the downregulation of the mRNA expression of proinflammatory cytokines, like NF-kB, IL-1beta and TNF-a [14]. Besides, *Lactobacillus casei* has shown beneficial effects in the celiac disease for GALT (gut-associated lymphoid tissue) recovery and homeostasis, restoring a healthy mucosal structure [15]. These unconventional and promising probiotics could further contribute to biotherapeutic strategies in the future [11, 15].

Butyrate

Butyric acid is a fatty acid, produced by colonic bacteria from the fermentation of dietary fiber [1]. Butyrate plays a significant role in intestinal health because of its anti-inflammatory and regenerative properties. Other than regulating the colon motility, blood flow, and pH, butyric acid also improves the mucosal function as well as the epithelial barrier of the intestine. Besides, it possesses anti-inflammatory, antioxidant, antimicrobial and antineoplastic properties and offers a significant energy source for the colonocytes [16].

Decreased butyrate oxidation is observed in the biopsy of large bowel mucosal specimens of quiescent ulcerative colitis (UC) patients. Recently, research studies have demonstrated that Sodium Butyrate (NaB) displays anti-inflammatory properties by inhibiting the production of interleukin (IL)-12, caused by the suppression of both IL-12p40 and IL-12p35 mRNA accumulation, and also enhances the release of IL-10 in *S. aureus*-induced human monocytes [17]. The lack of NaB in diets poor in carbohydrates could cause clinically relevant functional alterations [16, 18].

Butyrate and short-chain fatty acids (SCFA) are likely candidates for novel therapeutic approaches. Butyrate plays a vital role in mucosal repair by non-transgluta-

minase-mediated as well as transglutaminase-mediated pathways, dilatation of arterioles, increase in oxygen uptake and mucosal blood flow, reduction of mucosal permeability as well as increase in mucosal production and its release. The effectiveness of sodium butyrate in enhancing repair of the mucosal lesions and in decreasing associated symptoms further strengthens its role in treating and managing UC as well as other mucosal disorders of the colon [19].

The amount of BCoAT (butyryl-CoA acetate CoA-transferase) has been found to be significantly decreased in Crohn's disease (CD) patients, suggesting a genetic inability of the microbes to synthesize butyrate in CD subjects. Therefore, many interventional studies as well as randomized clinical trials have analyzed and established butyrate effectiveness in reducing various disease symptoms, especially underlying inflammation. Previously, UC patients were given butyrate as enemas, while CD patients were given butyrate as oral tablets, with reduced intestinal surface diffusion capacity. Recently, researchers have highlighted that the absence of butyrate might change gut homeostasis by increasing lumen oxygen concentration, thus decreasing the concentration of butyrate-producing bacteria [20-22].

Recently, a novel oral formulation of butyrate (ButyroseR Lsc Microcaps-BLM) has been prepared where butyrate is enclosed in a lipophilic microcapsule, thus providing enhanced intestinal diffusion and facilitating the gradual release of the active ingredient [23]. This type of oral formulation allows butyrate absorption even in distal parts of the colon.

Previously, microencapsulated sodium butyrate (MSB) has been linked with regenerative and anti-inflammatory properties of the large bowel mucosa. MSB provides symptomatic relief from various colonic diseases, like IBD, malabsorption and diarrhea. Sodium butyrate (NaB) regulates intestinal environment, modulates intestinal permeability, decreases oxidative stress, and restores the colonic defense barrier, reducing mucosal inflammation and enhancing cell regeneration, as well as promoting tissue healing, without significant side effects [24, 25].

A randomized, double-blinded, placebo-controlled pilot study by Facchin et al. [20] analyzed the effectiveness of the oral formulation of microencapsulated sodium butyrate on gut microbiota in IBD patients. Butyrate administration enhances bacterial growth, which in turn leads to higher butyrate production. Exogenous butyrate could regulate gut bacteria and stimulate the growth of butyrogenic as well as SCFA genera, which in turn produce additional endogenous butyrate for intestinal homeostasis restoration.

Emmi et al. [26], in their proof-of-concept trial, showed for the first time that butyrate-enriched diets can regulate the blood redox status and stimulate fibrin degradation, which is diminished by a neutrophil-dependent mechanism (via ROS) among Behçet's syndrome patients. Similarly, butyrate-enriched supplementation resulted in a major reduction of blood inflammatory parameters, such as leukocyte ROS production and peroxidation of plasma lipids. Particularly, a significant improvement was observed in the susceptibility of fibrin for plasmin-induced lysis [26].

Many clinical studies have reported the decrease of visceral pain in IBD patients treated with NaB. The anti-inflammatory as well as the trophic effect of NaB could be beneficial for the patients suffering from IBD, diverticulitis, diarrhea, malabsorption, and cachexia. NaB is an essential component of gut hemostasis and it is crucial for natural regeneration within the intestinal epithelium. Several pathological processes affecting the large intestine may be linked with reduced endogenous levels of butyric acid. Several studies showed that the reduction in the frequency of irritable bowel syndrome (IBS) symptoms by the administration of microencapsulated sodium butyrate (MSB) might be caused by the reduction of intestinal receptors oversensitivity, leading to lower intraintestinal pressure amplitude [27]. The absence of side effects shows that MSB treatment is well tolerated and safe as a supplemental treatment for standard IBS therapy [25, 27, 28].

Phosphatidylcholine

Any disturbance in the mucosal barrier acts as an initiating factor that leads to attacks from commensal colonic bacterial flora, causing mucosal inflammation. Phospholipids, among the main components of the mucosa, consists of almost 90% phosphatidylcholine (PC) as well as lysophosphatidylcholine (LPC). Phosphatidylcholine plays a major role in the mucosal defense by creating a protective hydrophobic layer; whereas a defective phosphatidylcholine layer might lead to inflammation or even ulceration [29]. Additionally, phosphatidylcholine along with other lipids inhibits proinflammatory signaling in macrophages-derived phagosome model systems. On the other hand, the intrinsic mucus phosphatidylcholine content in UC was significantly reduced, regardless of the degree of mucosal inflammation [30]. Moreover, a local increase in the concentration of phosphatidylcholine in colonic mucus might improve the functions of the intestinal barrier, reducing inflammation in UC patients [29]. Thus, phosphatidylcholine supplementation might be helpful in order to restore the structure as well as the density necessary for the mucus to act as a protective mechanical barrier. Moreover, phosphatidylcholine could also be integrated in the mucosal cell membrane, where it affects the signaling processes associated with inflammation. The latest *in vitro* studies, involving a phagosomal analysis model system, validate the involvement of phosphatidylcholine in the signaling networks linked to the inhibition of proinflammatory signaling [29, 31, 32]. Phosphatidylcholine supplementation is considered as one of the most promising therapeutic strategies for colonic mucus. Stremmel et al. [33] showed that in 80% of the steroid-refractory UC patients, phosphatidylcholine successfully replaced steroid treatment. Almost half of the patients supplemented with phosphatidylcholine had shown more than 50% improvement in their clinical

activity index, which is linked with histologic and endoscopic activities and ultimately improves the quality of life. Phosphatidylcholine exhibits an enhanced safety profile and seems to be effective in shorter time as compared to 6-mercaptopurine or azathioprine [33].
The oral ingestion of phosphatidylcholine is completely absorbed in the upper intestinal tract, while the topical rectal administration of phosphatidylcholine fails to integrate in the mucus. This is why the best way for administering phosphatidylcholine was to encapsulate it with Eudragit-S100 (Rohm Pharma, Darmstadt, Germany), to offer pH-dependent release within the distal region of the intestinal tract [33, 34].
The basic idea behind this retarded release phosphatidylcholine (rPC) preparation was that the absence of phosphatidylcholine in the colonic mucus induces inflammation within ulcerative colitis. Two earlier studies have provided evidence of the therapeutic effectiveness of rPC in controlling inflammation in chronic UC patients with active non-steroid treatment, as well as steroid refractory UC. The results of the first study revealed 70% improvement in disease activity among chronic-active UC patients with a non-steroid treatment. The results of the second study showed clinical remission among 50% of the chronic steroid-refractory UC patients, and they could eventually discontinue the steroid therapy [30, 33, 34]. Some animal studies have also reported that when phosphatidylcholine is topically applied on the colon region, it protects those animal models against trinitrobenzenesulphonic or acetic acid-induced colitis [29].
In a prospective, double blind, randomized, placebo-controlled study Stremmel et al. [33], evaluated the clinical effectiveness of retarded release of an oral phosphatidylcholine preparation among 60 patients having chronic, non-steroid dependent and active UC. Their results strongly suggest that phosphatidylcholine supplementation has a significant therapeutic potential against UC [29]. Long-term supplementation of phosphatidylcholine might be helpful in maintaining the clinical remission without the adverse effects that are usually observed after immunosuppressive and steroid therapies [29].

Lactoferrin

Lactoferrin is a multifunctional iron-binding glycoprotein that is found in significant concentrations in different human mucosal secretions, especially breast milk. Lactoferrin accounts for almost 25% of the breast milk protein content (1-3 g/mL), it chelates iron and inhibits bacterial growth. The antimicrobial properties of lactoferrin have been established *in vitro* as well as *in vivo*, proving its potential benefits on intestinal health. Lactoferrin improves the hematocrit levels and reduces the rate of lower respiratory tract and gut infections in infants, without adverse effects [35].
A research study focusing on the administration of transgenic milk supplementation comprised of human lactoferrin to malnourished pigs resulted in decreased intestinal permeability and beneficial effects on the jejunal structure, along with significant weight gain [36]. Lactoferrin, in combination with lysozyme, acts as a bactericidal agent for both Gram-positive and Gram-negative bacteria; in fact, lactoferrin, thanks to its ability to chelate iron and to bind with membrane lipopolysaccharides, disrupts the outer membrane, whereas lysozyme acts upon the inner peptidoglycan cell wall. Notably, both lactoferrin as well as lipopolysaccharides show resistance against proteolytic degradation, which is an essential requirement for the therapeutic use of any oral agent in humans [37].
In a Peruvian study on children, ranging from 5 to 33 months of age, oral supplementation of lactoferrin containing rehydration solutions decreased the duration and severity of diarrheal episodes in patients with acute diarrhea and dehydration, with no adverse effects [38]. Because of these therapeutic properties, lactoferrin is considered as an attractive candidate for preventing as well as treating environmental enteric dysfunction (a chronic subclinical inflammatory condition of the gut). Generally, lactoferrin is considered a beneficial supplement for the neonates who could not have breast milk or those who are weaning but might still take advantage from its therapeutic properties [35].

Palmitoylethanolamide (PEA)

The activation of the intestinal immune system as well as an imbalance in the endocannabinoid system are involved in IBS pathophysiology, which is why the supplementation of endocannabinoid-like nutritional compounds might improve IBS symptoms, specifically abdominal pain. Particularly, palmitoylethanolamide (PEA) is an endogenous fatty acid amide, most abundant in peanuts and egg yolk, which has been repeatedly reported to perform anti-inflammatory as well as analgesic activities both *in vivo* and *in vitro* [39, 40].
The palmitoylethanolamide treatment was reported to be noticeably effective in decreasing abdominal pain severity in IBS. Palmitoylethanolamide and polydatin are nutritional compounds that act synergistically to decrease the activation of mast cells [39].
Palmitoylethanolamide has lower affinity for the cannabinoid receptors and regulates inflammation as well as nociception, which mainly occurs by the down-regulation of mast cell activity. Interestingly, the Nobel laureate Rita Levi-Montalcini recognized that palmitoylethanolamide might act as a mast cell modulator, as well as a potential agonist of PPAR-a (Peroxisome proliferator-activated receptor-alpha), CB2 (cannabinoid type 2) receptors, TRPV1 (transient receptor potential vanilloid type 1), and orphan GPCR (G protein coupled receptor). In addition, several research studies have statistically established that the palmitoylethanolamide-polydatin treatment was effective on abdominal pain severity, if not on its frequency [40, 41].

Silymarin

Silymarin, an extract from *Silybum marianum* seeds, is a natural source of flavanolignans complex that exhibits strong intracellular antioxidant properties. In addition to hepatoprotective effects, silymarin also possess beneficial effects on the regulation of immune-mediated murine colitis through bowel histology restoration and on the decrease of certain bowel inflammatory cytokines – specifically, NF-$\varkappa$B (nuclear factor $\varkappa$B), TNF-α, and IL-1β (interleukin-1β). Additionally, there is a correlation between NF-$\varkappa$B inhibition and colitis activity reduction in rat models after treatment with silibinin (the major silymarin flavanolignan component) and ursodeoxycholic acid [42, 43].

Recent research studies have reported that the use of Silymarin on UC patients has many beneficial effects: treated patients were reported to remain in a remission state and to tolerate the drug well. These beneficial effects are probably due to its anti-oxidative properties. The data from several clinical studies as well as animal studies have also reported beneficial effects of Silymarin on hemoglobin concentration (Hb), TNF-α, IL-1β, lipid peroxidation, erythrocyte sedimentation rate (ESR), bowel cell myeloperoxidase, and cell histology. These clinical trial results thus support the hypothesis that silymarin supplementation is useful to maintain remission in UC patients [44].

Omega 3

The significant and beneficial role of dietary omega-3 in inflammation is well established. There is epidemiological evidence of reduced incidence of inflammatory and autoimmune disorders in those who take these polyunsaturated fatty acids (PUFAs). Since TNF and IL-1 are the main inflammation mediators, a decrease in the production of these cytokines contributes to the improvement of inflammatory symptoms among patients treated with omega-3 supplements; however, the exact step of the inflammation pathway at which these therapeutic agents have their maximum effect is still to be defined [45].

A double–blind, placebo-controlled crossover study by Stenson et al. [46], involving fish oil supplementation as well as a placebo group, showed that fish oil supplementation increases B5 production, reduces B4 production and also decreases the contents of leukotrienes rectal dialysates. Also Belluzzi et al. [47] reported a decreased rate of relapse among Crohn's disease patients that are in remission after the supplementation of 2.7 grams of omega-3 enteric-coated fish oil preparation. Similarly, Endres et al. [48] have evaluated the therapeutic effect of omega-3 fatty acid in IBD patients and reported that many studies have mentioned a significant improvement in the clinical activity as well as steroid-sparing effects. However, other studies only showed a trend towards improvement, without reaching significance [45, 48].

Conclusion

Inflammatory bowel disease (IBD), comprising ulcerative colitis (UC) and Crohn's disease (CD), is characterized by chronic intestinal inflammation and is influenced by the composition of the gut microbiota. IBD greatly affects the patients' quality of life and most of the currently used drugs show non-responsiveness after some time, failing to achieve remission in more than 50% of patients. As diet is a major factor in the maintenance of gut microbiota, several components of the Mediterranean diet, especially extra-virgin olive oil (EVOO), are being studied and show anti-inflammatory properties and benefits for intestinal health. Recently, the use of various supplementations containing probiotics, butyrate, phosphatidylcholine, lactoferrin, palmitoylethanolamide (PEA), silymarin and omega-3 has been proposed to treat patients with inflammatory gut diseases. Since these supplements are known to exert anti-inflammatory effects within the gut, to improve the stability of the intestinal microbial cohort, to strengthen the barrier function of the mucosa and to have no side effects, they should be considered for the treatment and management of intestinal inflammation.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; AKK, GB, KD: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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Review

Dietary supplements for the management of COVID-19 symptoms

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Keywords

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Summary

SARS-CoV-2, the etiological agent of COVID-19, caused a pandemic in 2020, which is only recently slowing down. The symptoms of COVID-19 range from cough to fever and pneumonia and may persist beyond the active state of the infection, in a condition called post-COVID syndrome. The aim of this paper is to review the relationship between COVID-19 and nutrition and to discuss to most up-to-date dietary supplements proposed for COVID-19 treatment and prevention. Nutrition and nutritional dysregulations, such as obesity and malnutrition, are prominent risk factors for severe COVID-19. These factors exert anti-inflammatory and proinflammatory effects on the immune system, thus exacerbating or reducing the immunological response against the virus. As for the nutritional habits, the Western diet induces a chronic inflammatory state, whereas the Mediterranean diet exerts anti-inflammatory effects and has been proposed for ameliorating COVID-19 evolution and symptoms. Several vaccines have been researched and commercialized for COVID-19 prevention, whereas several drugs, although clinically tested, have not shown promising effects. To compensate for the lack of treatment, several supplements have been recommended for preventing or ameliorating COVID-19 symptoms. Thus, it is critical to review the dietary supplements proposed for COVID-19 treatment. Supplements containing α-cyclodextrin and hydroxytyrosol exhibited promising effects in several clinical trials and reduced the severity of the outcomes and the duration of the infection. Moreover, a supplement containing hydroxytyrosol, acetyl L-carnitine, and vitamins B, C, and D improved the symptoms of patients with post-COVID syndrome.

COVID-19 pandemic

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is the causative agent of coronavirus disease-2019 (COVID-19), which has been depicted as a public health emergency of global concern by the World Health Organization (WHO). The contagion probably started in Wuhan city, Hubei Province, China, in December 2019 and has infected over 428 million people and has caused the death of over 6 million (WHO website, accessed on March 29th, 2022). The high spread of SARS-CoV-2 is mainly because of its method of transmission: it spreads between people during close contact via small droplets produced during talking, sneezing, and coughing [1].

Coronaviruses

Coronaviruses are positive-sense single-stranded RNA viruses with crown-shaped peplomers and are members of the family *Coronaviridae*. Apart from the RNA, coronaviruses consist of spike protein (S-protein), envelope protein (E-protein), membrane glycoprotein (M-protein), and nucleocapsid protein (N-protein) [2]. The RNA of coronaviruses is 26-32 kilo bases (kb) in length and is the largest viral genome known. Specifically, the viral RNA of SARS-CoV-2 is 29 kb in length and encodes approximately 9860 amino acids [2, 3]. SARS-CoV-2 exhibits 50% similarity with the Middle East respiratory syndrome coronavirus (MERS-CoV) and 80% phylogenetic identity with severe acute respiratory syndrome coronavirus (SARS-CoV), the causative agents of global outbreaks in 2011 and 2002, respectively. Coronaviruses are divided into four main genera: α-coronavirus, β-coronavirus, γ-coronavirus, and δ-coronavirus. SARS-CoV-2, MERS-CoV, and SARS-CoV are all β-coronaviruses [2, 3].

The N protein forms the nucleocapsid and binds the RNA genome. This protein is involved in viral genome replication and in the host's cellular response to the virus [4]. The S protein is a type 1 membrane glycoprotein that is divided into two subunits, namely, S1 and S2. S1

is involved in receptor binding, whereas S2 mediates the fusion of viral and cellular membranes [2]. The M protein acts on the cellular membranes and facilitates the formation of new viral particles [5]. Finally, E protein is yet to be fully understood. This protein is highly expressed in host cells, but only a portion is incorporated into the new viral particles. E protein has three main roles: participation in viral assembly, action during the release of virions, and implication in viral pathogenesis [6, 7]. Binding of the S protein to its receptor initiates the attachment of the viral particle to the host cell. The specificity is permitted by the receptor binding domain of the S1 subunit, which varies depending on the coronavirus. SARS-CoV-2 binds to angiotensin converting enzyme 2 (ACE2). Following receptor binding, the cathepsin TMPRRS2 cleaves the S protein, and the viral membrane fuses with the cellular membrane [2].

Symptoms

SARS-CoV-2 infection is asymptomatic in 40% of the cases [8] but causes severe and very severe symptoms in 14 and 5% of the infected patients, respectively [9]. The most common symptoms are, in order, cough, weakness, taste disorder, myalgia, and fever [10], resulting in dyspnea and pneumonia. Additionally, severe complications, such as acute respiratory distress syndrome, acute heart injury, and secondary infections, are seen in the infected individuals, thus worsening the outcome of the infection. Symptoms are more severe in the elderly, while the infection and mortality rates are low in children [9]. Higher viral loads are found in symptomatic patients, thus contributing to a higher chance of secondary transmission of the disease [11]. The symptoms may appear gradually and can disappear spontaneously, with the incubation period lasting 3-15 days [9]. Severe symptoms are related to alterations in circulating leukocytes and to the activation of the cytokine cascade. SARS-CoV-2 antigens are presented by antigen-presenting cells, such as macrophages and dendritic cells, to the T cells, thereby leading to their activation, differentiation, and cytokine release [12]. The dysregulated secretion of IL-6, IL-1β, IL-10, and TNF-α alters the lung tissue and causes respiratory distress [13]. IL-6 appears to be relevant for the immunopathology of SARS-CoV-2. Indeed, IL-6 exacerbates the chronic inflammation at the base of COVID-19 [14, 15]. The cytokine storm can trigger systemic inflammation and result in multiple organ failure. Moreover, helper T cells can activate T-dependent B cells, thus stimulating the production of virus-specific antibodies, whereas cytotoxic T cells can kill viral-infected cells [16].

COVID-19 treatment and prevention

Several compounds have been tested against coronaviruses during the SARS-CoV pandemic and the MERS-CoV outbreaks. Although it was expected that one or more compounds could serve as an effective antiviral therapy, this approach has so far had little impact on the clinical outcomes of patients with COVID-19 globally [17]. Antiviral treatments are critical for people awaiting vaccines as well as for immunocompromised people who do not respond well to vaccination. Antiviral compounds can be divided into four main groups based on their mode of action: compounds that inhibit SARS-CoV enzymes, compounds that inhibit viral entry, interferons, and drugs that inhibit host processes involved in viral replication [17].

Compounds that inhibit SARS-CoV enzymes can be nucleoside analogs that act on the polymerase enzyme (remdesivir [18] and molnupiravir [19]) or can be directed against the proteases Mpro and PLpro (ebselen [20] and GC-376 [21]). Entry inhibitors act on the viral spike glycoprotein, and they can be monoclonal antibodies, single-domain antibodies, polyclonal antibodies, fusion inhibitors, or soluble ACE2. Interferons stimulate antiviral responses in human cells, slowing cellular metabolism, interfering with membrane formation, and inducing cytokine release. Finally, host-targeting compounds act on cellular proteins, signaling pathways, or cellular organelles exploited by viruses for their replication. These compounds can regulate host proteases, nucleotide and protein synthesis, and endosomal trafficking, whereas the mechanism of action of some compounds is unknown [17].

Over 20 vaccines against SARS-CoV-2 were approved all over the world by November 2021. Although their modes of action varied, they proved to exhibit a high efficiency in preventing COVID-19 [22-24]. Some vaccines contain nucleoside-modified messenger RNA, such as BNT16b2 developed by Pfizer/Biontech and mRNA-1273 developed by Moderna. Moreover, the vaccines can be vectors that contain SARS-CoV-2 antigens, such as AZD1222 developed by AstraZeneca and Sputnik V. Another group of vaccines comprises protein subunits, such as NVX-CoV2373. Finally, CoronaVac developed by Sinovac Life Sciences uses the conventional method of inactivated viruses [23, 24]. The outbreak of SARS-CoV-2 variants suggested the use of vaccine mixing, with a hope of increasing the effectiveness. This method has so far been successful, without severe side effects [23].

COVID-19 and nutrition

Nutrition is one of the main factors that influence health. Good nutrition can improve well-being and mitigate or even prevent most chronic diseases (e.g., diabetes, hypertension, and obesity). COVID-19 is no different, and the nutritional status is a crucial determinant for the prognosis of patients with the infection [25]. Upon observing mortality rates in population clusters, it becomes clear that people with comorbidities related to nutrition, such as type 2 diabetes and obesity, are at an increased risk for severe symptoms and mortality [26]. These risk factors are exacerbated by the consumption

of the typical Western diet, which contains high amounts of saturated fatty acids, refined carbohydrates, and sugars [27, 28]. The Western diet chronically activates the innate and adaptive immune responses, thus stimulating a chronic inflammatory state. This dietary pattern activates macrophages, neutrophils, and dendritic cells via toll-like receptor 4. Moreover, the diet inhibits T and B lymphocyte function and maturation, possibly via oxidative stress [27]. Finally, Western diet lowers the host defense against viruses [26]. Bad nutrition is correlated with low social status. Minorities face barriers in embracing healthy food choices mainly because of a high rate of poverty and a decreased quality of healthcare [29]. As for the dietary recommendations, several countries have released their own guidelines through their health organizations, which are in agreement on most recommendations [25]. They encourage the consumption of whole grain, fruits, and vegetables. These foods provide vitamins, minerals, and water, which are important for a healthy status. Vitamins A, C, D, E, B_6, and B_{12} and zinc have a role in the maintenance of physical barriers as well as in the differentiation and functioning of innate immune cells [30]. Moreover, vitamins and micronutrients act as scavengers of reactive species of oxygen, thus decreasing oxidative stress [30]. An adequate hydration is also suggested [25]. Water is essential for several cellular and physiological processes, among which body temperature regulation and heart functioning are pertinent [31]. The Mediterranean diet has been proposed to be beneficial in preventing and ameliorating COVID-19 symptoms. The diet is characterized by a high intake of fruits, vegetables, legumes, olive oil, and nuts, which are anti-inflammatory foods that are rich in vitamins and minerals [16, 32]. These foods contain bioactive compounds, such as polyphenols, which exhibit anti-inflammatory, antithrombotic, and antioxidant properties. Hence, the Mediterranean diet has beneficial effects on immune health and offers protection against several infections as well as noncommunicable diseases [16].

COVID-19 and obesity

Obesity is among the most prominent risk factors for severe COVID-19, together with type 2 diabetes, cardiovascular diseases, chronic respiratory diseases, hypertension, and cancer [33]. The increased risk is attributed to several factors: impaired respiratory mechanics, low respiratory muscle strength and lung volumes, weakened immune system, and chronic basal inflammation [25, 33]. Chronic inflammation is a common feature in obesity and results from metabolic tissue stress and adipose tissue dysfunction. Hypertrophic adipocytes evolve in their proinflammatory state, stimulating the release of chemotactic mediators and the recruitment of leukocytes. The leukocytes, in turn, release proinflammatory cytokines, stimulating local and systemic chronic inflammation states [34]. Adipose tissue is involved in regulating the immunity owing to the release of leptin and adiponectin. Leptin influences hematopoiesis in the bone marrow and also regulates the expression of proinflammatory cytokines by innate immune response cells. Adiponectin stimulates inflammatory resolution because of its anti-inflammatory and insulin-sensitizing properties. Obesity has been shown to be correlated with decreased functioning and activation of T cells. Furthermore, excess fat increases the probability of developing cytokine storm, a severe complication of SARS-CoV-2 infection [35]. People with obesity show disrupted lung mechanics and physiology, reduced lung volumes, and decreased compliance and respiratory muscle efficiency [34]. Finally, ACE2, the SARS-CoV-2 receptor, is highly expressed in mature adipocytes; thus, its expression is high in people with obesity. Higher expression of ACE2 could contribute to the increased risk of severe complications in patients with COVID-19 who are obese [34]. Obesity prevalence varies between different countries (40% in USA, 20% in Italy, and 6.2% in China) and between different social status, being usually higher in poor people [28, 34]. Moreover, the pandemic influenced the physical activity and nutritional habits of people to a great extent. People reduced the level of their physical activity and preferred staying home; also, they consumed more of processed foods that are high in sodium, sugar, and fats. These new habits contributed to weight gain, increasing the risk of obesity and, thus, the risk of COVID-19 complications [34, 36].

COVID-19 and malnutrition

Another common risk factor for COVID-19 is malnutrition [1, 37]. Therefore, prevention, diagnosis, and treatment of malnutrition have been proposed to be included in the routine management of patients with COVID-19 [38]. Several physiological effects explain the role of malnutrition in worsening the outcomes of SARS-CoV-2 infection. Reduced adipose tissue causes a reduction in the release of adipocytokines and leptins, thus leading to immunosuppression. Malnutrition causes the T cells to produce less of IL-2 and IFN-γ and further impairs complement activation and induces thymic atrophy [39]. Malnutrition is common in elderly patients with COVID-19 because of several disease outcomes: inflammatory state, which catabolizes the muscle proteins, worsening frailty; gastrointestinal disorders, which reduce nutrient absorption; and anxiety, which lowers the appetite [1]. Moreover, diabetes, a typical risk factor for COVID-19, can influence the metabolism of macronutrients, thereby leading to malnutrition [40]. Considering the correlation of malnutrition with COVID-19 and the fact that prolonged hospitalization can cause a decrease in body weight and muscle mass, patients with COVID-19 should increase their protein and micronutrient levels by consuming oral nutrient supplements [1, 16].

COVID-19 and natural molecule supplementation

Supplements could be used to prevent COVID-19. Indeed, some vitamins and minerals improve immunity, and supplements are recommended for individuals who cannot meet dietary requirements owing to specific challenges [25]. As new therapeutics are still being explored and tested for COVID-19, natural molecules could fill the gap. Many natural molecules can influence the viral endocytic pathway and could be used to treat SARS-CoV-2 infection [3]. For instance, methyl-β-cyclodextrin is a macromolecule that can inhibit SARS-CoV-2 attachment to host cells. The molecule can lower the cholesterol content in the cell membranes and decrease viral infectivity, reducing the binding of the viral spike glycoproteins to the ACE protein. Furthermore, the molecule redistributes cholesterol among the raft and nonraft cell membrane regions and influences the expression of ACE2 [3]. Phytosterols, natural plant sterols with a cholesterol-like structure, mimic the action of methyl-β-cyclodestrin. These sterols lower the cholesterol level in the cell membrane and further reduce the infectivity of several viruses, such as hepatitis B virus and HIV [3]. Regular phytosterol intake can decrease LDL cholesterol by up to 10% [41]. Flavonoids are other natural molecules with antiviral properties. These molecules can block viral attachment and entry, inhibit viral replication, and disrupt the translation and processing of viral proteins. Examples include kaempferol, which inhibits coronaviruses by blocking the assembly and release of virions, and luteolin, which interferes with SARS-CoV entry by binding to the S2 protein. Flavonoids also regulate the main protease of SARS-CoV-2, but more research is needed to confirm this hypothesis [3]. Flavonoids have been proven to lower SARS-CoV-2 infectivity by targeting two-pore channels [42]. Myoinositol is the precursor for inositol-3-phosphate, a second messenger of G-protein coupled receptors. Myoinositol exerts beneficial effects in pneumology, promoting surfactant maturation. Furthermore, the molecule reduces the IL-6 cascade by acting on phophatidyl-inositol-3-kinase, thus inhibiting many other inflammatory processes. Catechins from green tea have also been recently tested and have been proven to inhibit viral replication, probably by acting on proteases [43]. Another natural molecule, hydroxytyrosol, has been tested against SARS-CoV-2. The molecule is extracted from olive oil and leaves and can modify the composition of the plasma membrane, thereby affecting viral entry. Hydroxytyrosol is antiviral, acting on the viral envelope, and anti-inflammatory, decreasing the production of IL-6 and TNF-α, which have been correlated with severe cases of COVID-19 [44-46]. Furthermore, acetyl L-carnitine can downregulate the production of proinflammatory cytokines and is an amino-acid-derived compound that transports long-chain fatty acids into the mitochondria, thus permitting oxidation and energy production. Acetyl L-carnitine is used for treating several diseases, including diabetes and neurological disorders, and it has been associated with the overall improvement of health and reduction of fatigue [47].

Vitamin D, which comes from the diet and is also synthetized by the human body, is a key molecule that offers protection against viral infections because the activation of its receptor regulates the innate and acquired immune systems. Vitamin D3 is produced in the skin by exploiting ultraviolet B radiation. Subsequently, vitamin D3 or oral vitamin D is converted to the active form calcitriol as a result of metabolic reactions in the liver and kidneys [48]. Respiratory epithelial cells express the vitamin D receptor, and vitamin D decreases the expression of IL-8. Furthermore, vitamin D increases the expression of antimicrobial substances by macrophages and other leukocytes. However, up to 50% of the population has vitamin D deficiency, which is linked to an increased susceptibility to acute viral infections [9]. Moreover, hypo-vitamin D is an independent risk factor in the overall population for total mortality [48]. Vitamin D status has been associated with the severity of COVID-19. A study has shown that countries south of latitude 35° north have a lower mortality rate, which can be attributed to increased vitamin D production [49]. Vitamin B has also been recently tested for COVID treatment owing to its beneficial effects on mitochondrial function, inflammation, digestion, and toxin elimination [47]. Finally, other molecules, such as vitamin C, zinc, and selenium, have also been proposed for COVID-19 supplementation mainly because of their antioxidant properties (Tab. I) [25].

A COVID-19 supplement was tested in 2020 and yielded promising results. Its main therapeutic molecules were α-cyclodextrin and hydroxytyrosol, and it was supplemented in the form of a spray. The supplement's *in vitro* antioxidant properties were proved, and it was found to be safe at all the tested doses [50, 51]. It was tested on a group of 149 healthy volunteers without any side effects, and none of them were infected by the virus although many were at a high risk for the infection because of their job. The supplement was also tested on positive subjects, who became negative in half the time of a control group of positive nontreated patients with a comparable viral load. Another new supplement composed of hydroxytyrosol, acetyl L-carnitine, and vitamins B, C, and D was proposed in 2021 for the treatment of post-COVID syndrome [47]. Although it was a pilot observational study, the use of the supplement increased the self-reported levels of energy and decreased self-reported tiredness and tension, with few side effects, in twenty subjects [47]. Despite the limitations of these studies, the final results suggest the use of these supplements to prevent SARS-CoV-2 infection, to reduce the viral load, to shorten the duration of the treatment, or to ameliorate the symptoms of post-COVID syndrome [47, 52].

Conclusion

Nutrition and nutritional dysregulations, such as obesity and malnutrition, seem to be involved in SARS-CoV-2

Tab. I. Natural molecule supplementation in COVID-19.

Molecules	Action mechanism	Outcomes
Methyl-β-cyclodextrin	Inhibit SARS-CoV-2 attachment to host cells	Reduction of the cholesterol content in the cell membranes and decrease viral infectivity
Phytosterols	Cholesterol-like structure, mimic the action of methyl-β-cyclodestrin	Reduction of the cholesterol level in the cell membrane and further reduce the infectivity of several viruses
Flavonoids	Block viral attachment and entry, inhibit viral replication, and disrupt the translation and processing of viral proteins	Reduction SARS-CoV-2 infectivity by targeting two-pore channels
Myoinositol	Reduces the IL-6 cascade by acting on phophatidyl-inositol-3-kinase	Inhibition og many other inflammatory processes
Hydroxytyrosol	Decreasing the production of IL-6 and TNF-α	Antiviral and anti-inflammatory: acting on the viral envelope
Acetyl L-carnitine	Downregulate the production of proinflammatory cytokines	Increase oxidation and energy production
Vitamin D	Activation of receptors that regulates the innate and acquired immune systems	Increases the expression of antimicrobial substances by macrophages and other leukocytes
Vitamin B	Mitochondrial function	Inflammation, digestion, and toxin elimination
Catechins	Acting on proteases	Inhibit viral replication

pathogenesis. Indeed, nutrition influences the immune system, modulating its responses. Several supplements have been proposed for preventing or ameliorating COVID-19 symptoms. Dietary supplements containing hydroxytyrosol reduced the severity of the infection and improved the symptoms of patients with post-COVID syndrome in many clinical trials. New research and clinical studies will help identifying other effective natural molecules against SARS-CoV-2 infection.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; GB, MCM, FF, MF, SN, LL, GMT, GF, FB, PG, STC: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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REVIEW

An overview of the genetic aspects of hair loss and its connection with nutrition

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Keywords

Androgenetic alopecia • Hair loss • Mediterranean Diet • Nutrition

Summary

Hair loss is a widespread concern in dermatology clinics, affecting both men's and women's quality of life. Hair loss can have many different causes, which are critical to identify in order to provide appropriate treatment. Hair loss can happen due to many variables, such as genetic factors or predisposition, vitamin and mineral deficiencies, skin problems, hair growth disorders, poor diet, hormonal problems, certain internal diseases, drug use, stress and depression, cosmetic factors, childbirth, and the chemotherapy process. Treatment for hair loss varies depending on the type of alopecia, deficiency, or excess of structures such as vitamins and minerals, and also on hair and skin structure.
The Mediterranean diet is characterized by low amounts of saturated fat, animal protein, and high amounts of unsaturated fat, fiber, polyphenols, and antioxidants. The main nutrients found in the Mediterranean Diet are rich in antioxidant, anti-inflammatory components. It also has an important place in hair loss treatment, since recently treatment strategies have included polyphenols and unsaturated oils more and more frequently. The goal of this work was to review published articles examining alopecia and its types, the many micronutrients that affect alopecia, and the role of the Mediterranean diet in alopecia. The literature shows that little is known about hair loss, nutritional factors, and diet, and that the data collected are conflicting. Given these differences, research into the function of diet and nutrition in the treatment of baldness is a dynamic and growing topic.

Introduction

Hair loss or alopecia, a clinical condition that is frequently seen in dermatology clinics, can be caused by many etiological factors and it significantly affects the patients' quality of life [1]. This group of diseases is basically divided in two subgroups: cicatricial alopecias and non-cicatricial alopecias. While cicatricial alopecia may progress with loss of follicles, thus causing irreversible hair loss, this condition is usually reversible in non-cicatricial ones. Many causes are known to have a role in non-cicatricial alopecia, including emotional issues, chronic disorders, dietary inadequacies, trace elements, and vitamin deficiencies [2]. Other factors can be stress, drug use, immune system, endocrine disorders, and genetic and epigenetic changes [3].
A balanced and regular diet is very important for healthy hair: sudden weight loss, low-caloric diets, unbalanced diet, obesity, and excessive intake of vitamin and mineral supplements can cause hair loss. Micronutrients, which are the main elements of the hair follicle cycle, are very important in alopecia, which is why dietary supplements (mostly vitamin and mineral) are among the preferred methods to prevent hair loss. Given the frequency of hair loss in current times and its impact on the patients' social lives, finding effective alopecia treatments impacts a huge portion of the population [2].
The role of diet in the development and treatment of alopecia has recently been a hot topic of research. It has been found that plant-rich diets – such as the Mediterranean Diet (MD), whose main nutrients are rich in antioxidants, anti-inflammatory, and estrogenic components – include chemicals that stimulate hair growth and reduce hair loss. These diets contain phytochemicals that promote hair development by lowering the generation of reactive oxygen species in the dermal papilla cells, causing growth hormones to be secreted [4].
This is why dietary practices characterized by a large intake of anti-inflammatory, antioxidant and estrogenic activities are emphasized as additional treatments for alopecia. This review aims to explain the general background of alopecia, to emphasize the role of nutritional and dietary supplements in the treatment of alopecia, and to consider the effects of herbal treatment methods on alopecia patients.

Alopecia and types of alopecia

Alopecia is the partial or complete loss of hair, which can be caused by a disruption in the hair development

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cycle or by damage to the hair follicles as a result of systemic or local factors such as genetics, hormone imbalances, and infection [5-7].
The hair growth cycle consists of four stages: the anagen phase (the growth phase, which is also the longest, lasting 2-7 years); the catagen phase (the transitional phase, lasting up to 2 weeks, which includes hair follicle involution due to apoptosis); the telogen phase (the resting phase, lasting up to 12 weeks); and the exogenous phase (the release phase of telogen hair) [8].
The fact that alopecia is affected by many factors causes the existence of a wide number of types of alopecia. Androgenic alopecia, alopecia areata, chemotherapy-induced alopecia, anagen effluvium, telogen effluvium, traction alopecia, and trichotillomania are some of the most common kinds. The clinical classification system of Rook and Dawber divides all types of alopecia into two categories: cicatricial alopecia and non-cicatricial alopecia [6, 7].

Cicatricial alopecias

Cicatricial alopecias (CAs) are clinical pathological conditions that describe permanent hair loss caused by the replacement of damaged hair follicles with fibrotic scar tissue. They are classified into two groups, primary cicatricial alopecia (PCA) and secondary cicatricial alopecia (SCA) [9, 10].
The PCA group includes multiple inflammatory diseases with distinct clinical and histopathological features and unknown and irreversible causes, primarily affecting and destroying hair follicles. It is responsible for 7% of all hair loss cases. PCA is subdivided into lymphocytic, neutrophilic, or mixed subtypes [11, 12]. Chronic cutaneous lupus erythematosus, lichen planopilaris, classic Brocq pseudopelade, folliculitis decalvans, and dissection folliculitis are some of the diseases with clinical conditions [13].
The SCA group includes inflammatory and neoplastic conditions and physical traumas that usually affect primarily the dermis and cause secondary follicular destruction [9]. While in PCAs the disease directly affects hair follicles, in SCAs they disappear due to secondary reasons. Factors affecting SCA formation include genodermatoses, permanent alopecia due to developmental defects, physical and chemical injuries, infections, inflammatory dermatoses, drug uses, and neoplastic conditions [14]. The classification of cicatricial alopecias is summarized in Table I.

Non-cicatricial alopecias

Non-cicatricial alopecia is reversible alopecia, characterized by an altered hair cycle and by the fact that hair follicles are preserved [5].
Non-cicatricial alopecias are divided in local alopecias and diffuse alopecias [17]. Local alopecias include alopecia areata, tinea capitis, and trichotillomania. Diffuse alopecias include alopecia totalis or universalis, telogen or anagen effluvium. The classification of non-cicatricial alopecias is summarized in Table II. The most common alopecia in this group is androgenic alopecia.

Androgenic alopecia, caused by the miniaturization of hair follicles, is the most common type of progressive hair loss, affecting 30-50% of men and approximately 30% of middle-aged women. Alopecia areata, the second most common type of non-cicatricial alopecia, is known to be associated with autoimmune problems [5].

Common causes of hair loss

As previously stated, the hair growth cycle consists of four phases: anagen, catagen, telogen, and exogen. Hair loss differs according to the affected stage. Although there are many different reasons for hair loss, androgen and stress-related causes are usually underlying. Other systemic causes of hair loss include telogen effluvium, nutrition, endocrine imbalances, drugs, infections, special diseases, malignancies, problems with the immune system, environmental factors, age, and genetic factors [3].

Androgen-related alopecia

The most common type of hair loss is androgenetic alopecia, which affects 80% of men and 50% of women. In this kind of alopecia, genetic factors and age-related causes affect the androgen mechanism, which is the one allowing vellus hair to turn into longer, thicker, and darker terminal hair: the defect in this mechanism makes it function differently by stimulating the regression of hair follicles to turn into vellus hairs. Androgenetic alopecia is a dynamic and continuous hair loss problem, in which mast cells and lymphocytes are defined around the miniaturized follicle, rich in stem cells [18-20].
Although androgens play an important role in androgenetic alopecia, genetic predisposition is also important: genetic background plays an important role, with a rate of 0.81 in twin studies, thus showing familial clustering. Having a polygenic feature, its genetic background is complex. However, changes in the androgen receptor (AR) gene and 5-alpha reductase gene were found to be effective in androgenetic alopecia. In addition, triple repeat polymorphisms have been associated with single nucleotide polymorphisms. It has been stated that the AR gene on the X chromosome and the ectodysplasin A2 receptor (EDAR2) gene are related and that polymorphisms in this gene are associated with androgenic alopecia, but its exact role has not been fully defined. Androgenetic alopecia is associated with genetic alterations in the WNT signaling pathway, which regulates dermal papilla cells and androgen metabolism. Expression studies and epigenetic studies are limited, as scalp biopsy is difficult to obtain. Overexpression of prostaglandin synthase (PGDS) and the PGDS product prostaglandin D2 (PGD2) restrict hair development by generating an early catagen phase, according to the limited study [8, 21].

Stress-induced alopecia

Stress is one of the most common reasons of hair growth disorders and hair loss, since it leads to an increase in the level of cortisol released into the body. It has been proven

Tab. I. Classification of cicatricial alopecias [9, 14-16].

Primary Cicatricial Alopecia (working classification of PCA by North American Hair Research Society)	Secondary Cicatricial Alopecia		
Lymphocytic	Chronic cutaneous lupus erythematosus, Lichen planopilaris, Frontal fibrosing alopecia, Graham Little syndrome, Classic pseudopelade (Brocq), Central centrifugal cicatricial alopecia, Alopecia mucinosa, Keratosis follicularis spinulosa decalvans	Physical or chemical trauma	Burns, Toxic/corrosive substances (e.g. acid or alkali burns), Ischemia/pressure, Traction/ trichotillomania (end stage)
		Ionizing radiation	Radiation
		Infections	Bacterial infections, Viral infections, Fungal infections (tinea capitis, in particular, deep trichophytosis caused by zoophilic pathogens)
Neutrophilic	Folliculitis decalvans, Dissecting cellulitis/folliculitis	Malignant and benign tumors	Primary tumors, Metastases, Lymphoproliferative diseases, Epidermal and organoid nevi
		Genodermatoses	Aplasia cutis congenita, Ectodermal dysplasia, Ichthyosis, Epidermolysis bullosa, Darier's disease, Incontinentia pigmenti, Hyalinosis cutis et mucosae
		Granulomatous diseases	Sarcoidosis, Necrobiosis lipoidica
Mixed	Folliculitis (acne) keloidalis, Folliculitis (acne) necrotica, Erosive pustular dermatosis	Autoimmune diseases	Graft–versus–host disease, Scleroderma (en coup de sabre), Lichen sclerosus, Blistering dermatoses (cicatricial pemphigoid)
Non-Specific	Idiopathic scarring alopecia with inconclusive clinical and histopathological findings, End stage of various inflammatory scarring alopecias	"Deposition" dermatoses	Amyloidosis, Mucinosis
		Inflammatory diseases	Psoriasis, Pityriasis amiantacea

that cortisol has a negative effect on the formation mechanism of the hair follicle by breaking down hyaluronan and proteoglycans, which are integrating substances in the extracellular matrix and skin. Acute and chronic stress is known as the main cause of telogen effluvium. Stress also can aggravate the types of alopecia that are primarily caused by endocrine imbalances, immunological responses, and toxic causes. In addition, the stress that occurs in response to hair loss causes hair loss to continue. In animal studies, chronic stress has been associated with hair growth arrest, increased granulation of mast cells, and perifollicular inflammation. Further studies have also shown that certain stress mediators, such as substance P, adrenocorticotropic hormone, prolactin, and cortisol, inhibit hair growth [22].

Telogen effluvium

Telogen effluvium is a scarless hair loss condition caused by physiological stress (such as delivery, rapid weight loss, mental stress, long-term drug usage), medical conditions like hypo/hyperthyroidism, post-diet effects, and prolonged fasting. Although it is generally reversible, it affects the psychosocial status of the patients, and its mechanism has not been discovered yet. Telogen effluvium is difficult to diagnose and treat because there are many factors at play in the etiology of the disease [23, 24].

Endocrine imbalances

The formation of hair follicles occurs as a result of a molecular genetic process regulated before birth. Many hormones and their receptors play important roles in the healthy progression of this process: thyroid hormones, glucocorticoids, insulin-like growth factor-I, and prolactin are all hormones that influence hair development, although androgens are the most important ones. In addition, vitamin D receptors and retinoid X receptors are also effective in postpartum hair growth [21, 25].

Immune System

Alopecia areata and primary cicatricial alopecia are two common forms of immune-mediated alopecia. Alopecia areata is a T-cell-mediated autoimmune illness that affects hair follicles in the anagen stage. The JAK-STAT (Janus kinase-signal converter and transcription activator) signaling pathway is involved in its development [26].

The concept of immune privilege is the phenomenon of protection of organs or tissues that can be seen in the corneal tissue, testis, placenta, liver, intestine, brain, hair follicle, and mucosal tissues from autoimmune threats developed by the host's immune system [27].

Hair follicles in the anagen stage are protected from unwanted immune responses by exhibiting immune privilege from the protrusion level to the bulb. The key mechanisms are the lack or reduction of MHC class I and MHC class II expression, signaling that there is no risk with the CD200 signal, and the absence of antigen presentation. In the development of alopecia areata, collapse is observed in the anagen hair bulb. While NK-G2D+ T cells and natural killer (NK) cells rapidly increase in the medium, perifollicular mast cells exhibit proinflammatory properties and interact with CD8+ T cells that recognize autoantigens [26-28].

The intense presence of CD8+CD3+ cytotoxic T cells in anagen stage hair follicles and the more targeting of pigmented hair suggest that the targets of cytotoxic T cells are melanocytes and melanogenesis-related autoantigens. The fact that over 100 single nucleotide polymorphisms are associated in studies indicates the importance of the genetic background [26, 28].

Studies have shown that in primary cicatricial alopecia there is a loss of immune privilege in the protrusion region. Although it is not exactly known what causes the loss of immune privilege, it is thought that among the causes might be perifollicular/intrafollicular increase in skin microtrauma, infectious or psycho-emotional stressors, bacterial superantigens, mast cell degranulation, and ectopic interferon γ (IFN-γ) secretion [26].

Other causes of alopecia

Drug-induced alopecia is a type of non-scarring alopecia involving the scalp, which occurs when one of the two main mechanisms of the normal hair growth cycle, anagen effluvium or telogen effluvium, is stopped. When the drug is stopped, the hair loss improves. Other body hairs are rarely affected by drugs [29]. Anti-hypertensives, anti-arrhythmics, statins, anti-metabolites, psychotropic agents, anti-convulsants, anti-coagulants, antiretrovirals, and H2 blockers are among the drugs that cause hair loss [3].

Hair loss is also known to be caused by agents such as bacterial infection Treponema pallidum, viral infection Epstein-Barr virus, HIV, hepatitis C, varicella-zoster virus, and cytomegalovirus [3].

Hair loss and subsequent alopecia are seen as a result of diseases such as liver and kidney failure, rheumatological disorders, discoid lupus erythematosus, connective tissue disorders, amyloidosis, cutaneous sarcoidosis, systemic sarcoidosis [3]. Alopecia can also be seen in systemic and cutaneous hematological malignancies. Although it is usually associated with mycosis fungoides, hair loss is common in cutaneous T-Cell lymphoma [3, 30].

Nutrition and hair loss

The integrity of the normal function of healthy skin and hair depends largely on an adequate and balanced diet. Whether a nutritional imbalance is a general or specific deficiency, an excessiveness of one component over another can compromise the organism's balance. When looking at the studies published in the literature, it is apparent that the nutritional elements that cause hair loss in healthy adults are still to be thoroughly determined.

In the first published reports in this domain, that are animal studies carried out in the first half of the XX century to explain the relationship between nutritional factors and hair loss, Cunningham identified iron deficiency in rats in 1932, Sullivan and Nicholls found riboflavin deficiency in 1941, and Day studies reported that zinc deficiency causes hair loss in both rats and mice [31-35]. The discovery and identification of vitamins related to dietary deficits have had a profound impact on the evolution of nutrient medicine since the first clinical trials. People frequently look for information on vitamin and mineral supplements and diets to prevent or treat many skin disorders, including hair loss. Hair loss greatly affects a person's appearance and personality in physical and social aspects. As a result, hair loss has a negative impact on the standard of living of men and women alike, therefore making the treatment of alopecia essential [36, 37]. Among the about 100,000 hair follicles on the human scalp, 90% of them are in the anagen phase without alopecia, requiring critical nutrients including proteins, vitamins, and minerals to create healthy hair [38]. As a result, trace elements, such as vitamins, are crucial dietary components [39]. Vitamins and trace elements are micronutrients that are critical elements of the diet, even though they are only required in minute amounts. The normal cycle of hair follicles is dependent on micronutrients as they play a role in cell renewal, which is common in rapidly dividing hair follicles [40]. Furthermore, several micronutrients are suggested to minimize oxidative stress because they play a role in alopecia pathogenesis [41]. As a result, a detailed understanding of the significance of these micronutrients could aid research into alopecia prevention and therapy [40].

Micronutrients

Vitamin A, which is made up of a set of unsaturated chemical compounds such as retinol, retinal, and retinoic acid, is an essential nutrient that humans cannot generate and thus it must be taken from diet [42]. Vitamin A and its compounds are required for a variety of functions throughout life, including immune function, cell differentiation and proliferation, reproduction, growth, maintenance of epithelial cell integrity, vision, and em-

bryogenesis [43, 44]. Generally, excessive vitamin A consumption can lead to hair loss in most people [45].
In one study, dietary vitamin A activates follicle stem cells by activating the hair cycle developmental and growth phases in a dose-dependent manner (anagen) [46, 47]. For healthy hair, it is important to maintain homeostasis and, accordingly, to maintain an appropriate concentration of the active metabolite [48]. A sufficient level of vitamin A may usually be obtained via a well-balanced diet. One case reported of a 60-year-old man who took too much vitamin A and had non-scarring fronto-central alopecia and a decrease in pubic and axillary hair. These changes were accompanied by drug toxicity associated with excessive intake of vitamin A by the patient [49]. In conclusion, these studies show that there are some optimal levels of vitamin A that are suitable, but very little or too much of this molecule contributes to the formation, maintenance, or advancement of alopecia [40].
The only vitamin B produced by the body is B7 (biotin). The recommended daily amounts of the vitamin B complex, which are pantothenic acid, riboflavin, thiamine, niacin, B6, B12, and folate, can be taken through a balanced diet. Lack of biotin is extremely uncommon in people who eat a healthy, well-balanced diet, and biotin supplements are not necessary [50]. Hair loss has only been linked to B2 (riboflavin), B7 (biotin), B12, and folate deficits. Biotin (vitamin H or B7) is a vitamin B complex with five carboxylases (acetyl-CoA carboxylase, 3-methylcrotonyl-CoA carboxylase, pyruvate carboxylase, and propionyl-CoA carboxylase) [50]. In addition, biotin is effective in cell signaling, histone modifications, and gene regulation [51]. Biotin lack is a condition that can be inherited or acquired; the inheritance pattern is autosomal recessive and there can be neonatal and infantile forms. It is caused by a lack of the enzyme holocarboxylase synthetase and appears from the first six weeks of life; it can be a potentially fatal disease in newborns. Diffuse dermatitis and alopecia are common among survivors. The scalp may be devoid of vellus and terminal follicles, and may also lack lanugo hairs, eyebrows, and eyelashes. Hair on the scalp, eyebrows, and eyelashes may be present but sparse or completely lost [52, 53].
Despite the popularity of biotin, there is still insufficient evidence in randomized controlled trials to support that supplementing this micronutrient prevents or treats hair loss. There are no clinical studies showing that biotin supplements are effective in treating hair loss unless people are deficient [54]. Hair loss, rashes on the skin, and fragile nails are all signs of biotin deficiency. In one study, 38% of women suffering from hair loss had low levels of biotin [55].
Humans cannot produce folate. Folate is essential for cell division and maintenance, and for synthesis of the nucleotide (thymidine), which is required for DNA repair. Folate also plays an important role in the 'site-specific' methylation of the cytosine base in gene expression. Remethylation of plasma homocysteine to methionine is another action of folate/folic acid. This water-soluble vitamin's demand is satisfied in part by dietary folate and in part with the use of synthetic folic acid [56]. Hair, skin, and nail changes may indicate a lack of folate [38].
Vitamin B12 (also known as cobalamin) is needed to activate folate, which is essential for DNA synthesis. Inadequate nutrition causes impaired nerve transmission and insufficient erythrocyte and other hematological cell production. B12 is an essential nutrient in the transfer of a methyl group in a reaction that requires methionine synthase, which converts homocysteine to methionine. This reaction activates folate, which can be used in DNA synthesis. Myelin production, and thus the preservation and repair of nerve axons, both require B12. In addition, B12 is required for energy synthesis in mitochondria and erythropoiesis in the bone marrow [57].
Due to the effect of B12 and folate on nucleic acid, it has been highlighted that they could play a role in the proliferation of hair follicles [38]. Additionally, a case-control review of dietary research on alopecia areata found no changes in vitamin B12 values in patients with and without alopecia areata. Although vitamin B12 appears to not affect hair growth and repair, research on the subject is scarce [53].
Ascorbic acid, often known as vitamin C, is a water-soluble vitamin produced as a product of glucose metabolism. Vitamin C has a chelating and reducing effect. This effect helps intestinal absorption and mobilization of iron [58, 59]. Accordingly, vitamin C supplementation is critical for patients suffering from hair loss due to iron deficiency [38]. The enzyme l-gulonolactone oxidase, which is necessary for the production of vitamin C, is evolutionarily deficient in humans, which is why vitamin C must be received from diet [60]. The pathophysiological role of vitamin C in the hair cycle is poorly known [53]. Citrus juices contain large amounts of vitamin C, and patients with iron deficiency are recommended to take oral supplements such as orange juice [61]. While vitamin C insufficiency has been linked to anomalies in body hair [59], there's still no evidence of a link between vitamin C values and hair loss.
Vitamin D is a steroid hormone that is fat-soluble. Vitamin D plays a regulatory role in various pathways, such as calcium absorption, bone mineralization, immune system, and DNA transcription [62, 63]. Vitamin D, whether obtained from diet or through the skin, is inactive and must be activated by enzymes [38]. Vitamin D insufficiency is a typical occurrence [64]. It was observed that vitamin D has an effect on the hair cycle in animal research. By attaching to the nuclear vitamin D receptor (VDR), vitamin D regulates keratinocyte development and differentiation. VDR immunoreactivity

Tab. II. Classification of Non-Cicatricial Alopecias [17].

Diffuse Alopecia	Local Alopecia
Female pattern hair loss	Alopecia areata
Male pattern hair loss	Tinea capitis
Diffuse alopecia areata	Traction alopecia
Alopecia totalis or universalis	Trichotillomania
Telogen effluvium	
Anagen effluvium	

is highest in the anagen phase in murine hair follicle keratinocytes [38, 53]. Vitamin D thus plays a function in alopecia and may be the cause of hair loss in vitamin D-deficient rickets patients [65]. Alopecia, hypocalcemia, rickets, hyperparathyroidism, and osteomalacia develop in mice lacking the vitamin D receptor (VDR). Normalizing mineral ion homeostasis prevents all these abnormalities, except alopecia. Hair regeneration experiments in athymic nude mice demonstrate that the lack of VDR in keratinocytes results in a defect in anagen initiation, similar to the one observed in mice without VDR. Although these studies show that expression of the VDR in keratinocytes is necessary, they do not prove that it is sufficient to maintain the normal hair cycle [66]. Patients with VDR mutations who develop vitamin D resistance also suffer from severe alopecia of the body and scalp [53, 65]. In two patients with inherited vitamin D-resistant rickets and baldness, Forghani et al. discovered novel nonsense mutations in the VDR gene [67, 68]. Daroach et al. compared patients with alopecia areata to healthy controls in a prospective study to correlate VDR expression in alopecia areata patients, finding higher vitamin D deficiency levels in the alopecia group [69]. To better understand the effects of vitamin D in alopecia, more large-scale research is required [68].

Iron is vital for all cellular functions because of its role in electron transport, oxygen delivery, and enzyme activity. It includes a wide range of enzymes, from hydroxylases – regulating cellular metabolism – to demethylases – changing DNA chromatin and consequently gene expression [70]. Iron is vital in rapidly proliferating cells, such as those in the hair follicle matrix, since it is a cofactor for ribonucleotide reductase, the rate-limiting enzyme in DNA synthesis. Some studies have demonstrated that various genes in the hair follicle are regulated by iron [71]. Iron deficiency, which also causes telogen effluvium, is the most frequent dietary deficiency worldwide [72, 73]. The level of serum ferritin, also known as iron-binding protein, is an important correlate of total body iron stores and is utilized in hair loss research as an indicator. Iron deficiency is frequent in women who are experiencing hair loss [31]. A mutation in the *TMPRSS6* gene caused the "mask mouse" to lose body hair and develop iron deficiency anemia, according to a study conducted by Du et al. A mutation in *TMPRSS6* was linked to an inability to downregulate *HAMP* expression in mice, and elevated hepcidin levels were linked to reduced dietary iron absorption and, as a result, iron insufficiency. Iron treatment, interestingly, corrected iron deficiency and stimulated hair growth in these animals [74]. Further research is needed to develop iron supplementation guidelines, therapy markers, and cures for patients suffering from hair loss due to iron deficiency.

Selenium (Se) is primarily involved in thyroid hormone metabolism, redox homeostasis, and enzymatic activity like glutathione peroxidases (GPx) as an important microelement. The antioxidant potential of other selenoproteins is thought to have a significant impact on human health [75]. Hair loss has been observed in selenium-deficient rats [76] and in knock-out mice lacking a selenium cofactor protein [77]. Although these findings suggest a link between selenium deficiency and hair loss, the role of selenium in human hair follicles is still not fully understood. Hair loss can be caused by selenium toxicity [53]. Among people who have been diagnosed with selenium toxicity, 72 percent was affected by hair loss affected, losing 10 percent to 100 percent of their hair [78]. After starting daily selenium therapy in 6 infants with pseudoalbinism alopecia-like findings, alopecia and pseudoalbinism improved when serum selenium levels reverted to normal [79]. Another clinical study showed that there was a significant reduction in hair loss and other gastrointestinal symptoms in ovarian cancer patients who received selenium supplements during chemotherapy as compared to controls. This result shows that selenium has a beneficial effect during chemotherapy [80]. Selenium is thought to play a role in the hair cycle, and healthy people can obtain enough through their diet. Supplementation is not suggested unless there are known deficits, particularly given the risk of hair loss due to toxicity.

Zinc is a trace element that is essential for a variety of cellular functions through proteins, enzymes, and zinc fingers, and also acts as a multitude of transcription factors important in gene regulation [81, 82]. Zinc deficiency can cause telogen effluvium, thin white and brittle hair, as well as many dermatological problems [83]. Superoxide dismutase is one of the zinc-dependent enzymes, which is thought to be one of the contributing factors to alopecia areata [84]. The function of antioxidant processes in alopecia areata is a popular issue in the scientific community. Zinc has also been linked to the Hedgehog signaling pathway, which has been linked to hair follicle morphogenesis [53]. Although zinc homeostasis impairment has been linked to a number of issues, its diabetic effects and function in metabolic syndrome are still being researched [85]. Zinc is a cofactor for enzymes involved in dopamine transport [86], cell membrane stabilization [87], and prostaglandin metabolism. Data relating zinc levels to telogen effluvium and androgenetic alopecia are not homogeneous. In another study comparing 312 people with hair loss with 32 controls, patients with alopecia areata and telogen effluvium showed low zinc levels [88].

In summary, nutritional supplement can damage the hair, when taken without a cause: a surplus dosage of micronutrients, including vitamins A and E, as well as selenium, has been linked to hair loss [83]. Hypervitaminosis A can cause hair loss, and findings on the effects of isotretinoin on hair loss back up this theory. Even though the connection between vitamin D values and telogen effluvium or androgenetic alopecia is still contested, most researchers concur that patients having alopecia and vitamin D insufficiency should take vitamin D supplements. On the other hand, people with iron-deficient alopecia need vitamin C. There is no evidence that vitamin E has a function in androgenetic alopecia or telogen effluvium. Women with hair loss are more likely to be lacking iron. In individuals with iron insufficiency and/

or low ferritin, many researchers deem that iron supplementation is necessary. Data on zinc concentrations in telogen effluvium and androgenetic alopecia are not yet clear and zinc screening is not advisable. It can be also a cause of hair loss, selenium toxicity, and riboflavin deficiency. Nevertheless, there are still no comprehensive studies to make any recommendations about screening for riboflavin or selenium. Hair loss can be caused by a lack of biotin. However, there is no proof that biotin supplementation increases hair growth. Exogenous biotin may also interfere with several laboratory tests, resulting in erroneous negative or positive results. Although some research has been carried out on the link between hair loss and B12 or folate/folic acid, currently there is not enough data on these dietary supplements.

Mediterranean diet and hair loss treatment

The Mediterranean Diet (MD), among the healthiest nutrition models accepted by the whole world, was put forward by Prof. Ancel Keys and his friends from examining the typical diet in Southern Italy and Greece. The MD is characterized by a low intake of saturated fat and animal protein, by a high intake of unsaturated fats, fiber, and antioxidants, and by appropriate intake of omega-6/omega-3 fatty acids. It is generally rich in vegetables, fruits, legumes, nuts, grains, fish, and unsaturated fats, with small amounts of meat and dairy products [89-91]. In the Seven Country Study, the MD has been linked to a lower risk of coronary artery disease. In addition, several studies support its effectiveness in preventing diabetes, obesity, and even various types of cancer [89, 91]. This is thought to be related to an adequate intake of polyphenols, which play a critical role in the MD. Polyphenols have genomic effects that play a part in the inactivation and activation of regulatory genes. They also exert epigenetic effects by modulating the expression of microRNAs, regulating mechanisms such as DNA methylation and histone modification, and chromatin rearrangement [91, 92]. The genetic and epigenetic effects of polyphenols, one of the main components of the MD, on the genome have recently been the focus of attention for researchers.

In addition, polyphenols protect the body against harsh climatic conditions and oxidative stress, which are involved in the defense against ultraviolet radiation and pathogen attacks in plants. Diets rich in these secondary metabolites found in fruits, vegetables, fiber foods, cereals, and beverages protect against the development of chronic and neurodegenerative diseases, diabetes, cancer, cardiovascular diseases, aging, and hypertension in humans [93]. Polyphenols can act as antioxidants, stimulate cell signaling pathways, and reduce inflammation by blocking gene activity. Studies have proven that the MD plays an important role in reducing inflammation [94]. The presence of over 8,000 polyphenols identified so far and the differences between the regions of the MD are considered limiting factors in the study of the effects of this diet. However, the protective effect of polyphenols has been proven by many studies [91, 93]. Plants containing flavonoids are promising in terms of reducing hair loss or stimulating hair growth [95].

The effects of the main components of the foods consumed in the MD are examined in hair loss and in many other diseases. Studies have shown that the polyphenolic compounds in tea significantly increase hair growth. In addition, it has been reported that epigallocatechin-3-gallate, the main component of tea polyphenols, can reduce the risk of androgenetic alopecia by inhibiting 5α-reductase, which increases hair growth in humans [96].

Essential fatty acids (linolenic, linoleic, oleic, myristoleic, palmitoleic, and stearic acids) found in olive oil, which has an important place in the MD, have inhibitory effects on 5α-reductase (5AR) that provide hair regrowth [4, 18, 97]. It has been reported that hair loss and depigmentation are seen in hair and eyebrows in people affected by linoleic acid and alpha-linolenic acid deficiency [83]. Mice with testosterone-induced alopecia showed a significant increase in hair growth after day 16 of topical applications of hydroalcoholic extracts of rosemary, which is a polyphenol. In vitro tests showed strong inhibition of the 5AR enzyme binding to the dihydrotestosterone (DHT) receptor [98, 99]. Although not fully proven, topical applications of coconut oil for hair loss treatment continue to be used [100].

Polyunsaturated fatty acids are thought to be absorbed into mast cell membranes, altering membrane-associated enzymes such phospholipases, which affect mast cell exocytosis. In a monkey study, it was shown that supplementing polyunsaturated fatty acids reduced hair loss in slightly alopecic monkeys; however, the cause of the hair loss was unknown [101].

A plant nanoparticle of safflower oil body loaded with human fibroblast growth factor 10 (hFGF10) in mice with androgenetic alopecia was found to accelerate hair regeneration by targeting hair follicles and reducing inflammation without any toxicity It has also been found to reduce hair follicle inflammation by inhibiting the overproduction of TNF-α, IL-1β, and IL-6 in macrophages and increasing the proliferation of dermal papilla cells. In this respect, it can be seen as a viable treatment option in androgenetic alopecia [102]. The standardized para rubber seed oil has been proven to be a safe and efficient bio-oil to stimulate hair growth or reduce/suppress hair loss [103].

Although there are many products for the treatment of alopecia, their effectiveness is controversial; research thus focuses on more effective pharmaceutical product development. As a result, the bioactivity of oligomeric procyanidin, a dimeric derivative from apples that can stimulate hair epithelial cell proliferation and induce anagen phase in vitro and in humans, is a popular application [104].

In one study, it was observed that resveratrol, an antioxidant, anti-inflammatory, and anti-apoptotic agent, when applied to mice, stimulated the transition of the hair cycle from the telogen phase to the anagen phase, delayed the transition to the catagen phase, and protected the hair

follicle from oxidative damage. Resveratrol is considered to be a potential candidate drug for the treatment of alopecia [105]. In addition, a topical lotion containing Redensyl and Sepicontrol A5 polyphenols is used as an alternative treatment for androgenetic alopecia, as it is very safe and provides patient satisfaction [106]. When given to rabbits, *Ficus benghalensis* leaf extracts were found to be effective in increasing hair follicle development by lowering the action of 5-reductase II [107].

As a result, a lack of certain nutrients, such as vitamins, minerals, vital fatty acids, and proteins, can cause hair loss, structural anomalies, and color changes, albeit the exact causes remain unknown [83]. Hair loss is prevalently treated by dermatologists, and it has a significant psychological and emotional impact on patients. Micronutrients like vitamins and minerals have a vital, though not completely understood, role in hair follicle formation and in immune cell activity. A lack of these micronutrients could be a modifiable risk factor for the development, prevention, and treatment of alopecia.

It has been proven by studies that those who follow the MD have less risk of alopecia. As the use of polyphenol-based phytochemicals – frequently used in the MD– increases, the effect of polyphenols in the treatment of alopecia is taken into consideration. In addition, in several studies essential oils were used in the treatment of alopecia, leading to positive results [4, 91].

Considering all the data, more research is needed soon on the role of nutrients in the hair cycle, its association with known alopecia diseases, and effective supplementation regimens. The fact that the incidence of alopecia is low in those fed the MD is promising for the development of the most appropriate preventive and supportive treatments by further researching the active ingredients frequently used in the MD.

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Conflicts of interest statement

The authors declare no conflict of interest.

Author's contributions

MD, MB, and TB developed the study design and conceptualization of the research methodology. NG, NB, and SK contributed to the manuscript's writing. MD, YO, HA, and MCE contributed to manuscript reviewing and editing processes. All authors have read and approved the final manuscript

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Review

Dietary supplements for improving nitric-oxide synthesis

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Keywords

Nitric oxide supplementation • Nitric oxide synthesis • L-arginine supplementation • L- citrulline supplementation • Dietary supplements

Summary

Nitric oxide (NO) is an essential component of the human body, involved in blood vessel dilation, stimulation of hormone release, signaling and regulation of neurotransmission. Nitric oxide is synthesized by nitric-oxide-synthase-dependent and -independent pathways. Nitric oxide supplementation improves cardiac health, enhances performance during exercise, reduces high blood pressure during pregnancy, reduces erectile dysfunction and improves healing processes and respiratory response. Nitric-oxide-associated benefits are mostly apparent in untrained or moderately trained individuals. L-arginine and L-citrulline supplementation contributes to nitric oxide levels because L-arginine is directly involved in NO synthesis, whereas L-citrulline acts as an L-arginine precursor that is further converted to NO by a reaction catalyzed by NO synthase. L-arginine supplements increase respiratory response and enhance performance during exercise, while L-citrulline with malate and other molecules increase working capacity. Various studies involving beetroot juice have reported a significant increase in plasma nitrite levels, regarded as markers of NO, after intake of beetroot juice. Although NO supplementation may have mild to moderate side-effects, using smaller or divided doses could avoid some of these side-effects. Since nitric oxide supplementation may worsen certain health conditions and may interfere with certain medicines, it should only be taken under medical supervision.

Nitric oxide

Nitric oxide or nitrogen monoxide (NO) is a component of the human body that may cause dilation of blood vessels and release of hormones such as human growth hormone (GH) and insulin [1]. Shortly after the role of NO as a signaling molecule was established, it emerged that particular nitric oxide synthases (NOS) catalyze the intricate enzyme reactions that lead to synthesis of NO from the substrates L-arginine and molecular oxygen. An alternative pathway of NO synthesis, known as NOS-independent, was subsequently discovered: it is based on simple reduction of nitrate and nitrite, major NO oxidation products. Nitric oxide acts as a mediator and regulates significant non-cholinergic and noradrenergic neurotransmission functions involving memory, learning, neuroprotection and synaptic plasticity [2].
Although nitric oxide supplementation has been used for decades, little scientific evidence is available to support its health benefits [1]. The composition of NO-stimulating supplements is mostly a cocktail of amino acids, creatine, minerals, carbohydrates, vitamins, and so forth. Studies have also shown that components like creatine, amino acids and carbohydrates may have ergogenic effects among themselves [2, 3].
Many studies have used NO-donors and other components like malate, aspartate and glutamate to enhance NO-donor bioavailability [2]. Benefits associated with NO-donors have been reported in moderately trained individuals, whereas recent scientific data indicates that well-trained athletes benefit less from NO supplements. Hence it appears that the training status of an individual is a significant factor for dietary NO-donor effectiveness. One logical explanation of the association of exercise with the effectiveness of NO supplementation may be due to the positive effect of physical exercise on the regulation of NO metabolism. Although short-term physical training promptly increases the bioactivity of NO, if training is continued, the short-term functional changes are followed by NO-dependent structural changes that lead to arterial re-modelling and the structural normalization of shear [4].
Two of the most prevalent NO supplements are L-arginine and L-citrulline. Scientists have conducted various clinical trials on the effectiveness and side-effects of NO supplements [5].

L-arginine

L-arginine is a metabolically versatile amino acid involved in building proteins and in the synthesis of NO. It is found naturally in dairy products, red meat, fish and poultry. Commercial manufacturers produce it as a

pill, cream or powder. According to a recently published article, a normal person typically consumes almost 5 g L-arginine per day without NO supplementation. The human body converts consumed L-arginine into NO for different body functions [6].

Different research studies have reported an increase in exercise performance after consumption of supplements containing L-arginine and other components by moderately trained and untrained subjects. For example, in their recent study Bailey et al. reported that 6 g/day L-arginine for 3 days with vitamins and other amino acids led to a reduction in VO2 (rate of oxygen consumption) in low to moderate exercise sessions and an extension of exhaustion time (L-arginine: 707-232 seconds; placebo: 562-145 seconds) during the cycling incremental exercise test [7].

In another study, high-dose L-arginine supplementation (14.2 g/day) was given to females for almost 6 months. At the end of the period, a significant increase in relative maximum power (power/kg), measured as peak jump power in the countermovement jump test, was observed [8]. Certain male studies show that L-arginine supplements may increase respiratory response. Likewise Koppo et al. showed significantly higher speed in phase II of the VO2 (rate of oxygen consumption) during moderate intensity endurance cycling after 6 g/day L-arginine supplementation for 14 days [8, 9].

Chen et al. found that L-arginine supplementation 5.2 g/day for 21 days with L-citrulline and antioxidants increased the power output of elderly males by almost 21% during the incremental cycle ergometer exhaustion test [10]. These results have been associated with an increase in the gas exchange threshold after L-arginine supplementation. Researchers have also suggested that attenuation of metabolic products like potassium, lactate and ammonia may be caused by increased clearance from the circulation due to NO synthesis and increased blood flow [10, 11].

Several other studies have also established the benefits of L-arginine supplementation in strength and power performance among moderately-trained individuals. Campbell et al. reported that L-arginine supplements (6 g/day for 56 days) with a-keto-glutarate significantly increased the value of the one-repetition-maximum bench press test and peak power in a 30-second Wingate anaerobic test [12]. Stevens et al. and Buford & Koch, measured the acute dose of L-arginine, namely 6 g L-arginine as a-keto-isocaproic supplements, that increases the work sustained in continuous isokinetic eccentric/concentric knee extension repeats and mean power performance during the Wingate anaerobic test for 10 seconds, respectively [13, 14].

Bailey et al. [24] analysed plasma levels of nitrite and reported a significant increase after L-arginine supplements. In animal models, L-arginine-supplemented diets caused a 1-2% increase in intramuscular concentrations of creatine phosphate, which may lead to increased response in anaerobic exercise [24]. These results support Buford and Koch's finding of increased performance during recurrent bouts of anaerobic cycling after taking glycine-arginine-a-ketoisocaproic acid supplements [13].

L-citrulline

L-citrulline is a non-essential amino acid found naturally in nuts, meat, watermelon and legumes [15]. It is manufactured commercially as a powder or pill. In the body, L-citrulline is synthesized by conversion of L-arginine to NO in a NOS-catalyzed reaction. In healthy populations, normal plasma concentrations of L-citrulline are reported to be almost 25 mmol/L, although lower values were recently measured (10-15 mmol/L) in professional cyclists [16, 17].

In recent years, dietary interest in L-citrulline has arisen due to its significance as a L-arginine precursor. Interestingly, unlike L-arginine, L-citrulline bypasses hepatic metabolism and is not catalyzed by arginase enzymes. Physiologists have therefore established that systemic administration of L-citrulline alone could be an efficient way to raise extracellular levels of L-arginine. Enterocytes take up dietary L-citrulline and release it into the portal circulation, bypassing metabolization by periportal hepatocytes and transporting it to the kidneys where almost 80% is catabolized to L-arginine by proximal tubule cells [18-20].

Various studies have analysed the effects of L-citrulline in combination with malate, an intermediate of the tricarboxylic acid cycle. Initial studies calculated adenosine triphosphate (ATP) production rates during finger flexion exercise by 31-phosphorus magnetic resonance spectroscopy. The results showed that taking 6 g/day L-citrulline with malate for almost 16 days led to a significant increase (almost 34%) in the oxidative production rate of ATP during exercise and a 20% increase in the phosphocreatine recovery rate after exercise [21].

Lately a research group conducted two studies that showed increased plasma levels of NO metabolites in well-trained athletes at the end of a cycling competition; 2 hours before the race, the athletes had taken a single 6-g dose of L-citrulline with malate [16]. An increase in the availability of plasma arginine has also been linked to availability of substrate for NO synthesis and to the activity of polymorphonuclear neutrophils. Another current study by Perez-Guisado and Jakeman confirmed that a single 8-g dose L-citrulline with malate increased working capacity by almost 19%, as measured by the number of repetitions completed until exhaustion in a bench-press fitness test at 80% of one-repetition-maximum [16, 22].

Nitric oxide synthesis by the NO-synthase-dependent pathway

The nitric oxide synthase (NOS)-dependent pathway of NO synthesis involves the amino acid L-arginine which participates in a reaction catalyzed by particular NOS enzymes. Extracellular L-arginine is rapidly taken up

by endothelial cells in the presence of molecular oxygen and NADP (nicotinamide adenosine dinucleotide phosphate), and L-arginine is then oxidized to NO [23]. This complex reaction is catalyzed by NOS enzymes containing an L-arginine binding site. Three nitric oxide synthase isoforms have so far been recognized, including type I or neuronal NOS (nNOS), type II or inducible NOS (iNOS) and type III or endothelial NOS (eNOS). Both iNOS and eNOS are constitutive enzymes regulated by intracellular calcium/calmodulin, although expression of nNOS may be induced at gene transcription level by aging processes, muscle activity, macrophages and other tissues responding to inflammatory mediators, independent of Ca2+ levels. L-citrulline may also be considered an alternative NO-donor, because it may bring about an increase in L-arginine levels [24-26].

Nitric oxide synthesis by the NO-synthase-independent pathway

In the 1990s, two distinct research groups discovered a NOS-independent pathway for NO synthesis [27, 28]. This alternative pathway used nitrate and nitrite as its main precursors. The NOS-dependent pathway is oxygen dependent, whereas the NOS independent nitrate/nitrite pathway of NO synthesis is activated gradually as oxygen tension falls [29].

Circulating nitrate has a half-life of almost 5 hours and is distributed to different tissues. Although the full mechanism is not yet completely defined, various studies have revealed that nitrate circulating in plasma is actively absorbed by salivary glands where its concentration is enhanced in saliva (10 to 20 times higher than in blood) [30]. In the oral cavity, certain facultative anaerobic bacteria that live on the tongue surface reduce this nitrate to nitrite by means of nitrate reductase. Hence in the absence of oxygen, these anaerobic bacteria utilize nitrate as an alternative electron-acceptor to produce ATP. After swallowing, half the nitrite present in saliva is transferred to the acidic stomach environment and is metabolized to NO, while the other half remains intact and is reabsorbed to increase circulating concentrations of nitrite in plasma. Under suitable physiological conditions, this nitrite may be converted into NO and other biologically active nitrogen oxides in the tissues and blood. These findings establish the presence of a completely reverse/alternative pathway (nitrate–nitrite–NO) in mammals [28, 31].

Nitrate supplementation

The alternative NO synthesis pathway has also been well studied with reference to exercise physiology. Nitrate and nitrite modulate mitochondrial respiration through NO synthesis. Two recent studies by the same research group showed that plasma NO metabolites like nitrate and nitrite increased significantly after nitrate treatment. The efficiency of human mitochondria, assessed in vitro by oxygen consumption per molecule of ATP produced (phosphate/oxygen ratio), was significantly enhanced with respect to a placebo group after intake of 0.1 mmol/kg sodium nitrate for 3 days [32-34].

Interestingly, studies involving beetroot juice have also reported a significant increase in plasma nitrite levels (regarded as markers of NO) after intake of beetroot juice. Larsen et al. reported it as an alternative metabolic pathway for mitochondrial respiration which might explain the decrease in oxygen demand during exercise after ingestion of nitrate-rich food [34].

In five other studies by the same research group, nitrate supplementation was taken as beetroot juice and its effects on human performance were assessed. In one of these studies, Bailey et al. reported a significant increase in oxygen uptake kinetics (VO2) after dietary supplementation with nitrate-rich beetroot juice (500 ml/day for 6 days) [35].

Beetroot juice supplements brought about a 19% decrease in pulmonary response amplitude during low to moderate-intensity exercise, such as cycle ergometer. Beetroot supplementation also caused a 23% decrease with respect to a placebo group in the slow component of VO2 and a 19% extension of exhaustion time in the incremental cycle ergometer test [35]. The effect of nitrate provided by beetroot juice supplementation was also observed to be faster and acute intake affected cardiovascular response within a few hours [36]. Vanhatalo et al. validated this effect of beetroot juice supplementation. They gave a single dose of beetroot juice (500 mL beetroot juice equivalent to 434 mg sodium nitrate) to subjects 2.5 hours prior to a cycle-based ergometer test involving two moderate work-loads with 90% gas exchange threshold, followed by the ramp test [7]. In another study, Lansley et al. used the same dose of beetroot juice 2.5 hours prior to exercise and observed significant improvement in mean completion time (2.8%) and average power output (5%) during 4-16.1 km of cycle ergometer time trials, with respect to the placebo group [37].

Glycine propionyl-L-carnitine

Glycine propionyl-L-carnitine (GPLC) is a carnitine propionyl ester with an extra glycine. It is used as a dietary supplement to increase blood levels of nitrate/nitrite which are surrogate markers of NO. It improves metabolism of NO in two ways: 1) as reported in certain animal studies, the protective action of GPLC is due to its antioxidant properties which may prevent peroxidative damage of vessels. On this basis, other researchers proposed that reduced release of reactive oxygen species could be associated with lower breakdown of NO; 2) a substantial increase in expression of the *eNOS* gene was observed in culture medium of human endothelial cells after incubation with carnitine. Hence, researchers suggested that GPLC could also stimulate NO synthesis through *eNOS* gene expression [38-42].

A number of recent studies have analysed the effect of GPLC as a NO donor in exercise and sport with different conclusions. Bloomer et al. reported a substantial increase in the plasma metabolites of NO, like nitrate and nitrite, in active male athletes after supplementation with GPLC (4.5 g/day for 4 weeks) [42]. Another study by the same research group further validated these results. [43] Results obtained by Jacobs et al. revealed that a single dose of GPLC (4.5 g) 90 minutes prior to a performance test, including 5-10-seconds of Wingate sprints on a cycle ergometer separated by active recovery periods of one minute, caused a significant increase in peak power (5.2%) and a decrease in power decrement (5.2%) in sprints, with respect to the placebo group [44].

2-(Nitroxy) ethyl 2-amino-3-methylbutanoate

The 2-(nitroxy) ethyl 2-amino-3-methylbutanoate molecule was recently recognized to increase the delivery of NO in the human body and is considered to be more effective and efficient than other traditional NO donors. A study of the effects of 2-(nitroxy) ethyl 2-amino-3-methylbutanoate on plasma NO markers quantified nitrate/nitrite levels among moderately resistance-trained males. Several other studies also suggest that NO supplementation increases blood flow in the human body, enhancing individual performance in exercise and sport, and promoting cardiac health, healing and other potential benefits [45].

Benefits

Various researchers believe that NO causes body relaxation and widens or dilates the blood vessels [6].

Improving heart health

Nitric oxide supplementation has many beneficial and boosting effects on the heart. These effects include reduction of blood pressure and arterial stiffness and improvement of blood flow in the carotid artery. However, most of these studies are based on animal models. Further human studies are required to validate these effects of nitric oxide supplementation on the human heart [5].

Enhancing exercise and recovery

The significance of NO is quite prominent in exercise physiology and NO supplementation is considered an ergogenic aid. Nitric oxide proves to be a significant blood flow and mitochondrial respiration modulator in physical exercise [46]. Various researchers have suggested that nitric oxide supplementation could increase oxygen delivery to the muscles. This could enhance athletic performance and reduce soreness after workouts [2]. Moreover, genetic variants in several genes, among which nitric oxide synthase 3, the enzyme responsible for NO synthesis, are correlated to endurance performance and trainability [47-49].
Several studies published in the journal Sports Medicine suggest that NO supplements may increase exercise tolerance. However, this effect is more prominent in subjects who exercise at a moderate rate and not regularly. The studies were mostly conducted on young males and therefore do not predict or offer insights into the effect of NO supplementation on older subjects or women [2]. It is also proposed that the increase in the blood flow caused by NO synthesis may improve the recovery and healing of activated tissues. Most commercial NO supplements claim to provide all these benefits and to increase the performance of support related individuals [45].

Reducing erectile dysfunction

As NO supplements increases blood flow, they have been studied for erectile dysfunction. Some published studies show that NO supplementation may reduce erectile dysfunction in mild to moderate cases [50].

Reducing high blood pressure in pregnancy

Preeclampsia, also known as gestational hypertension, may endanger both mother and fetus. In 2005, a study reported that pregnant women who took L-arginine supplements for prolonged periods had lower blood pressure than those who did not. Although more studies are required to understand these findings, it is a promising result [51].

Other reported benefits

There are some further benefits of nitric oxide supplementation. Research studies have reported that nitric oxide supplementation increases weight loss [52]. Improvement in lung function was also observed after nitric oxide supplementation in cystic fibrosis patients undergoing treatment for altitude sickness. Studies conducted on male subjects report remarkable enhancement of respiratory response after L-arginine supplementation. Koppo et al. [12] observed significant enhancement of speed during phase II of pulmonary oxygen consumption (VO2) at the start of moderately intense endurance exercise after L-arginine supplements (6 g/day for 14 days) [9]. Better recovery was observed after major injury or trauma with nitric oxide supplementation, which also played role in prevention of the common cold. Nitric oxide supplementation is suggested to reduce the side-effects of memory loss. Other studies propose that nitric oxide supplementation could help the healing of diabetic foot ulcers [53].

However, most of these benefits lack scientific support and are generally not based on research. The claims should be properly investigated before accepting or rejecting them.

Side-effects

Although dietary supplements with L-arginine and L-citrulline are considered safe, they may nevertheless have mild to moderate side-effects, including gastrointestinal disturbances like vomiting, nausea, bloating, diarrhea, stomach pain, as well as headache, heartburn and palpitations [54]. Tolerance of these amino acids differs greatly between individuals and higher doses (>9 g/day) may increase the risk of gastrointestinal distress. It is suggested that smaller or divided doses may cause fewer adverse effects [55]. Existing or prior prosecretory or proabsorptive intestinal states may increase susceptibility. A secretory intestinal state combined with the extra stimulation of higher doses (> 9 g/day) of L-arginine, L-citrulline or both may overwhelm the capacity of the colon to re-absorb [54].
Nitric oxide supplementation could have a higher risk of side-effects in persons with certain health conditions. Such health conditions include cirrhosis, guanidinoacetate methyltransferase deficiency and low blood pressure. Individuals suffering from liver scarring or cirrhosis should be cautious about taking nitric oxide supplements as they could worsen liver function [56]. Guanidinoacetate methyltransferase deficiency (GAMT) is a genetic condition associated with lack arginine to creatine converting enzyme. People with GAMT should therefore not take NO supplements [57]. Persons with low blood pressure should stop nitric oxide supplementation before undergoing any surgery. Medical practitioners have expressed concern that nitric oxide supplementation could aggravate other health conditions including kidney disease, heart conditions and viral infections such as herpes [51, 54].
In 2006, a study reported that individuals taking L-arginine after suffering a heart attack were at high risk of gastrointestinal motility, repeated heart attack and hospitalization than heart patients who did not [58].
Nitric oxide supplements may interfere with certain medications, such as blood pressure and diabetes medication. Before taking nitric oxide supplements, individuals should consult their health practitioners to avoid side-effects or interference with their medication [51, 59].
Another interesting fact is that in most research assessing the effect of NO supplementation on athletic performance and during sport or exercise, the amount of nitric oxide obtained from supplements could easily have been obtained from the natural food sources like green leafy vegetables and beetroot juice. Obtaining nitric oxide from natural food sources is a better option for avoiding certain side-effects [2].

Conclusions

Nitric oxide is a physiological compound of the human body that dilates blood vessels, stimulates hormone release, regulates neurotransmission and acts as a signaling molecule. Nitric oxide is synthesized by NOS-dependent and -independent pathways. Nitric oxide supplementation improves cardiac health, enhances performance during exercise, improves healing, reduces erectile dysfunction, reduces high blood pressure during pregnancy and improves respiratory response.
The benefits associated with NO have mostly been obtained in moderately trained individuals. Two prevalent NO supplements are L-arginine and L-citrulline. L-arginine is involved in NO synthesis. L-arginine supplements increase respiratory response and enhance performance during exercise. L-citrulline is a L-arginine precursor that is converted into NO by a reaction catalyzed by NOS. L-citrulline combined with malate and other molecules increase working capacity. Many studies on beetroot juice report a significant increase in plasma nitrite levels, a NO marker, after intake of beetroot juice. Similarly, glycine propionyl-L-carnitine and 2-(nitroxy) ethyl 2-amino-3-methylbutanoate are dietary supplements that act as nitric oxide donors, enhance nitric oxide synthesis and may also stimulate NO synthesis through expression of the *eNOS* gene.
Nitric oxide supplementation may have mild to moderate side-effects including gastrointestinal disturbances like vomiting, nausea, bloating, diarrhea, stomach pain, as well as headache, heartburn and palpitations. To avoid these side-effects, smaller or divided doses of these supplements are suggested. Nitric oxide supplements should not be taken by persons with certain health conditions and may interfere with medication. They should therefore only be taken under medical supervision.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; AKK, MCM, PC, PM, FF, SN, STC: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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REVIEW

Polyphenols and *Lactobacillus reuteri* in oral health

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Keywords

Mediterranean diet • Polyphenols • Oral health • *Lactobacillus reuteri*

Summary

Oral health is one of the necessary preludes to the overall quality of life. Several medical procedures and therapies are available to treat oral diseases in general and periodontal diseases in particular, yet caries, periodontitis, oral cancer, and oral infections remain a global concern. Natural molecules, with their anti-oxidant, anti-inflammatory, and anti-microbic properties, are one of the main sources of oral health and dental health care, and should be supplemented to exploit their beneficial effects. A possible way to improve the intake of these molecules is adhering to a diet that is rich in fruits, vegetables, and probiotics, which has many beneficial properties and can improve overall health and wellbeing. The Mediterranean diet, in particular, provides several beneficial natural molecules, mainly because of the precious nutrients contained in its typical ingredients, mainly plant-based (olives, wine, citrus fruits, and many more). Its beneficial effects on several diseases and in increasing the overall wellbeing of the population are currently being studied by physicians. Among its nutrients, polyphenols (including, among other molecules, lignans, tannins, and flavonoids) seem to be of outmost importance: several studies showed their anticariogenic properties, as well as their effects in decreasing the incidence of non-communicable diseases. Therefore, plant-derived molecules – such as polyphenols – and probiotics – such as Lactobacillus reuteri – have shown a significant potential in treating and curing oral diseases, either alone or in combination, owing to their antioxidant and antimicrobial properties, respectively.

Introduction

Plant-based foods, such as the typical fruits and vegetables of Mediterranean diet, are rich in many important phytochemicals that confer several health benefits. Among these, an important role is played by Polyphenols, a group of chemicals having at least one phenol moiety, which are particularly beneficial for human health [1]. Dietary polyphenols are a diverse group of phytochemicals, having approximately 8000 types of phenolic structures that are naturally present in cereals, fruits, vegetables, and beverages; based on the number of phenolic rings they contain, they can be classified into five subclasses, which are: phenolic acids, lignans, tannins, stilbenes, and flavonoids (Fig. 1, Tab. I) [2]. Epidemiological studies have shown that polyphenols – more specifically flavonoids – have strong antioxidant, anti-inflammatory, anti-cancerous, and anticariogenic properties [3], which can reduce the severity and incidence of non-communicable diseases such as diabetes, cancer, and cardiovascular problems.

Phenolic acids

Abundantly present in fruits and vegetables, phenolic acids are derivatives of cinnamic and benzoic acids. Hydroxycinnamic acids (such as ferulic, p-coumaric, caffeic, rosmarinic, chlorogenic, and sinapic acids) are more common than hydroxybenzoic acids, which for example are contained in high amounts in black radish, onions, and red fruits [4]; hydroxybenzoic acids include gallic, syringic, vanillic, protocatechuic, and salicylic acids. The major dietary sources of phenolic acids include cocoa, wholegrains, fruits, nuts, coffee, and beer [5]. Observational studies have shown an inverse relationship between phenolic acid consumption and non-communicable diseases, such as metabolic syndrome [6], type-2 diabetes [7], hypertension [8], and non-alcoholic fatty liver disease [9]. In addition, phenolic acid consumption had a profound effect in the management of depressive disorders and in sleep quality [10, 11]. However, studies related to cardiovascular effects of these phenolic acids are scarce.

Flavonoids

Flavonoids are perhaps the most powerful antioxidants naturally present in plants. They can be structurally distinguished by the presence of a di-phenyl-propane moiety (C6-C3-C6) and can be further classified into isoflavones, flavones, flavanones, flavonols, anthocyanidins, and flavanols [12]. Flavonoids have strong antioxidant, antimutagenic, anti-proliferative (against tumor cells), cardioprotective, radio-protective, antiatherosclerosis, and antimicrobial properties. In addition, these molecules also help in maintaining hormonal balance in menopausal women [13-15]. Among them, quercetin and genistein manifest a highly beneficial property of

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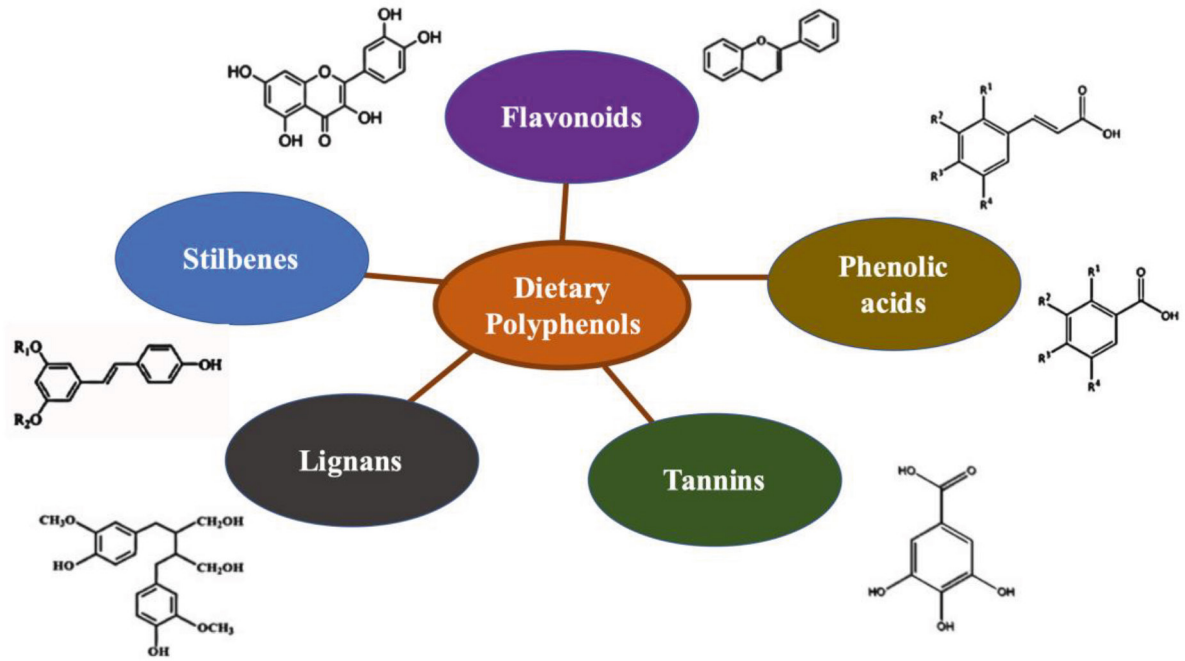
Fig. 1. Classification of dietary polyphenols.



Tab. I. Classes and properties of polyphenols.

Polyphenols	Subclasses and examples	Food sources	Health benefits	References
Phenolic acids	Benzoic acid and cinnamic acid derivatives (e.g. caffeic acid)	Cherries, black radish, onion, kiwi, berries, coffee	Antimicrobial, anticancer, anti-inflammatory, anti-mutagenic	[22]
Flavonoids	Flavonols (e.g. quercetin)	Leek, ginger, broccoli, onion, leafy greens, berries, tea	Antioxidant and radical-scavenging activities, inhibiting cell migration of hepatocyte growth factor-induced medulloblastoma	[23]
	Flavanols (e.g. catechins)	Grapes, chocolate, red wine, cocoa, apricots, black beans, green tea	Antioxidants, antimalarial, anticancer, antiviral, anti-inflammatory, anti-allergenic, UV protective	[24]
	Flavanones (e.g. hesperetin)	Citrus fruits (orange, grapefruit, lemon) and their juices	Antioxidative e anti-inflammatory. Ameliorate memory impairment and Aβ pathology	[25]
	Flavones (e.g. luteolin)	Oregano, celery, parsley, capsicum pepper	Neuroprotective, cardioprotective, antioxidant, anti-inflammatory, antiallergic	[26]
	Isoflavonones (e.g. genistein)	Milk, tofu, soy, tempeh miso	Anti-cancerous activity by inhibiting DNA topoisomerases and tyrosine kinases and inducing apoptosis, modulating PI3K/Akt and Wnt/β-catenin signal conduction	[27]
	Anthocyanidins (e.g. delphinidin)	Aubergine, red cabbage, rhubarb, red wine, black grapes, berries, cherries	Antioxidative, antidiabetic, anti-inflammatory	[28]

▶

Tab. I. *Continues.*

Polyphenols	Subclasses and examples	Food sources	Health benefits	References
Tannins	Dense tannins (e.g. pro-cyanidins)	Cocoa, chocolate, apples, grapes	Prevention, delay in the onset and treatment of cardiovascular diseases	[29]
	Hydrolyzable tannins (e.g., gallotannins)	Mango, pomegranate	Anti-inflammatory activity	[30]
Stilbenes	Resveratrol	Grapes, wine	Cardioprotective, antioxidant, antiplatelet, anti-inflammatory, anticancer activities, lowering blood glucose levels	[31]
Phenolic alcohols	Hydroxytyrosol	Olive	Antioxidant, anti-inflammatory, anticancer, protecting skin and eyes	[32, 33]

inhibiting ATP binding to tyrosine kinases, thus preventing proliferative diseases like cancer and psoriasis [15].

Stilbenes

This class of flavonoids is present in low amounts in various food sources, but some of its members are widely studied because their intake brings a myriad of health benefits [16]. For instance, resveratrol, which is contained in high quantities in red grapes and grape juice (both fermented and non-fermented), manifests antioxidant, anti-inflammatory, antibacterial, and anticancer properties [17, 18]. Besides being a strong antioxidant, resveratrol also has antidiabetic and cardioprotective activity and, because of its several molecular targets, its usage is very promising in the development of novel remedial approaches against metabolic syndrome, atherosclerosis, ischemic heart disease, and heart failure [19].

Tannins

Tannins are complex, water-soluble phenolic compounds derived from phenolic acids. They have a strong free radical-scavenging capability, which gives them antimutagenic and antibacterial activities. An example of powerful anticarcinogenic tannins are Ellagitannins derivatives, such as Ellagic acid, which can be found in many fruits and nuts like cranberries, strawberries, red grapes, raspberries, pomegranates, peaches, walnuts, and pecans [20].

Lignans

Lignans are diphenolic compounds, defined as phytoestrogens, which are derived from phenolic acids by the dimerization of two cinnamic acid residues. Lignans confer several health benefits, such as lowering the risk of cancer and cardiovascular diseases. In women, lignans help in alleviating the symptoms of menopause and osteoporosis. Wholegrain cereals and seeds – such as linseed, flaxseed and legumes – are rich sources of lignans [21].

Potential Effects of Polyphenols on Health

Polyphenols manifest a wide variety of health benefits, owing to their antioxidant, immunomodulator, anti-inflammatory, and radical-scavenging properties. For instance, resveratrol, curcumin, and epigallocatechin gallate (EGCG) have neuroprotective properties against neurodegenerative diseases (e.g. Alzheimer's-like diseases and dementia). Furthermore, these polyphenols inhibit the neurotoxic effects of the beta-amyloid protein that accumulated due to Alzheimer's disease [34, 35]. Moreover, the iron-chelating effects of EGCG, ginkgetin, curcumin, ginsenosides, and myricetin prevent neurotoxicity, thus protecting against Alzheimer's, Parkinson's, and Huntington's [35, 36].

Besides that, phenolic compounds manifest strong anti-inflammatory properties against systemic and localized inflammation by mitigating the cytokine pathway and reducing oxidative stress [37]. For instance, flavonoid-rich foods and resveratrol reduce inflammation, lower blood pressure, reduce platelet activity, block cholesterol oxidation, reduce LDL, and improve ventricular health, thereby preventing cardiovascular diseases [38].

The free radical-scavenging activity of Flavonoids such as catechins, flavanols, flavones, anthocyanins, flavanones, and isoflavones, prevents cellular growth in tumours, thereby decreasing the risk of oncogenesis [39]. Polyphenols have been observed to be beneficial in breast, endometrial, colon, prostate, and epithelial cancer [40].

Several polyphenols can inhibit lipid, starch, and protein digestion in the gastrointestinal tract by binding to the respective digestive enzymes, thus inhibiting it [41]. For instance, anthocyanins slow down the digestion of starch and can regulate and alter glucose transport, thus providing better glycaemic control in type 2 diabetes [42, 43]. Curcumin, catechins, and resveratrol exhibit anti-obesogenic effects by reducing inflammation, inhibiting lipogenesis, oxidating adipocyte and increasing energy expenditure, thus resulting in enhanced weight loss and improved weight maintenance [41].

In addition to that, polyphenols have been indicated in wound healing, but perhaps the most apparent effect of

polyphenols is maintaining oral health. Several researchers have reported health benefits of polyphenols in relieving periodontal diseases, maintaining oral health, and preventing oral cancer.

Polyphenols in the oral cavity

Oral mucosa is continuously under food- and environment-mediated oxidative stress. Polyphenols come in contact to the oral cavity directly and, due to their antioxidant and antimicrobial properties, prevent several diseases of the oral cavity, from infections to cancers [44]. In addition, polyphenols can be used as "processing cofactors" to enhance the mechanical and functional characteristics of the biomaterials that are used in dental tissue engineering [45]. For instance, grape seed extract, which is rich in proanthocyanidins (PAs), has been used in various dental applications, e.g. resin-dentin binding, because of its dual action of cross-linking with the collagen and of inhibiting the metalloprotein [46].
Polyphenols make stable complexes with proline-rich proteins and histatins in the oral cavity, which remain stable during their course from the oral cavity to the gastrointestinal tract and so on [47].
Polyphenols play multiple roles in dental diseases: for example, some polyphenol hydroxyls are very reactive, while others are very protective against microorganisms, yet another group acts as disinfectants by producing hydrogen peroxide and inhibiting bacterial proteins and enzymes [48]. This antimicrobial activity of polyphenols is concentration-dependent: for instance, at low concentration polyphenols interfere with specific sites, thus inhibiting the enzymes, whereas at high concentrations they cause enzyme denaturation [49]. In addition, polyphenols such as flavonoids affect the bacterial membrane permeability by interacting with membrane proteins, enzymes, and lipids, thus mediating loss of macromolecules, protons and ions [50].

Polyphenols in Oral cancer

Oral cancer is one of the most important medical issues and necessitates the development of effective strategies to reduce its incidence and mortality rates. Polyphenols are well known for their anti-cancerous activities against various types of cancers, based on their capability to inhibit enzymes and tumour development [51]. They are also known for their roles in preventing oxidative stress and DNA damage, modulating carcinogens metabolism and inhibiting DNA adduct formation [52-54]. For instance, catechins extracted from tea induce apoptosis, arrest cell growth, inhibit metalloproteinase synthesis, reduce metastasis risk by inhibiting the invasion and proliferation in both oral lekoplakia cell lines and in oral cancer [54, 55].
Two different case control studies, conducted in Uruguay and Italy, have reported an inverse relationship between flavonoids consumption and risk of oral cancer [56]. The Italian study demonstrated a significant inverse relationship of flavanones, flavonols, and total flavonoids, with risk of oral cancers, while this is not the case with isoflavones, anthocyanidins, flavan-3-ols, and flavones [57]. This indicates that different types of polyphenols have different effects on the risk and development of oral cancers. For instance, some polyphenols are particularly useful in human papillomavirus-mediated oral cancers because they considerably reduce the development of HPV-induced cancers by inhibiting DNA adduct formation and reducing cell proliferation, as well as preventing the invasion of affected cells into unaffected cells, exhibiting cytotoxic activity, apoptosis induction, and cell differentiation [58].

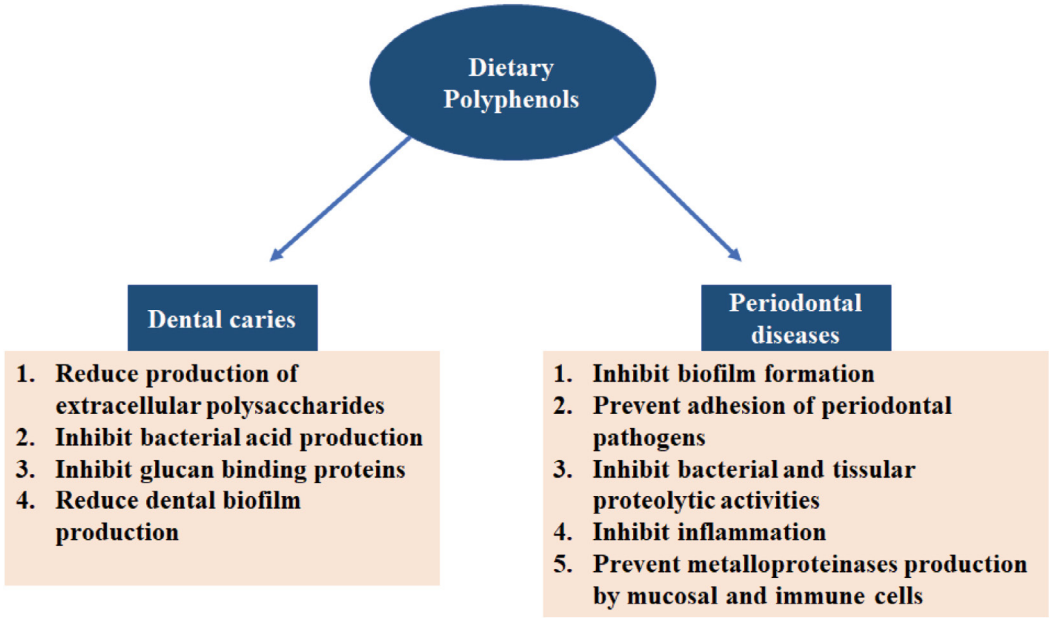
Polyphenols in Dental Caries and Periodontal Diseases

Containing over 700 bacterial species, the oral cavity is one of the most complex microbial ecosystems of the human body [59]. Integrated in an extracellular matrix of polysaccharides, the dental biofilm formed on hard and soft tissues of the oral cavity (containing food debris, epithelial cells, proteins, enzymes, and microbial cells) is the source of dental caries and periodontal diseases – the two main problems of oral cavity of bacterial origin [60]. For instance, the cariogenic *Streptococcus mutans* and *Streptococcus sobrinus* ferment sugars contained in food particles stuck to the teeth or other parts of the oral cavity, resulting in the production of organic acids that reduce the pH to 5.5, demineralising tooth enamel and thus causing dental caries. Dental caries is the most common infectious, multifactorial disease that results in the dissolution of tooth enamel [61]. Therefore, fruits and vegetables (and their extracts) that are rich in polyphenols not only reduce the number of harmful pathogens in the oral cavity, but also maintain oral hygiene (Tab. II).
Periodontal diseases are the diseases of the tissue that supports and surrounds the teeth; they can manifest episodically. Periodontal diseases can be further classified into gingivitis and periodontitis, which result in the inflammation of the free gingiva and the progressive destruction of all of the tooth-supporting tissue, including periodontal ligament and alveolar bone, respectively. These diseases follow a pattern of active destruction, latency and healing periods, and are dependent on accumulation of gram-negative anaerobes in the subgingival region and immune-destructive response of the host.
Dietary polyphenols play an important role in preventing the disequilibrium between oxidative stress and antioxidant activities in the oral cavity, thereby preventing periodontal tissue destruction. This implicates the effectiveness of these dietary phytochemicals in fighting periodontal diseases, especially when the oral cavity is exposed to oxidative stress from environment and food sources. For instance, tea polyphenols enhance the antioxidant capacity of saliva if the (green or black) tea is held in the mouth for 2-3 minutes. Localised application

Tab. II. Beneficial effects of polyphenols in oral cavity pathogens.

Polyphenols/food sources	Beneficial effects	References
Tea Polyphenols	Inhibition of glucosyltransferase (GTF), acid production, adherence to hard surfaces	[62]
Polymeric polyphenols	Inhibition of *Streptococcus mutans* polysaccharide synthesis	[63]
Extracts of unfermented cocoa, epicatechin, red grape seed, and green tea	Bacteriostatic against *S. mutans*, inhibit acid production, and reduce adherence of the bacterium against glass	[64]
Tannins from grapes	Salivary alpha-amylase inhibition in humans	[65]
Cocoa flavonols	Enhance interleukin 5 secretion by peripheral blood mononuclear cells and trigger IgA production against *S. mutans*	[66]
Oolong tea extract and its polyphenols	Significant reductions in caries and plaque development in rats infected with *S. mutans*	[67]
Barley coffee, coffee, wine, and tea	High consumption accompanies lower *Lactobacilus sp.* and *S. mutans* in plaque and saliva, thus lowering dental plaque scores	[68]
Oolong tea extract	Cariostatic activity against *S. sorbinus* in the oral cavity	[69]
Hydroxytyrosol from olives and olive tree	Reduces the viral load of the oral and nasal cavity mucosa during SARS-CoV-2 infections	[70-73]

Fig. 2. Effect of dietary polyphenols on dental caries and periodontal diseases.



of fruit extracts prevents biofilm formation and reduces pocket depth. Furthermore, increased phagocytic activity of polymorphonuclear leucocytes in the gingival crevicular fluid was observed upon daily intake of two fresh grapefruits for 2 weeks. The effects of dietary polyphenols on oral cavity diseases are presented in Figure 2. Perhaps the most widely studied polyphenols in connection to periodontal conditions are those from various kinds of tea: they not only enhance the proliferation of human periodontal ligament fibroblasts, but also inhibit virulence manifestation of the periodontic anaerobic pathogen *Porphyromonas gingivalis*. Therefore, regular consumption of polyphenol-rich diets presents an effective method of fighting periodontal diseases.

Lactobacillus reuteri in oral health

Lactobacillus reuteri (*L. reuteri*) is one of the most widely studied bacteria, having a huge repertoire of beneficial effects on human health. It colonizes a variety of niches in human body such as the oral cavity, the gastrointestinal tract, the skin and the urinary tract [74]. Owing to its antimicrobial activities (production of antimicrobial organic acids, ethanol, and reuterin), *L. reuteri* can inhibit the colonization of pathogenic microbes in its vicinity and even reshape the microbial communities in the host [75]. Second, *L. reuteri* can have beneficial impact on the immune system of the host. For instance, it has strong immunomodulatory and anti-inflammatory properties, as manifested by the synthesis of pro-inflam-

matory cytokines and promotion of regulatory T cell growth and function and strengthening of the intestinal barrier, which prevents transfers of gut microbiota from the lumen to tissues [76]. As periodontal diseases are caused by bacteria inhabiting these niches, one of the basic methods of prevention and cure is to decrease the pathogen load by scaling and root planning (SRP), involving the probiotic bacteria. Alternatively, deploying probiotic bacterial strains as a method of biological control of pathogenic ones holds promise in treating the periodontal diseases. Many studies have shown the efficacy of the probiotic bacterium *Lactobacillus reuteri Prodentis* (LrP) as a useful therapeutic supplement, as part of periodontal maintenance regime post-intervention. This not only replenishes the oral cavity with useful bacteria, but also decreases the harmful ones. For instance, daily oral administration of *L. reuteri* strains – DSM 17938 and PTA 5289 – in human subjects for 12 weeks resulted in changes in oral microbiota in a randomized controlled trial, while keeping the bacterial species richness unaltered throughout the duration of the trial [77]. In addition, oral *L. reuteri* treatment suppressed the growth of periodontal pathogens in the subgingival microbiota [78]. Studies have also shown that *Lactobacillus reuteri Prodentis* can sustainably increase the population of beneficial bacteria in the oral cavity, thus restoring the natural oral flora lost during infection [79].

Several in vitro studies showed *L. reuteri*'s inhibitory effects on periodontopathogens, which are likely due to its by-products: an example is reuterin, a non-protein broad-spectrum antibiotic that can inhibit the growth of a variety of gram-positive/negative bacteria, yeast, and fungi [80]. Many periodontopathogens, such as *P. gingivalis* ATCC 33277, *P. intermedia* ATCC 25611, and *F. nucleatum* ATCC 25586, are effectively inhibited by *L. reuteri* ATCC PTA 5289, except for *A. actinomycetemcomitans* ATCC 33384 [81].

Both live *L. reuteri* PTA 5289 and DSM 17938 and their CFS show inhibition on *P. gingivalis* ATCC 33277 and *F. nucleatum* ATCC 25586; however, only the live form of the two *L. reuteri* inhibited the growth of *A. actinomycetemcomitans* ATCC 29522 in vitro [82, 83]. Another subspecies, *L. reuteri* ATCC 55730, inhibited the growth of *F. nucleatum* ATCC 10953, *P. gingivalis* ATCC 33277, and *A. actinomycetemcomitans* ATCC 33384, also preventing the mortality of HOK cells infected with periodontal pathogens [84].

Exopolysaccharide (EPS) is a substance that *L. reuteri* DSM 17938 produces to enhance in adherence to epithelial cells and to compete with pathogenic bacteria for adhesion sites [85]. The release of IL-6 triggered by *F. nucleatum* in KB cells was shown to be suppressed by *L. reuteri* KCTC 3594. *L. reuteri* was used in clinical trials to inhibit *P. gingivalis*, supragingival plaque, subgingival plaque, and *P. intermedia* in saliva [86, 87]. In patients with peri-implant mucositis, *L. reuteri* DSM 17938 and PTA 5289 for peri-implant diseases could only reduce the load of *P. gingivalis* [88]. In animal investigations, it was discovered that live *L. reuteri* DSM 17938 and PTA 5289 improved immune reactions and increased hemocyte density in *Galleria mellonella* infected with *P. gingivalis* ATCC 33277 [81, 82]. The immunomodulatory properties of *L. reuteri* may help to manage the imbalance between MMP and TIMP or to inhibit the effectiveness of pro-inflammatory cytokines, which may minimize the inflammation and degeneration of periodontal tissues [89]. This implicates that *L. reuteri* has a repertoire of beneficial traits that can be deployed to successfully manage periodontal pathogens and subgingival microbiota.

Conclusion

The benefits of polyphenols and probiotics in management and cure implicate that these molecules should be given due consideration in terms of clinical evaluation. Although there are numerous in vitro and in vivo studies demonstrating the potential benefits of polyphenols in oral health, strong evidence from well-designed clinical trials is still lacking. On the other hand, *L. reuteri* is already well known for its probiotic effects and bio-antagonism against oral pathogens. It would be interesting to see how these two can act synergistically in combating oral diseases and maintaining oral health.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; ZN, MCM, Kristjana D, Kevin D, STC, FB, PG: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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Review

Ethical considerations regarding animal experimentation

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Keywords

Animal experimentation • Animal model • Bioethics • 4Rs principle • Animal welfare

Summary

Animal experimentation is widely used around the world for the identification of the root causes of various diseases in humans and animals and for exploring treatment options. Among the several animal species, rats, mice and purpose-bred birds comprise almost 90% of the animals that are used for research purpose. However, growing awareness of the sentience of animals and their experience of pain and suffering has led to strong opposition to animal research among many scientists and the general public. In addition, the usefulness of extrapolating animal data to humans has been questioned. This has led to Ethical Committees' adoption of the 'four Rs' principles (Reduction, Refinement, Replacement and Responsibility) as a guide when making decisions regarding animal experimentation. Some of the essential considerations for humane animal experimentation are presented in this review along with the requirement for investigator training. Due to the ethical issues surrounding the use of animals in experimentation, their use is declining in those research areas where alternative in vitro or in silico methods are available. However, so far it has not been possible to dispense with experimental animals completely and further research is needed to provide a road map to robust alternatives before their use can be fully discontinued.

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Introduction

Animal model-based research has been performed for a very long time. Ever since the 5th century B.C., reports of experiments involving animals have been documented, but an increase in the frequency of their utilization has been observed since the 19th century [1]. Most institutions for medical research around the world use non-human animals as experimental subjects [2]. Such animals might be used for research experimentations to gain a better understanding of human diseases or for exploring potential treatment options [2]. Even those animals that are evolutionarily quite distant from humans, such as *Drosophila melanogaster*, Zebrafish (*Danio rerio*) and *Caenorhabditis elegans*, share physiological and genetic similarities with human beings [2]; therefore animal experimentation can be of great help for the advancement of medical science [2].

For animal experimentation, the major assumption is that the animal research will be of benefit to humans. There are many reasons that highlight the significance of animal use in biomedical research. One of the major reasons is that animals and humans share the same biological processes. In addition, vertebrates have many anatomical similarities (all vertebrates have lungs, a heart, kidneys, liver and other organs) [3]. Therefore, these similarities make certain animals more suitable for experiments and for providing basic training to young researchers and students in different fields of biological and biomedical sciences [3]. Certain animals are susceptible to various health problems that are similar to human diseases such as diabetes, cancer and heart disease [4]. Furthermore, there are genetically modified animals that are used to obtain pathological phenotypes [5]. A significant benefit of animal experimentation is that test species can be chosen that have a much shorter life cycle than humans. Therefore, animal models can be studied throughout their life span and for several successive generations, an essential element for the understanding of disease progression along with its interaction with the whole organism throughout its lifetime [6].

Animal models often play a critical role in helping researchers who are exploring the efficacy and safety of potential medical treatments and drugs. They help to identify any dangerous or undesired side effects, such as birth defects, infertility, toxicity, liver damage or any potential carcinogenic effects [7]. Currently, U.S. Federal law, for example, requires that non-human animal research is used to demonstrate the efficacy and safety of any new treatment options before proceeding to trials on humans [8]. Of course, it is not only humans benefit from this research and testing, since many of the drugs and treatments that are developed for humans are routinely used in veterinary clinics, which help animals live longer and healthier lives [4].

COVID-19 and the need for animal models

When COVID-19 struck, there was a desperate need for research on the disease, its effects on the brain and body and on the development of new treatments for patients with the disease. Early in the disease it was noticed that those with the disease suffered a loss of smell and taste, as well as neurological and psychiatric symptoms, some of which lasted long after the patients had "survived" the disease [9-15]. As soon as the pandemic started, there was a search for appropriate animal models in which to study this unknown disease [16, 17]. While genetically modified mice and rats are the basic animal models for neurological and immunological research [18, 19] the need to understand COVID-19 led to a range of animal models; from fruit flies [20] and Zebrafish [21] to large mammals [22, 23] and primates [24, 25]. And it was just not one animal model that was needed, but many, because different aspects of the disease are best studied in different animal models [16, 25, 26]. There is also a need to study the transmission pathways of the zoonosis: where does it come from, what are the animal hosts and how is it transferred to humans [27]?

There has been a need for animal models for understanding the pathophysiology of COVID-19 [28], for studying the mechanisms of transmission of the disease [16], for studying its neurobiology [29,30] and for developing new vaccines [31]. The sudden onset of the COVID-19 pandemic has highlighted the fact that animal research is necessary, and that the curtailment of such research has serious consequences for the health of both humans and animals, both wild and domestic [32] As highlighted by Adhikary et al. [22] and Genzel et al. [33] the coronavirus has made clear the necessity for animal research and the danger in surviving future such pandemics if animal research is not fully supported. Genzel et al. [33], in particular, take issue with the proposal for a European ban on animal testing. Finally, there is a danger in bypassing animal research in developing new vaccines for diseases such as COVID-19 [34]. The purpose of this paper is to show that, while animal research is necessary for the health of both humans and animals, there is a need to carry out such experimentation in a controlled and humane manner. The use of alternatives to animal research such as cultured human cells and computer modeling may be a useful adjunct to animal studies but will require that such methods are more readily accessible to researchers and are not a replacement for animal experimentation.

Pros and cons of animal experimentation

Arguments against animal experimentation

A fundamental question surrounding this debate is to ask whether it is appropriate to use animals for medical research. Is our acceptance that animals have a morally lower value or standard of life just a case of speciesism [35]? Nowadays, most people agree that animals have a moral status and that needlessly hurting or abusing pets or other animals is unacceptable. This represents something of a change from the historical point of view where animals did not have any moral status and the treatment of animals was mostly subservient to maintaining the health and dignity of humans [36].

Animal rights advocates strongly argue that the moral status of non-human animals is similar to that of humans, and that animals are entitled to equality of treatment. In this view, animals should be treated with the same level of respect as humans, and no one should have the right to force them into any service or to kill them or use them for their own goals. One aspect of this argument claims that moral status depends upon the capacity to suffer or enjoy life [37].

In terms of suffering and the capacity of enjoying life, many animals are not very different from human beings, as they can feel pain and experience pleasure [38]. Hence, they should be given the same moral status as humans and deserve equivalent treatment. Supporters of this argument point out that according animals a lower moral status than humans is a type of prejudice known as "speciesism" [38]. Among humans, it is widely accepted that being a part of a specific race or of a specific gender does not provide the right to ascribe a lower moral status to the outsiders. Many advocates of animal rights deploy the same argument, that being human does not give us sufficient grounds declare animals as being morally less significant [36].

Arguments in favor of animal experimentation

Those who support animal experimentation have frequently made the argument that animals cannot be elevated to be seen as morally equal to humans [39]. Their main argument is that the use of the terms "moral status" or "morality" is debatable. They emphasize that we must not make the error of defining a quality or capacity associated with an animal by using the same adjectives used for humans [39]. Since, for the most part, animals do not possess humans' cognitive capabilities and lack full autonomy (animals do not appear to rationally pursue specific goals in life), it is argued that therefore, they cannot be included in the moral community [39]. It follows from this line of argument that, if animals do not possess the same rights as human beings, their use in research experimentation can be considered appropriate [40]. The European and the American legislation support this kind of approach as much as their welfare is respected.

Another aspect of this argument is that the benefits to human beings of animal experimentation compensate for the harm caused to animals by these experiments.

In other words, animal harm is morally insignificant compared to the potential benefits to humans. Essentially, supporters of animal experimentation claim that human beings have a higher moral status than animals and that animals lack certain fundamental rights accorded to humans. The potential violations of animal rights during animal research are, in this way, justified by the greater benefits to mankind [40, 41]. A way to evaluate when the experiments are morally justified was published in 1986 by Bateson, which developed the Bateson's Cube [42]. The Cube has three axes: suffering, certainty of benefit and quality of research. If the research is high-quality, beneficial, and not inflicting suffering, it will be acceptable. At the contrary, painful, low-quality research with lower likelihood of success will not be acceptable [42, 43].

Impact of experimentations on animals

Ability to feel pain and distress

Like humans, animal have certain physical as well as psychological characteristics that make their use for experimentation controversial [44].

In the last few decades, many studies have increased knowledge of animal awareness and sentience: they indicate that animals have greater potential to experience damage than previously appreciated and that current rights and protections need to be reconsidered [45]. In recent times, scientists as well as ethicists have broadly acknowledged that animals can also experience distress and pain [46]. Potential sources of such harm arising from their use in research include disease, basic physiological needs deprivation and invasive procedures [46]. Moreover, social deprivation and lack of the ability to carry out their natural behaviors are other causes of animal harm [46]. Several studies have shown that, even in response to very gentle handling and management, animals can show marked alterations in their physiological and hormonal stress markers [47].

In spite of the fact that suffering and pain are personalized experiences, several multi-disciplinary studies have provided clear evidence of animals experiencing pain and distress. In particular, some animal species have the ability to express pain similarly to human due to common psychological, neuroanatomical and genetic characteristics [48]. Similarly, animals share a resemblance to humans in their developmental, genetic and environmental risk factors for psychopathology. For instance, in many species, it has been shown that fear operates within a less organized subcortical neural circuit than pain [49, 50]. Various types of depression and anxiety disorders like posttraumatic stress disorder have also been reported in mammals [51].

Psychological capabilities of animals

Some researchers have suggested that besides their ability to experience physical and psychological pain and distress, some animals also exhibit empathy, self-awareness and language-like capabilities. They also demonstrate tools-linked cognizance, pleasure-seeking and advanced problem-solving skills [52]. Moreover, mammals and birds exhibit playful behavior, an indicator of the capacity to experience pleasure. Other taxa such as reptiles, cephalopods and fishes have also been observed to display playful behavior, therefore the current legislation prescribes the use of environmental enrichers [53]. The presence of self-awareness ability, as assessed by mirror self-recognition, has been reported in magpies, chimpanzees and other apes, and certain cetaceans [54]. Recently, another study has revealed that crows have the ability to create and use tools that involve episodic-like memory formation and its retrieval. From these findings, it may be suggested that crows as well as related spe-

cies show evidence of flexible learning strategies, causal reasoning, prospection and imagination that are similar to behavior observed in great apes [55]. In the context of resolving the ethical dilemmas about animal experimentation, these observations serve to highlight the challenges involved [56, 57].

Ethics, principles and legislation in animal experimentation

Ethics in animal experimentation

Legislation around animal research is based on the idea of the moral acceptability of the proposed experiments under specific conditions [58]. The significance of research ethics that ensures proper treatment of experimental animals [58]. To avoid undue suffering of animals, it is important to follow ethical considerations during animal studies [1]. It is important to provide best human care to these animals from the ethical and scientific point of view [1]. Poor animal care can lead to experimental outcomes [1]. Thus, if experimental animals mistreated, the scientific knowledge and conclusions obtained from experiments may be compromised and may be difficult to replicate, a hallmark of scientific research [1]. At present, most ethical guidelines work on the assumption that animal experimentation is justified because of the significant potential benefits to human beings. These guidelines are often permissive of animal experimentation regardless of the damage to the animal as long as human benefits are achieved [59].

Principle of the 4 Rs

Although animal experimentation has resulted in many discoveries and helped in the understanding numerous aspects of biological science, its use in various sectors is strictly controlled. In practice, the proposed set of animal experiments is usually considered by a multidisciplinary Ethics Committee before work can commence [60]. This committee will review the research protocol and make a judgment as to its sustainability. National and international laws govern the utilization of animal experimentation during research and these laws are mostly based on the universal doctrine presented by Russell and Burch (1959) known as principle of the 3 Rs. The 3Rs referred to are Reduction, Refinement and Replacement, and are applied to protocols surrounding the use of animals in research. Some researchers have proposed another "R", of responsibility for the experimental animal as well as for the social and scientific status of the animal experiments [61]. Thus, animal ethics committees commonly review research projects with reference to the 4 Rs principles [62].

The first "R", Reduction means that the experimental design is examined to ensure that researchers have reduced the number of experimental animals in a research project to the minimum required for reliable data [59]. Methods used for this purpose include improved experimental design, extensive literature search to avoid duplication of experiments [35], use of advanced imaging techniques, sharing resources and data, and appropriate statistical data analysis that reduce the number of animals needed for statistically significant results [2, 63].

The second "R", Refinement involves improvements in procedure that minimize the harmful effects of the proposed experiments on the animals involved, such as reducing pain, distress and suffering in a manner that leads to a general improvement in animal welfare. This might include for example improved living conditions for research animals, proper training of people handling animals, application of anesthesia and analgesia when required and the need for euthanasia of the animals at the end of the experiment to curtail their suffering [63].

The third "R", Replacement refers to approaches that replace or avoid the use of experimental animals altogether. These approaches involve use of *in silico* methods/computerized techniques/software and *in vitro* methods like cell and tissue culture testing, as well as relative replacement methods by use of invertebrates like nematode worms, fruit flies and microorganisms in place of vertebrates and higher animals [1]. Examples of proper application of these first "3R2 principles are the use of alternative sources of blood, the exploitation of commercially used animals for scientific research, a proper training without use of animals and the use of specimen from previous experiments for further researches [64-67].

The fourth "R", Responsibility refers to concerns around promoting animal welfare by improvements in experimental animals' social life, development of advanced scientific methods for objectively determining sentience, consciousness, experience of pain and intelligence in the animal kingdom, as well as effective involvement in the professionalization of the public discussion on animal ethics [68].

Other aspects of animal research ethics

Other research ethics considerations include having a clear rationale and reasoning for the use of animals in a research project. Researchers must have reasonable expectation of generating useful data from the proposed experiment. Moreover, the research study should be designed in such a way that it should involve the lowest possible sample size of experimental animals while producing statistically significant results [35].

All individual researchers that handle experimental animals should be properly trained for handling the particular species involved in the research study. The animal's pain, suffering and discomfort should be minimized [69]. Animals should be given proper anesthesia when required and surgical procedures should not be repeated on same animal whenever possible [69]. The procedure of humane handling and care of experimental animals should be explicitly detailed in the research study protocol. Moreover, whenever required, aseptic techniques should be properly followed [70]. During the research, anesthetization and surgical procedures on experimental animals should only be performed by professionally skilled individuals [69].

The Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines that are issued by the National Center for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) are designed to improve the documentation surrounding research involving experimental animals [70]. The checklist provided includes the information required in the various sections of the manuscript i.e. study design, ethical statements, experimental procedures, experimental animals and their housing and husbandry, and more [70].
It is critical to follow the highest ethical standards while performing animal experiments. Indeed, most of the journals refuse to publish any research data that lack proper ethical considerations [35].

Investigators' ethics

Since animals have sensitivity level similar to the human beings in terms of pain, anguish, survival instinct and memory, it is the responsibility of the investigator to closely monitor the animals that are used and identify any sign of distress [71]. No justification can rationalize the absence of anesthesia or analgesia in animals that undergo invasive surgery during the research [72]. Investigators are also responsible for giving high-quality care to the experimental animals, including the supply of a nutritious diet, easy water access, prevention of and relief from any pain, disease and injury, and appropriate housing facilities for the animal species [73]. A research experiment is not permitted if the damage caused to the animal exceeds the value of knowledge gained by that experiment. No scientific advancement based on the destruction and sufferings of another living being could be justified. Besides ensuring the welfare of animals involved, investigators must also follow the applicable legislation [74, 75].
To promote the comfort of experimental animals in England, an animal protection society named: 'The Society for the Preservation of Cruelty to Animals' (now the Royal Society for the Prevention of Cruelty to Animals) was established (1824) that aims to prevent cruelty to animal [76].

Animal welfare laws

Legislation for animal protection during research has long been established. In 1876 the British Parliament sanctioned the 'Cruelty to Animals Act' for animal protection. Russell and Burch (1959) presented the '3 Rs' principles: Replacement, Reduction and Refinement, for use of animals during research [61]. Almost seven years later, the U.S.A also adopted regulations for the protection of experimental animals by enacting the Laboratory Animal Welfare Act of 1966 [60]. In Brazil, the Arouca Law (Law No. 11,794/08) regulates the animal use in scientific research experiments [76].
These laws define the breeding conditions, and regulate the use of animals for scientific research and teaching purposes. Such legal provisions control the use of anesthesia, analgesia or sedation in experiments that could cause distress or pain to experimental animals [59, 76]. These laws also stress the need for euthanasia when an experiment is finished, or even during the experiment if there is any intense suffering for the experimental animal [76].
Several national and international organizations have been established to develop alternative techniques so that animal experimentation can be avoided, such as the UK-based National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) (www.caat.jhsph.edu), the European Centre for the Validation of Alternative Methods (ECVAM) [77], the Universities Federation for Animal Welfare (UFAW) (www.ufaw.org.uk), The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) [78], and The Center for Alternatives to Animal Testing (CAAT) (www.caat.jhsph.edu). The Brazilian 'Arouca Law' also constitutes a milestone, as it has created the 'National Council for the Control of Animal Experimentation' (CONCEA) that deals with the legal and ethical issues related to the use of experimental animals during scientific research [76].
Although national as well as international laws and guidelines have provided basic protections for experimental animals, the current regulations have some significant discrepancies. In the U.S., the Animal Welfare Act excludes rats, mice and purpose-bred birds, even though these species comprise almost 90% of the animals that are used for research purpose [79]. On the other hand, certain cats and dogs are getting special attention along with extra protection. While the U.S. Animal Welfare Act ignores birds, mice and rats, the U.S. guidelines that control research performed using federal funding ensure protections for all vertebrates [79, 80].

Living conditions of animals

Choice of the animal model

Based on all the above laws and regulations and in line with the deliberations of ethical committees, every researcher must follow certain rules when dealing with animal models.
Before starting any experimental work, thorough research should be carried out during the study design phase so that the unnecessary use of experimental animals is avoided. Nevertheless, certain research studies may have compelling reasons for the use of animal models, such as the investigation of human diseases and toxicity tests. Moreover, animals are also widely used in the training of health professionals as well as in training doctors in surgical skills [1, 81].
Researcher should be well aware of the specific traits of the animal species they intend to use in the experiment, such as its developmental stages, physiology, nutritional needs, reproductive characteristics and specific behaviors. Animal models should be selected on the basis of the study design and the biological relevance of the animal [1].
Typically, in early research, non-mammalian models are used to get rapid insights into research problems such as the identification of gene function or the recognition

of novel therapeutic options. Thus, in biomedical and biological research, among the most commonly used model organisms are the Zebrafish, the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*. The main advantage of these non-mammalian animal models is their prolific reproducibility along with their much shorter generation time. They can be easily grown in any laboratory setting, are less expensive than the murine animal models and are somewhat more powerful than the tissue and cell culture approaches [82].

Caenorhabditis elegans is a small-sized nematode with a short life cycle and that exists in large populations and is relatively inexpensive to cultivate. Scientists have gathered extensive knowledge of the genomics and genetics of *Caenorhabditis elegans*; but *Caenorhabditis elegans* models, while very useful in some respects, are unable to represent all signaling pathways found in humans. Furthermore, due to its short life cycle, scientists are unable to investigate long term effects of test compounds or to analyze primary versus secondary effects [6].

Similarly, the fruit fly *Drosophila melanogaster* has played a key role in numerous biomedical discoveries. It is small in size, has a short life cycle and large population size, is relatively inexpensive to breed, and extensive genomics and genetics information is available [6]. However, its respiratory, cardiovascular and nervous systems differ considerably from human beings. In addition, its immune system is less developed when compared to vertebrates, which is why effectiveness of a drug in *Drosophila melanogaster* may not be easily extrapolated to humans [83].

The Zebrafish (*Danio rerio*) is a small freshwater teleost, with transparent embryos, providing easy access for the observation of organogenesis and its manipulation. Therefore, Zebrafish embryos are considered good animal models for different human diseases like tuberculosis and fetal alcohol syndrome and are useful as neurodevelopmental research models. However, Zebrafish has very few mutant strains available, and its genome has numerous duplicate genes making it impossible to create knockout strains, since disrupting one copy of the gene will not disrupt the second copy of that gene. This feature limits the use of Zebrafish as animal models to study human diseases. Additionally they are rather expensive, have long life cycle, and genomics and genetics studies are still in progress [82, 84].

Thus, experimentation on these three animals might not be equivalent to experimentation on mammals. Mammalian animal model are most similar to human beings, so targeted gene replacement is possible. Traditionally, mammals like monkey and mice have been the preferred animal models for biomedical research because of their evolutionary closeness to humans. Rodents, particularly mice and rats, are the most frequently used animal models for scientific research. Rats are the most suitable animal model for the study of obesity, shock, peritonitis, sepsis, cancer, intestinal operations, spleen, gastric ulcers, mononuclear phagocytic system, organ transplantations and wound healing. Mice are more suitable for studying burns, megacolon, shock, cancer, obesity, and sepsis as mentioned previously [85].

Similarly, pigs are mostly used for stomach, liver and transplantation studies, while rabbits are suitable for the study of immunology, inflammation, vascular biology, shock, colitis and transplantations. Thus, the choice of experimental animal mainly depends upon the field of scientific research under consideration [1].

Housing and environmental enrichment

Researchers should be aware of the environment and conditions in which laboratory animals are kept during research, and they also need to be familiar with the metabolism of the animals kept in vivarium, since their metabolism can easily be altered by different factors such as pain, stress, confinement, lack of sunlight, etc. Housing conditions alter animal behavior, and this can in turn affect experimental results. By contrast, handling procedures that feature environmental enrichment and enhancement help to decrease stress and positively affect the welfare of the animals and the reliability of research data [74, 75].

In animals, distress- and agony-causing factors should be controlled or eliminated to overcome any interference with data collection as well as with interpretation of the results, since impaired animal welfare leads to more animal usage during experiment, decreased reliability and increased discrepancies in results along with the unnecessary consumption of animal lives [86].

To reduce the variation or discrepancies in experimental data caused by various environmental factors, experimental animals must be kept in an appropriate and safe place. In addition, it is necessary to keep all variables like humidity, airflow and temperature at levels suitable for those species, as any abrupt variation in these factors could cause stress, reduced resistance and increased susceptibility to infections [74].

The space allotted to experimental animals should permit them free movement, proper sleep and where feasible allow for interaction with other animals of the same species. Mice and rats are quite sociable animals and must, therefore, be housed in groups for the expression of their normal behavior. Usually, laboratory cages are not appropriate for the behavioral needs of the animals. Therefore, environmental enrichment is an important feature for the expression of their natural behavior that will subsequently affect their defense mechanisms and physiology [87].

The features of environmental enrichment must satisfy the animals' sense of curiosity, offer them fun activities, and also permit them to fulfill their behavioral and physiological needs. These needs include exploring, hiding, building nests and gnawing. For this purpose, different things can be used in their environment, such as PVC tubes, cardboard, igloos, paper towel, cotton, disposable masks and paper strips [87].

The environment used for housing of animals must be continuously controlled by appropriate disinfection, hygiene protocols, sterilization and sanitation processes. These steps lead to a reduction in the occurrence of

various infectious agents that often found in vivarium, such as Sendai virus, cestoda and Mycoplasma pulmonis [88].

Euthanasia

Euthanasia is a term derived from Greek, and it means a death without any suffering. According to the Brazilian Arouca Law (Article 14, Chapter IV, Paragraphs 1 and 2), an animal should undergo euthanasia, in strict compliance with the requirements of each species, when the experiment ends or during any phase of the experiment, wherever this procedure is recommended and/or whenever serious suffering occurs. If the animal does not undergo euthanasia after the intervention it may leave the vivarium and be assigned to suitable people or to the animal protection bodies, duly legalized [1].

Euthanasia procedures must result in instant loss of consciousness which leads to respiratory or cardiac arrest as well as to complete brain function impairment. Another important aspect of this procedure is calm handling of the animal while taking it out of its enclosure, to reduce its distress, suffering, anxiety and fear. In every research project, the study design should include the details of the appropriate endpoints of these experimental animals, and also the methods that will be adopted. It is important to determine the appropriate method of euthanasia for the animal being used. Another important point is that, after completing the euthanasia procedure, the animal's death should be absolutely confirmed before discarding their bodies [87, 89].

Relevance of animal experimentations and possible alternatives

Relevance of animal experiments and their adverse effects on human health

One important concern is whether human diseases, when inflicted on experimental animals, adequately mimic the progressions of the disease and the treatment responses observed in humans. Several research articles have made comparisons between human and animal data, and indicated that the results of animals' research could not always be reliably replicated in clinical research among humans. The latest systematic reviews about the treatment of different clinical conditions including neurology, vascular diseases and others, have established that the results of animal studies cannot properly predict human outcomes [59, 90].

At present, the reliability of animal experiments for extrapolation to human health is questionable. Harmful effects may occur in humans because of misleading results from research conducted on animals. For instance, during the late fifties, a sedative drug, thalidomide, was prescribed for pregnant women, but some of the women using that drug gave birth to babies lacking limbs or with foreshortened limbs, a condition called phocomelia. When thalidomide had been tested on almost all animal models such as rats, mice, rabbits, dogs, cats, hamsters, armadillos, ferrets, swine, guinea pig, etc., this teratogenic effect was observed only occasionally [91]. Similarly, in 2006, the compound TGN 1412 was designed as an immunomodulatory drug, but when it was injected into six human volunteer, serious adverse reactions were observed resulting from a deadly cytokine storm that in turn led to disastrous systemic organ failure. TGN 1412 had been tested successfully in rats, mice, rabbits, and non-human primates [92]. Moreover, Bailey (2008) reported 90 HIV vaccines that had successful trial results in animals but which failed in human beings [93]. Moreover, in Parkinson disease, many therapeutic options that have shown promising results in rats and non-human primate models have proved harmful in humans. Hence, to analyze the relevance of animal research to human health, the efficacy of animal experimentation should be examined systematically [94, 95]. At the same time, the development of hyperoxaluria and renal failure (up to dialysis) after ileal-jejunal bypass was unexpected because this procedure was not preliminarily evaluated on an animal model [96].

Several factors play a role in the extrapolation of animal-derived data to humans, such as environmental conditions and physiological parameters related to stress, age of the experimental animals, etc. These factors could switch on or off genes in the animal models that are specific to species and/or strains. All these observations challenge the reliability and suitability of animal experimentation as well as its objectives with respect to human health [76, 92].

Alternative to animal experimentation/ development of new products and techniques to avoid animal sacrifice in research

Certainly, *in vivo* animal experimentation has significantly contributed to the development of biological and biomedical research. However it has the limitations of strict ethical issues and high production cost. Some scientists consider animal testing an ineffective and immoral practice and therefore prefer alternative techniques to be used instead of animal experimentation. These alternative methods involve *in vitro* experiments and ex vivo models like cell and tissue cultures, use of plants and vegetables, non-invasive human clinical studies, use of corpses for studies, use of microorganisms or other simpler organism like shrimps and water flea larvae, physicochemical techniques, educational software, computer simulations, mathematical models and nanotechnology [97]. These methods and techniques are cost-effective and could efficiently replace animal models. They could therefore, contribute to animal welfare and to the development of new therapies that can identify the therapeutics and related complications at an early stage [1].

The National Research Council (UK) suggested a shift from the animal models toward computational models, as well as high-content and high-throughput *in vitro* methods. Their reports highlighted that these alternative methods could produce predictive data more affordably, accurately and quickly than the traditional *in vivo* or experimental animal methods [98].

Increasingly, scientists and the review boards have to assess whether addressing a research question using the applied techniques of advanced genetics, molecular, computational and cell biology, and biochemistry could be used to replace animal experiments [59]. It must be remembered that each alternative method must be first validated and then registered in dedicated databases.
An additional relevant concern is how precisely animal data can mirror relevant epigenetic changes and human genetic variability. Langley and his colleagues have highlighted some of the examples of existing and some emerging non-animal based research methods in the advanced fields of neurology, orthodontics, infectious diseases, immunology, endocrine, pulmonology, obstetrics, metabolism and cardiology [99].

In silico simulations and informatics

Several computer models have been built to study cardiovascular risk and atherosclerotic plaque build-up, to model human metabolism, to evaluate drug toxicity and to address other questions that were previously approached by testing in animals [100].
Computer simulations can potentially decrease the number of experiments required for a research project, however simulations cannot completely replace laboratory experiments. Unfortunately, not all the principles regulating biological systems are known, and computer simulation provide only an estimation of possible effects due to the limitations of computer models in comparison with complex human tissues. However, simulation and bio-informatics are now considered essential in all fields of science for their efficiency in using the existing knowledge for further experimental designs [76].
At present, biological macromolecules are regularly simulated at various levels of detail, to predict their response and behavior under certain physical conditions, chemical exposures and stimulations. Computational and bioinformatic simulations have significantly reduced the number of animals sacrificed during drug discovery by short listing potential candidate molecules for a drug. Likewise, computer simulations have decreased the number of animal experiments required in other areas of biological science by efficiently using the existing knowledge. Moreover, the development of high definition 3D computer models for anatomy with enhanced level of detail, it may make it possible to reduce or eliminate the need for animal dissection during teaching [101, 102].

3D cell-culture models and organs-on-chips

In the current scenario of rapid advancement in the life sciences, certain tissue models can be built using 3D cell culture technology. Indeed, there are some organs on micro-scale chip models used for mimicking the human body environment. 3D models of multiple organ systems such as heart, liver, skin, muscle, testis, brain, gut, bone marrow, lungs and kidney, in addition to individual organs, have been created in microfluidic channels, re-creating the physiological chemical and physical microenvironments of the body [103]. These emerging techniques, such as the biomedical/biological microelectromechanical system (Bio-MEMS) or lab-on-a-chip (LOC) and micro total analysis systems (lTAS) will, in the future, be a useful substitute for animal experimentation in commercial laboratories in the biotechnology, environmental safety, chemistry and pharmaceutical industries. For 3D cell culture modeling, cells are grown in 3D spheroids or aggregates with the help of a scaffold or matrix, or sometimes using a scaffold-free method. The 3D cell culture modeling conditions can be altered to add proteins and other factors that are found in a tumor microenvironment, for example, or in particular tissues. These matrices contain extracellular matrix components such as proteins, glycoconjugates and glycosaminoglycans that allow for cell communication, cell to cell contact and the activation of signaling pathways in such a way that the morphological and functional differentiation of these cells can accurately mimic their environment *in vivo*. This methodology, in time, will bridge the gap between *in vivo* and *in vitro* drug screening, decreasing the utilization of animal models during research [104].

Alternatives to microbial culture media and serum-free animal cell cultures

There are moves to reduce the use of animal derived products in many areas of biotechnology. Microbial culture media peptones are mostly made by the proteolysis of farmed animal meat. However, nowadays, various suppliers provide peptones extracted from yeast and plants. Although the costs of these plant-extracted peptones are the same as those of animal peptones, plant peptones are more environmentally favorable since less plant material and water are required for them to grow, compared with the food grain and fodder needed for cattle that are slaughtered for animal peptone production [105].
Human cell culture is often carried out in a medium that contains fetal calf serum, the production of which involves animal (cow) sacrifice or suffering. In fact, living pregnant cows are used and their fetuses removed to harvest the serum from the fetal blood. Fetal calf serum is used because it is a natural medium rich in all the required nutrients and significantly increases the chances of successful cell growth in culture. Scientists are striving to identify the factors and nutrients required for the growth of various types of cells, with a view to eliminating the use of calf serum. At present, most cell lines could be cultured in a chemically-synthesized medium without using animal products. Furthermore, data from chemically-synthesized media experiments may have better reproducibility than those using animal serum media, since the composition of animal serum does change from batch to batch on the basis of animals' gender, age, health and genetic background [76].

Alternatives to animal-derived antibodies

Animal friendly affinity reagents may act as an alternative to antibodies produced, thereby removing the need for animal immunization. Typically, these antibodies are obtained *in vitro* by yeast, phage or ribosome display.

In a recent review, a comparative analysis between animal friendly affinity reagents and animal derived-antibodies showed that the affinity reagents have superior quality, are relatively less time consuming, have more reproducibility and are more reliable and are cost-effective [106, 107].

Conclusions

Animal experimentation led to great advancement in biological and biomedical sciences and contributed to the discovery of many drugs and treatment options. However, such experimentation may cause harm, pain and distress to the animals involved. Therefore, to perform animal experimentations, certain ethical rules and laws must be strictly followed and there should be proper justification for using animals in research projects. Furthermore, during animal experimentation the 4 Rs principles of reduction, refinement, replacement and responsibility must be followed by the researchers. Moreover, before beginning a research project, experiments should be thoroughly planned and well-designed, and should avoid unnecessary use of animals. The reliability and reproducibility of animal experiments should also be considered. Whenever possible, alternative methods to animal experimentation should be adopted, such as *in vitro* experimentation, cadaveric studies, and computer simulations.
While much progress has been made on reducing animal experimentation there is a need for greater awareness of alternatives to animal experiments among scientists and easier access to advanced modeling technologies. Greater research is needed to define a roadmap that will lead to the elimination of all unnecessary animal experimentation and provide a framework for adoption of reliable alternative methodologies in biomedical research.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; AKK, DP, GH, RB, Paul S, Peter S, RM, BF, NC, SM, LL, DD, GMT, MCE, MD, SM, Daniele M, GB, AD, KD, MCM, TB, MS, STC, Donald M, AM, AB, KLH, MK, LS, LL, GF: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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Review

Methodology for clinical research

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Keywords

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Summary

A clinical research requires a systematic approach with diligent planning, execution and sampling in order to obtain reliable and validated results, as well as an understanding of each research methodology is essential for researchers. Indeed, selecting an inappropriate study type, an error that cannot be corrected after the beginning of a study, results in flawed methodology. The results of clinical research studies enhance the repertoire of knowledge regarding a disease pathogenicity, an existing or newly discovered medication, surgical or diagnostic procedure or medical device. Medical research can be divided into primary and secondary research, where primary research involves conducting studies and collecting raw data, which is then analysed and evaluated in secondary research. The successful deployment of clinical research methodology depends upon several factors. These include the type of study, the objectives, the population, study design, methodology/techniques and the sampling and statistical procedures used. Among the different types of clinical studies, we can recognize descriptive or analytical studies, which can be further categorized in observational and experimental. Finally, also pre-clinical studies are of outmost importance, representing the steppingstone of clinical trials. It is therefore important to understand the types of method for clinical research. Thus, this review focused on various aspects of the methodology and describes the crucial steps of the conceptual and executive stages.

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Introduction

According to epistemologists, who study the nature, origin and scope of knowledge, epistemic justification, the rationality of belief and related issues [1], there are six ways to obtain knowledge:

- authoritarianism;
- mysticism;
- rationalism and empiricism;
- pragmatism;
- scepticism.

Rationalism and empiricism, pragmatism and scepticism may be within the scope of the scientific method, whereas authoritarianism and mysticism are clearly pseudoscience or anti-science [2]. Science is characterized by systematic observation and experimentation, inductive and deductive reasoning, and the formation and testing of hypotheses and theories. The details of how these are carried out can vary greatly, but these characteristics are sufficient to distinguish scientific activity from non-science [3-8]

The choice and selection of a particular methodology depends on factors such as the hypothesis to investigate, the research question or statement of the problem, the objectives, the nature of the study, the study population and controls, intervention and variables [9-12]. The reliability and validity of the results therefore depend on an overall study design having well-defined objectives, reproducible methodology, diligent data collection and analysis to minimise errors and bias, and efficient reporting of the findings [9, 12]. Selecting an appropriate methodology is therefore essential to obtain valid results, and an understanding of research methodology is essential for researchers.

Medical research can be broadly categorised into primary and secondary research. Primary research involves conducting studies and collecting raw data that is then analysed and evaluated in secondary research [13]. Primary research can be further classified into three types as shown in Figure 1: basic or laboratory studies, also known as preclinical studies, clinical research, and epidemiological research. Both clinical and epidemiological research involve observational and experimental methods. Clinical research investigates the effects of specific interventions on individuals, while epidemiological research studies the causes and distribution of disease or mortality in human populations, especially the effects of exposure to single or multiple environmental agents [14]. Similar in essence, clinical research methods differ somewhat, depending on the type of study. Type is an integral element of study design and depends on the research question to answer. It should be specified before the start of any study [15]. Selecting an inappropriate study type results in flawed methodology, and if it occurs after commencement of the study, it is an error that cannot corrected.

Stages of clinical research

A clinical research project consists broadly of two stages: planning and action [16].

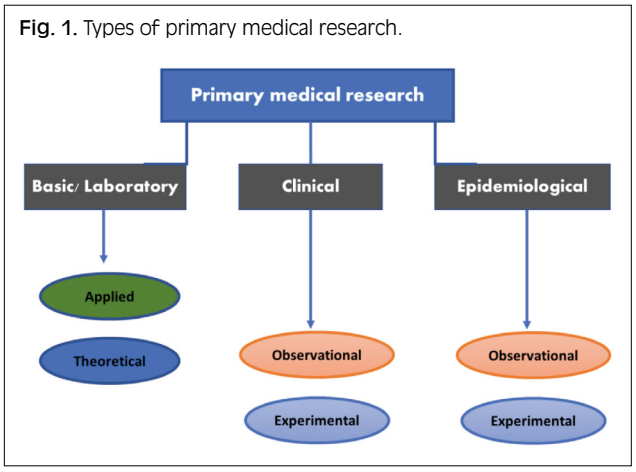


Fig. 1. Types of primary medical research.

Planning Stage

The planning stage consists of all the preliminary paperwork and search of the literature done before starting actual research. It includes identifying the problem, reviewing the literature, developing a research question, formulating a hypothesis, determining the type of study, selecting a study design, identifying the target/study population, and seeking informed consent to participation. It also includes establishing collaborations with experts and determining the overall feasibility of the proposed work [9, 16].

Before beginning the scientific investigation, the researchers should decide the data collection strategy, sampling techniques and statistical analysis. After choosing a working hypothesis and reformulating it as null and alternative hypotheses, the next step is to decide the type of study required to answer the research question and an appropriate method to implement it.

Action stage

This stage includes the actionable research, implementation of the method in coherence with the theoretical concept, randomisation, blinding, application of sampling techniques, data collection and statistical analysis [10, 11].

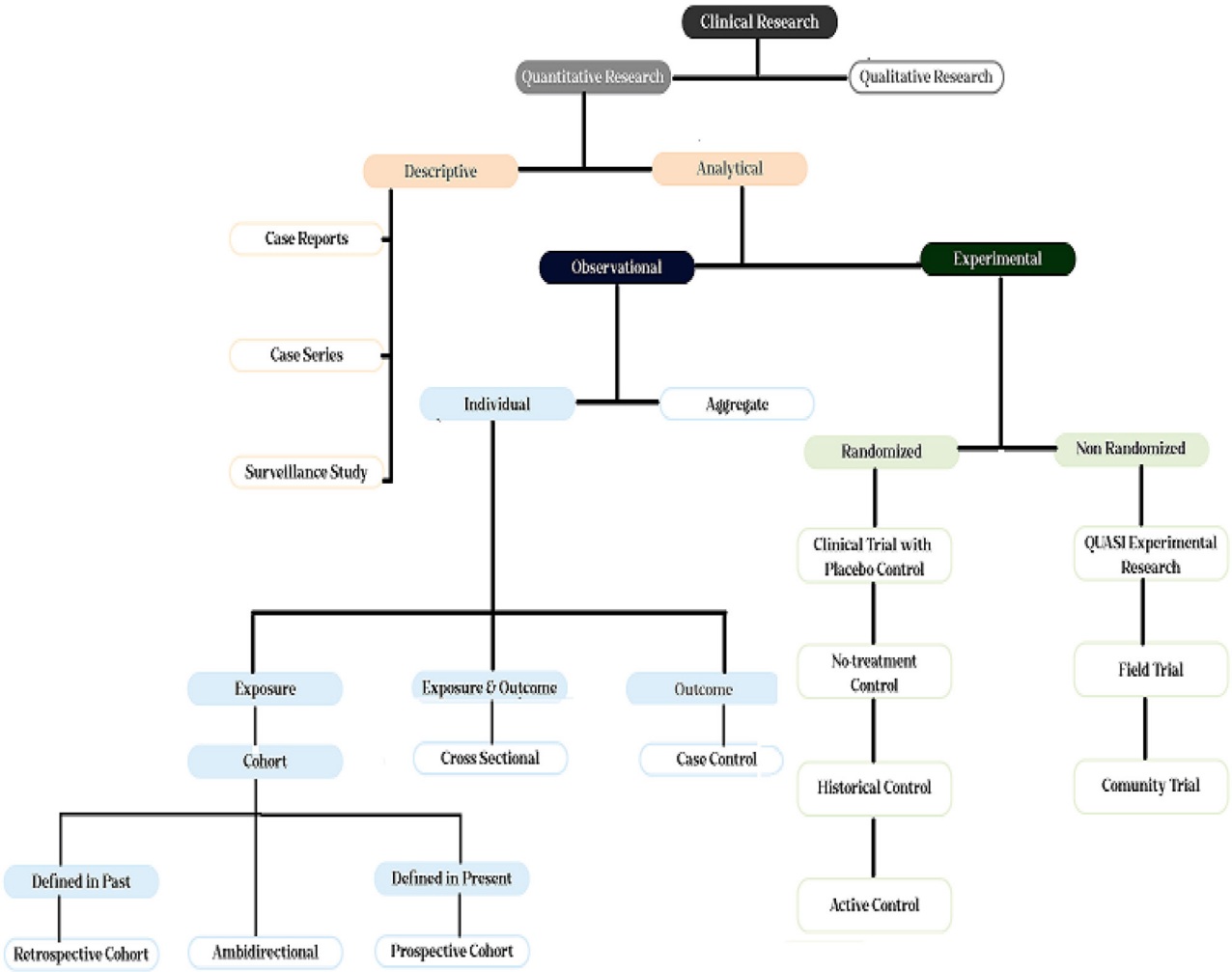
Classification of clinical research

Depending on the study design, clinical research can in principle be categorised as either quantitative or qualitative [9]. Further classification of clinical research methods may be based on data collection techniques and the direction of causality being investigated, as illustrated for example by time relationships. Clinical research can be classified as either descriptive or analytical, as illustrated in Figure 2 [9, 12, 17-20].

Descriptive research

Descriptive studies record and report unusual or new events, e.g. the prevalence of a disease or syndrome in a family, and correlate the events with possible expla-

Fig. 2: Classification of clinical research.



nations. This type of research is neither randomized nor pre-designed, and is presented as a case report, case series or surveillance study.

Case Reports

These are reports of individual patients with particular clinical characteristics. Such reports present baseline characteristics recorded and evaluated for single patients, compared with population values. Sometimes these studies may consist of observations recorded for administration of a certain treatment to an individual. They are essentially hypothesis generating, opening the way for more rigorous studies of an experimental nature [12].

Case series

Case series may include examination of successive clinical cases having common characteristics. They may, for example, present observations from patients exposed to a particular drug or group of drugs at regular intervals, and may include former histories of patients having similar outcomes, to detect possible cause-effect relationships.

Surveillance studies

This type of study involves continuous monitoring of disease occurrence in a population. Information related to a health problem of interest is collected in databases, analysed over a time period and inferences are made based on observed correlations.

Analytical/Explanatory Studies

The most significant difference between descriptive and analytical studies is the presence in the latter of control groups that enable comparative evaluations to be made. Analytical clinical studies can be further classified into experimental (intervention) studies and observational (non-intervention) studies.

Observational studies

Observational studies are non-intervention studies in which patients are prescribed a specified therapy based on diagnosis and therapeutic need. They include therapeutic, prognostic, observational drug studies, secondary data analyses, case series and single case reports,

and may be retrospective, prospective or ambidirectional [21]. In non-intervention studies, "knowledge from the treatment of persons with drugs in accordance with the instructions for use specified in their registration is analysed using epidemiological methods" [21]. "Diagnosis, treatment and monitoring are not performed according to a previously specified study protocol, but exclusively according to medical practice" [21].

Observational studies involve collecting data pertaining to study participants in their natural or real-world environments. They are usually diagnostic and prognostic studies, with a cross-sectional approach to data collection. The comparative-effectiveness study is the hallmark of non-experimental research [22], and involves comparison of comparable groups to interpret outcome effects. Such studies are also known as benchmarking-controlled trials because of the element of peer comparison [22].

Observational studies can be broadly categorised into individual and aggregate studies.

Aggregate observation studies

Individual level data aggregated by geographic area, year or any other parameter is termed aggregate data. Aggregate studies are conducted to record observations on pandemics and epidemics of communicable diseases and their treatment regimens, for example aggregate data on COVID-19 in a particular country, or the occurrence and effective treatment of malaria and its relapse in a particular geographical area. Data pertaining to non-communicable diseases is also aggregated in the same way to generate insights into the distribution of diseases in specified populations, as for example in cancer registries [23, 24].

Individual observation studies

Individual studies are based on disaggregated individual results and involve analysis to estimate differences between subgroups. In individual observational studies, subjects are observed individually and then gathered in groups based on outcomes or exposures or both. Based on grouping criteria, individual observational studies may take the form of case-control, cohort or cross-sectional studies.

a. Case-control studies
   Individual observational studies that involve grouping of subjects based on selected outcomes are termed case-control studies. In these studies, the exposure experience of the case group (subjects with the outcome of interest) is compared with that of the control group (subjects without the outcome), for instance occurrence or non-occurrence of renal failure in diabetic patients or heart attacks in hypertensive patients. The design of such studies is retrospective and evaluates possible associations between exposures and outcome. They are quick and inexpensive to perform, and the results are expressed as odds ratios (OR) and risk ratio/relative risk. Case control studies enable multiple exposure variables to be examined for a given outcome, but they do not allow correlation of sequential causes and effects with the outcome [12].
b. Cohort studies
   In this type of study subjects are grouped based on exposure. Cohort studies enable multiple outcomes to be studied for a given exposure. The exposure is well-defined, but the outcome may vary, thus providing an opportunity to monitor many outcomes of a single exposure [12]. Cohort studies can be retrospective, where the cohorts are defined on the basis of a past exposure, or prospective, where the cohorts are defined by a current exposure.

- Retrospective cohort studies
  In retrospective cohort observational studies, the researchers look back in time at archived or self-reported data in order to compare outcomes in exposed and non-exposed patients. The two groups are identified retrospectively and studied prospectively. This type of study is quick and inexpensive [25, 26], but is prone to recall-bias [27].
- Prospective cohort studies
  A prospective cohort study is a longitudinal cohort study in which cohorts differing in exposure to the factors being studied are followed up at predetermined time intervals to determine the effect on outcomes. This type of study helps to determine associations between a particular exposure and outcomes. For rare outcomes, large numbers of subjects and long follow-up periods are required, so such studies tend to be very expensive. In addition, if randomization and blinding are not conducted properly, the chances of bias and confounders increases [26].

c. Cross-sectional studies
   Cross-sectional studies have transverse study design and involve concurrent assessment of exposures and outcomes without any follow-up. These studies are essentially based on surveys, and are therefore appropriate for determining prevalence but cannot shed light on causation [12, 26].

Experimental studies

Experimental studies are intervention studies, and include preclinical trials on animals as well as clinical trials in humans. In these studies, the effect of an intervention is compared with that of another intervention or a placebo. Interventions studied may include, for example, use of medical devices, surgical, physical or psychotherapeutic procedures, psychosocial interventions, rehabilitation measures, acupuncture, physiotherapy training or diet [1, 14]. Experimental studies mostly aim to compare outcomes of treatment procedures in a group of patients exhibiting minimal internal differences. To avoid bias, patients are randomly allocated to treatment and control groups. Different countries have different procedures and legal and ethical requirements governing the conduct of such studies. For instance, the United Kingdom Medicines and Medical Devices Act 2021 requires that studies using medical devices be registered by the relevant authorities. In the European Union, interventional studies must be conducted in accordance with

the binding rules of Good Clinical Practice (GCP) [28]. In Germany, vaccine studies are considered to be intervention studies and are conducted as clinical studies according to the AMG [13]. Likewise, drug studies must seek approval from ethical committees. Informed consent must be obtained from the patient and an ethically defensible control group included. The control group is given another treatment regimen and/or placebo and should enable the central questions of the study to be answered [28].

Some experimental studies in biomedical research may focus on possible biomarkers, such as enzymes or genes, on evaluation of imaging techniques, such as magnetic resonance imaging and computed tomography, or on techniques such as gene sequencing in order to find correlations between genotypes and phenotypes. The development of statistical tests and mathematical models may also be regarded as experimental studies. Generally, the design of biomedical studies should be based on their purpose and objectives [13].

Design of experimental studies

The design of an experimental study depends on the type of information sought, the objectives of the study and the ultimate application. Designs can be characterized by interventions on selected groups of the study population under controlled environmental conditions compared with a control group without any interventions. The main designs employed in experimental studies are randomised controlled trials and non-randomised clinical trials, also known as quasi-experimental studies [9, 12, 26].

Non-randomized studies

In non-randomised studies, the study population is selected on the basis of pre-determined selection criteria; it is not randomized with respect to treatment(s) but is prescribed treatment based on the course of the disease. In many experimental studies involving surgical intervention which is only appropriate for particular patient groups, randomization is either not possible or not ethical. Generally, phase IV of a clinical trial has non-randomized design. Non-randomised studies can be further categorised as:

a. Quasi-experiment
   The investigator assigns exposure to the intervention as in a randomized controlled trial, but the subjects are not randomized [12].
b. Field trial
   These are large scale studies of therapeutic interventions, for example the efficacy of COVID-19 vaccines in combatting COVID-19. Many samples are required to determine efficacy, particularly when the incidence of a particular disease in the population is low [26].
c. Community trial
   In these trials, treatments are allocated to a community group. For instance, the effect of fluoridation of water was tested by exposing some communities to fluoride and comparing outcomes with those in unexposed communities.

Randomized controlled trials

Randomised controlled trials (RCTs) are trials in which the subjects are randomly assigned to experimental and control groups. The experimental group is given the treatment that is being tested and the control group is given an alternative treatment or a placebo or no treatment at all. Most experimental clinical studies are RCTs, and the subjects are either healthy volunteers or patients. After a new drug passes a pre-clinical trial, it is tested via RCTs. Various aspects of the RCT require careful consideration before the trial begins, for example study design, patient population, control group selection, randomization, sampling, blinding or open labelling of treatments and outcomes [12, 26].

a. Study design
   Study design is an important prerequisite for the success of the study. Randomised controlled trials commonly use parallel group design, matched pairs and cross-over designs [29].
- Parallel group design
  This design requires large number of subjects/patients who are enrolled, followed up and observed for outcomes on a parallel basis over a period of time.
- Matched pairs
  In this design, patients are matched for different variables. Matched subjects are assigned at random to intervention or control groups. Although this type of design is difficult to conduct, it helps overcome the influence of confounding variables on outcomes.
- Crossover design
  This design is used for drugs having reversible and transient effects. The effects of two interventions, administered sequentially, are assessed. The number of patients required is smaller than for the other designs [29].

b. Patient population
   In RCTs, the patient population is selected on the basis of predetermined selection criteria. This selection is carried out to avoid confounding variables and should be based on predefined inclusion and exclusion criteria. Withdrawal criteria, indicating the circumstances under which subjects should be withdrawn from the trial, should also be predefined.
- Inclusion criteria
  The criteria for selection of subjects (patients or healthy volunteers) are based on age, body mass index, gender, ethnicity, prognostic factors and diagnostic admission criteria. They are used to select the subjects and then randomly assign them to various treatments for comparison of outcomes [26, 30].
- Exclusion criteria
  These are criteria for excluding subjects from a particular trial, for example severity of disease, concurrent medication, allergies, underlying health conditions and many more [30].
- Withdrawal criteria

These indicate situations in which the trial is terminated for particular subjects and specify how and when the

subjects should be withdrawn from the study. When subjects are withdrawn, they are no longer subject to follow-up.

c. Control group
Perhaps the most important factor in any scientific research is identification and determination of a control group. Without successful deployment of a control group, a study cannot be authentic. Randomised controlled trials can include placebo, no-treatment, historical or active controls [26].

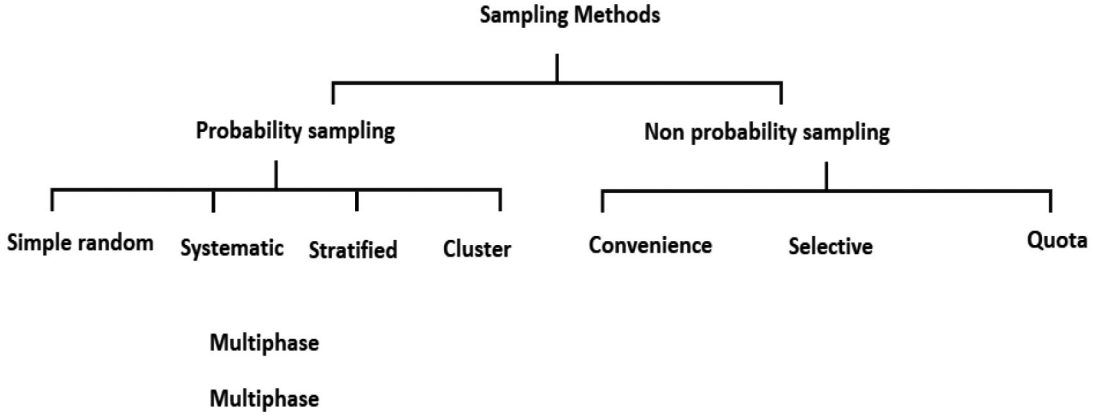
- Placebo control
A placebo is a fake or inert version of the drug under evaluation, with no pharmacological effect. Placebos help overcome any psychological impact of drug dispensing on disease progression, allowing the investigator to estimate the effectiveness of a treatment free from confounding psychological factors. However, placebo controls in drug research and sham surgery are ethically controversial, especially in cases where an effective treatment exists.
- No treatment controls
This is the least preferred type of control, where subjects are not given anything by way of treatment, not even a placebo. Such controls serve as a neutral reference group for the experimental groups receiving the treatment under investigation. This approach avoids bias due to psychological factors that may influence outcomes.
- Historical control
In some studies, concurrent controls are dispensed with and only historical control data is used. This is done specifically for studies involving rare diseases with high mortality. In such circumstances, withholding treatment from a control group would raise very considerable ethical implications [9]. Historical controls are controls used in previous studies. They help reduce the overall cost of the study, making drug developers more likely to invest. Historical controls also make enrolment in rare disease trials more feasible by reducing the number of patients required.

d. Randomization
Randomization is the optimal method of allocating subjects to the therapy arms of a trial. Random assignment of subjects to the treatment and control groups ensures equal distribution of all variables and confounding factors, such as genetic variabilities, risk factors and comorbidities, in all groups, thereby alleviating bias. Randomization is intended to ensure comparability between the groups, and it reduces the chance of allocating a specific therapy to patients with a particularly favourable prognosis. Randomization is carried out using random number tables, mathematical algorithms for pseudorandom number generation, physical randomization devices such as coins and cards, or sophisticated devices such as electronic random number indicator equipment [9-12, 26].
The main randomization techniques used in RCTs are simple randomization, cluster randomization and stratified randomization [31].

- Simple randomization
Randomization involving a single sequence of random assignments is known as simple randomization. It randomizes patients selected on the basis of selection criteria to various treatment groups.
- Cluster randomization
Cluster-randomized trials are used to compare treatments that are allocated to clusters (groups) of subjects, rather than to individuals. Groups of patients matching the selection criteria are randomly assigned to the group receiving the treatment or to a control group. Randomised controlled trials are used to evaluate complex interventions.
- Stratified randomization
This is a two-step procedure. As the name indicates, the subjects entering the clinical trial are first grouped in strata (groups) based on clinical features that might affect the outcome of their condition, and then undergo intra-group randomization to assign them to various treatment groups.

e. Sampling method
Sampling is the process of selecting a sample population from the target population. Sampling allows in-

Fig. 3. Sampling methods in clinical research.



formation to be obtained about the target population based on statistical analysis of a subset of the population, without any need to investigate the characteristics of every individual in the target population [32]. Sampling techniques are broadly categorised into probability and non-probability sampling, as shown in Figure 3.

- Probability sampling
  In this sampling technique, every element of the population has an equal chance of being selected. This helps create a sample truly representative of a given population [22]. Types of probability sampling techniques are:

Simple random sampling

In this type of sampling every experimental unit has an equal chance of being selected during sampling.

Systematic sampling

This sampling is used where a complete and up-to-date sampling frame is available. The first experimental unit is selected randomly, while the rest are selected randomly based on a predesigned pattern.

Stratified sampling

In this method the study population is divided into strata according to age, gender etc. and then sampling is carried out from these strata.

Cluster sampling

In this method the study population is divided into clusters and these clusters rather than individuals are taken as sampling units. The clusters are then randomly selected for inclusion in the study.

Multistage sampling

Multistage random sampling is conducted at several stages within population clusters. This sampling method is usually applied to large nationwide surveys.

Multiphase sampling

The sampling is conducted in two or more phases. In the first phase some data is collected from the whole sample and in the second, data is collected from a subset of the original sample.

- Non-probability sampling
  In this type of sampling technique, not all experimental units get an equal chance of being selected [22]. A non-representative sample which does not produce generalizable results is a possible result. Different types of non-probability sampling are:

Convenience sampling

This sampling is based on the convenience of the investigator.

Purposive/judgemental/selective/subjective sampling

This type is based on the judgement of the investigator.

Quota sampling

This method of sampling is used in studies involving interviews and is based on the judgment of the interviewers, depending on characteristics such as sex and physical status.

f. Blinding
   Blinding is defined as "concealing or masking the assignment of subjects to a study group from the participants of the study, i.e., patients/subjects, observers and researchers". Randomised clinical trials may be blinded or non-blinded [9, 12, 26].

- Non-blinded experiments
  Non-blinded experiments are also known as open-label studies. In this type of study, all participating patients, physicians, observers and researchers know the treatment used. This may result in bias, but is unavoidable where hiding a treatment raises ethical concerns. For instance, it is unethical to hide the treatment regime from patients with cancer, AIDS or organ failure. Patients may also be allowed to select the drug brand themselves.
- Blinded experiments
  In these experiments the blinding is done at the start of the experiment. Blinding can be single, double or triple.

Single-blind trials

The subjects (patients or healthy volunteers) do not know whether they are in the intervention or the placebo group.

Double-blind trials

Neither the subjects nor the researcher knows who has been assigned to the control and the test groups. Only the observer knows to which group the subjects have been assigned.

Triple-blind trials

In triple blind RCTs, personal or intentional bias is eliminated by none of the study participants (subjects, observer, researcher) knowing the label or nature of the treatment administered.

The information identifying treatment and subjects in double- and triple-blind experiments is held by another party and only made available to the researcher at the end of the trial.

Prospective, randomized, open-label, blinded-endpoint (PROBE) trials

Randomized controlled trials can be conducted as PROBE trials in which patients are randomly assigned to different treatment regimens and both patients and researchers are aware of the treatments administered. The PROBE trial is much easier to conduct than double- or triple-blind or doubled-blind placebo-controlled design, as it enables trials to be performed in conditions that resemble real-world practice. It is also economical and simplifies patient enrolment. However, it imposes certain conditions to avoid the bias associated with open label trials. PROBE designs are endpoint blinded, as the observer is unaware of the treatment being used. Since the subjects and researchers know the treatments, potential bias can be avoided by using so-called hard endpoints as primary endpoints. However, the results obtained by PROBE are less reliable than those obtained by double- or triple-blind studies [33].

g. Treatment considerations
   Another important prelude to a successful clinical study is the selection of treatment dosage, form, frequency, route of administration and concurrent medications for the test and active control groups. A drug may be available in various doses and in forms such as tablet, capsule or injectable. Since these factors

affect the plasma concentrations and effects of the drug, and ultimately the outcome; all these factors, except dose and frequency, are maintained constant throughout the study. If necessary, the dose and frequency of the drug may be changed gradually and sequentially. If the treatments involve more than one drug, their pharmacokinetic and pharmacodynamic interactions are kept under observation while determining dosage, in order to avoid any influence of these interactions on outcomes [9, 12, 33]. Another important consideration in treatment selection is patient compliance, since non-compliance may have adverse effects on outcome.

h. Outcome measures
Since the objectives of a clinical study indicate the possible outcomes, this is borne in mind in selecting the methods of monitoring and the data required for recording the outcomes of interest. In clinical experiments, outcomes are assessed in terms of efficacy endpoints, i.e. primary endpoints and surrogate or secondary endpoints. Primary endpoints are measures specified by the researcher at the start of the study in order to verify or refute the hypothesis, whereas surrogate endpoints are specified before commencement of the study but can be modified during its course. For instance, in an experiment estimating the efficacy of an antihypertensive drug, the primary endpoint would be to see whether or not the treatment reduces cardiovascular events, while a surrogate endpoint could be its ability to reduce blood pressure [26]. Many primary and secondary endpoints are prespecified before beginning a study. However, the main primary endpoint is the quality of life afforded by a particular treatment for individuals in the study group.

- Bias, chicanery and confounders
Bias is distortion of outcomes due to introduction of errors, voluntarily or involuntarily, at different stages of the research, e.g. the stages of design, population selection, calculation of number of samples, data entry and statistical analysis. Several types of bias can occur during clinical research (Tab. I).

Chicanery

Chicanery involves deliberate unethical changes to interventions, results and the data of patients. Copying data from other sources is also classified as chicanery.

Confounders

Confounders are factors, other than those being studied, that can affect an outcome parameter. These factors are not directly relevant to the research question but may possibly alter the outcomes [10, 11]. For example, while studying the effect of hypertension on renal failure, diabetes could be a confounder as it also affects kidney function. It is therefore essential to take all potential confounders into consideration when designing a study. If known, confounders can be controlled for by selection constraints or statistical adjustments, such as stratification and mathematical modelling, during study design. Various strategies are used during data analysis to adjust for confounders; these include stratified analysis using the Mantel-Haenszel method, a matched design approach, data restriction and model fitting using regression techniques [34].

Bias, chicanery and confounders can be avoided by randomization and blinding. The randomized controlled and blinded clinical trial with case number planning is there-

Tab. I. Types of bias in clinical research.

Type of Bias	Description
Investigator bias	Conscious or unconscious preference given to one group over another by the investigator
Evaluator bias	Introduced when an investigator making endpoint-variable measurements favours one group over another. Common with subjective endpoints
Performance bias/ set of Hawthorne effects	Introduced when participants know their allocation to a particular group and change their response or behaviour during a particular treatment
Selection bias	Introduced when samples (individuals or groups) are selected for data analysis without proper randomization; includes admission bias and non-response bias, in which case the sample is not representative of the population
Ascertainment or information bias	Errors in measurement or classification of patients; includes diagnostic bias and recall bias
Allocation bias	Systematic differences in the allocation of participants to treatment groups and comparison groups, when the investigator knows which treatment is going to be allocated to the next eligible participant
Confirmation bias	Information is processed in a manner consistent with someone's belief
Belief bias	The strength of arguments is judged on the basis of the plausibility of their conclusions rather than how strongly they support that conclusion.
Expectation bias	Introduced during publication by a personal preference for positive results over negative results when the results deviate from expected outcomes
Detection bias	Systematic errors in observation of outcomes in different groups results in detection bias when outcomes in one group are not as vigilantly sought as in the other.
Attrition bias/loss-to-follow-up bias	Preferential loss-to-follow-up in a particular group leads to attrition bias.
Commercial bias	Introduced for commercial reasons in the form of advertising or economic pressure on editors, particularly in studies involving new medical devices and drugs

fore accepted as the gold standard for evaluating the efficacy and safety of drugs and therapeutic regimes [35].

i. Validity
   The results of a clinical trial are said to be valid if the differences observed between the study and control groups are real and not influenced by bias or confounders (internal validity) and are applicable to a broader population (external validity). Placebo-controlled, double-blinded, randomised clinical trials have high internal validity, while external validity can be increased by broadening the eligibility criteria for enrolling subjects [36].

Preclinical studies for the development of biomedical products

Pre-clinical (or laboratory) studies form the basis of clinical trials. To reduce the time for, and to improve the chances of approval of a new drug, the choice of an appropriate preclinical model is of utmost importance. Preclinical studies evaluate the pharmacodynamics, pharmacokinetics and toxicology of a drug in *in vitro* and *in vivo* settings. Clinical trials are conducted when preclinical studies have demonstrated the efficacy and safety of a new drug. The results of clinical trials can improve preclinical studies and *vice versa*. Nonetheless, only a small fraction of drugs that pass the preclinical evaluation criteria are selected for clinical trials, and only a few are approved for use in humans, so optimization of standard preclinical procedures to mimic the complexity of human disease mechanisms is urgently needed [37].
In summary, preclinical studies involve the use of various *in vivo* and *in vitro* models and computer designs to evaluate the efficacy and safety of a new drug.

In vitro models (cell studies)

Advances in cell culture technology have made it possible to test new drugs on cell lines grown in vitro. These studies may involve testing of drugs on human or animal cancer cells [38].

In vivo models (animal studies)

Drugs that prove effective in vitro are then tested in vivo in live animals to ensure their safety in living systems. Animal models, and their critical validation, are of great importance in minimizing unpredicted adverse effects of a drug in clinical trial phases. Animal models are carefully selected on the basis of their advantages and limitations and on the objectives of the study, in order to mimic pathophysiological conditions in humans [38]. The validity of animal models is increased by following the relevant guidelines and standards in designing a study. Three types of models are used in preclinical studies:

Homologous models

Homologous models are animals which have the same causes, symptoms, and treatments of a particular disease that humans would have.

Isomorphic models

These animals have same symptoms and treatments of a particular disease as humans, but the cause may be different.

Predictive models

These models are only like humans in some aspects of a particular disease; however they provide useful information about the mechanisms of disease features.

In silico models (computer studies)

In silico models are based on computer simulations that complement or precede *in vitro* and *in vivo* studies. They predict how a drug might behave in these subsequent studies. In-silico studies require expertise in biochemistry, molecular biology, cheminformatics, and bioinformatics [38].
Pre-clinical studies provide useful information about the behaviour and safety of drugs. However, drugs do not necessarily behave in the same way in humans as they do in animal models. For example, human subjects and mice models differ sharply in absorption, processing, and excretion of certain drugs. Unexpected side-effects may therefore occur in humans that do not occur in animal models. Drugs which show promising outcomes in preclinical studies are then approved for testing in human subjects by regulatory authorities such as the Food and Drug Administration (FDA) in the US [37, 38].

Design, performance, and monitoring of clinical trials

Once preclinical studies on a new drug are completed and promising results are achieved, the next stage in biomedical research is testing the safety, efficacy and reproducibility of the drug's action on humans through clinical trials. Clinical trials are considered to be a safe and dependable method of evaluating the efficacy of a treatment. Clinical trials may be therapeutic or preventive [37-39].

Therapeutic trials

These trials are conducted to test experimental treatments, combinations of new or existing drugs, and new surgical interventions.

Preventive trials

These trials test the efficacy of interventions (drugs, vaccines) in preventing diseases and their outcomes.
In general, clinical trials aim to enhance the repertoire of information related to an intervention or lifestyle regime that might prove beneficial for patient management or treatment. They are designed to develop and test new diagnostic methods or treatments and their effects on humans, or new uses of existing diagnostic methods or treatment. They also help identify the most cost-effective and risk-free diagnostic methods or treatments.

Tab. II. Phases of a randomized controlled trial of a drug.

Phases	Aim	Number of participants
Phase 0	To check the a. capacity of a new drug to modulate its intended target in humans b. metabolisation of a drug	a few volunteers
Phase I	To check a. safety and unexpected effects b. safe dose c. absorption and elimination by the body	20-80 healthy volunteers or patients in an advanced stage of disease
Phase II	To assess a. safety and effects in a broader population	Hundreds of volunteers
Phase III	To a. assess efficacy b. check for collateral effects c. compare with pre-existing treatments d. determine whether approval and commercialization are possible	Hundreds to thousands of volunteers
Phase IV	To collect more information on a. risk b. benefits c. potential uses of an existing drug	Hundreds of thousands of volunteers

Randomized controlled trials are conducted to compare the safety and efficacy of two or more interventions in humans, and can often be based on clinical equipoise. Their phases [26] are shown in Table II.

Good clinical practice: guidelines and requirements

Clinical trials are the gold standard for evaluating the superiority or similarity of new drugs or surgical procedures with respect to existing ones. As clinical trials involve testing on humans, their design and conduct require careful planning, diligent execution and enormous resources to comply with regulations set by the regulatory authorities so that robust results can be attained. The good clinical practice (GCP) guidelines published by the International Council of Harmonization (ICH) is an international ethical standard that ensures that the design, conduct, performance, monitoring, auditing, recording, analysis and reporting of clinical trials takes place according to established values. It also ensures the reliability and precision of reported data, and protects the rights, integrity and privacy of subjects participating in a trial [28, 31]. Protection of the safety, wellbeing and rights of human subjects participating in a clinical trial is consistent with the principles of the Declaration of Helsinki [40] and with the ethical principles formulated by the World Medical Association [41]. The requirements for conducting clinical trials in the European Union, including GCP and good manufacturing practice and their respective inspections, are implemented in the Clinical Trial Directive (Directive 2001/20/EC) and the Good Clinical Practice Directive (Directive 2005/28/EC) [31].

The responsibility for GCP lies with all participants in the trial, from the site staff to the subjects and the ethical and monitoring committees. The roles and responsibilities of GCP participants are shown in Table III.

Conclusion

The planning and execution of clinical research is of vital importance for the advancement of medical science. The validity of clinical research findings depends on a variety of factors, such as study design, sampling techniques and statistical analysis. Choosing an appropriate study design

Tab. III. Clinical trial participants and their role in good clinical practice.

Participants	Role
Regulatory authorities	Review clinical data and conduct inspections for GCP and good manufacturing practice
Sponsor	Institution/organization responsible for initiation, management and finance of clinical trial
Project monitor	Monitors the project and is appointed by the sponsor
Investigator	Team leader responsible for conducting trial at trial site
Trial site pharmacist	In charge of maintaining, storing and dispensing drugs
Patients	Human subjects
Ethical review committee for the protection of subjects	Institutional or national regulatory authorities ensuring safety, well-being and protection of human subjects
Committee to monitor large trials	Overseas sponsors, drug companies

requires detailed knowledge of the types of clinical study, the situations where they are applied and the possible outcomes, so that a methodology befitting the hypothesis is adopted. Careful implementation of study design eliminates the chances of bias, provides quality assurance of the data collected and increases the validity of the results, adding value to the findings. Successful preclinical studies, basic research and pilot scale intervention studies pave the way for more sophisticated clinical trials. Randomised, double-blind clinical trials with case number planning are accepted as the gold standard for evaluating the efficacy and safety of drugs and therapeutic regimes and in evaluating the superiority or similarity of new drugs or surgical procedures to existing ones. As clinical trials involve testing on humans, their design and conduct require careful planning, diligent execution and enormous resources to comply with the rules set by the regulatory authorities, necessary to achieve robust results.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; AKK, DP, GH, RB, Paul S, Peter S, RM, BF, NC, SM, LL, DD, GMT, MCE, MD, SM, Daniele M, GB, KD, MCM, TB, MS, STC, Donald M, AM, AB, KLH, MK, LS, LL, GF: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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ORIGINAL ARTICLE

Validating methods for testing natural molecules on molecular pathways of interest in silico and in vitro

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Keywords

Gene expression • Bioinformatics tools • Biochemical pathways • In vitro • Natural molecules

Summary

Differentially expressed genes can serve as drug targets and are used to predict drug response and disease progression. In silico drug analysis based on the expression of these genetic biomarkers allows the detection of putative therapeutic agents, which could be used to reverse a pathological gene expression signature. Indeed, a set of bioinformatics tools can increase the accuracy of drug discovery, helping in biomarker identification. Once a drug target is identified, in vitro cell line models of disease are used to evaluate and validate the therapeutic potential of putative drugs and novel natural molecules. This study describes the development of efficacious PCR primers that can be used to identify gene expression of specific genetic pathways, which can lead to the identification of natural molecules as therapeutic agents in specific molecular pathways. For this study, genes involved in health conditions and processes were considered. In particular, the expression of genes involved in obesity, xenobiotics metabolism, endocannabinoid pathway, leukotriene B4 metabolism and signaling, inflammation, endocytosis, hypoxia, lifespan, and neurotrophins were evaluated. Exploiting the expression of specific genes in different cell lines can be useful in in vitro to evaluate the therapeutic effects of small natural molecules.

Introduction

The post-genomic era is marked by several discoveries in the discipline of molecular medicine that have enabled the recognition of disease-related genes and the subsequent development of targeted therapeutic strategies. Next generation RNA sequencing clearly demonstrates that genes do not function alone, but rather constantly interact with each other. These genetic interactions are crucial for regulating gene expression, and downstream biochemical, and signal transduction pathways [1, 2]. Genes that function in the same biological pathway are regulated by the same transcription factors and tend to show similar expression levels under a particular stimulus [3]. Networking amongst genes can be identified by pathway and network analysis models [4]. The topology of the networks is used to predict gene expression under different circumstances. For instance, under a particular environmental condition the expression of a central or 'hub gene' that acts as a transcription regulatory factor helps determine the expression of the genes that are regulated by it [5-7]. This means that this regulatory gene is acting as a switch to control the expression of its interacting genes. On the other hand, gene network connections might represent a set of genes activated in a particular pathway by a biological process or pathogenic condition, termed gene expression signatures [8]. These signatures indicate a difference in cellular activity and depict the interchange of different biological pathways. The gene expression signatures and contrasting networks can explain how aberrations in gene-gene and gene-environment interactions result in pathological conditions [3]. Consequently, one of the most powerful uses of high throughput genomic, transcriptomic, proteomic, and metabolomics data is the unravelling of the mechanisms underlying diseases by comparing biological pathways in control versus disease states [9]. This makes clear the importance of pathway analyses in deciphering the etiology of a specific disease, in the identification of potential biomarkers, and in targeted drug discovery [9, 10].

Biological pathways from a bioinformatics viewpoint

Biological pathways include are a set of genes or molecules that act in a synergistic fashion to accomplish a biological function. Biological pathways play a vital part in the advancement and survival of an organism and failure in functioning of a pathway results in the onset of disease [11]. Based on the cellular requirements at a particular time, the products of a pathway can manifest differently as structural or functional responses.
Biological pathways can be broadly categorized into metabolic, genetic and cell signalling pathways. These pathways interact with one another, forming a network of interconnected pathways that deal with complex cellular functions and with the regulation of gene expression [12, 13].

Biologic pathway analysis integrates gene ontology and pathway structure information to identify pathways whose activation/inactivation is linked with a specific condition or disease. This makes pathway analysis an important tool in deciphering mechanisms underlying a disease and consequent drug discovery [9, 12]. In fact, it is now clear that complicated diseases are a consequence of dysregulated pathways rather than the dysregulated expression of an individual gene. In fact, a variety of gene pathways may combine to manifest the same condition [14]. In such cases, responses to these disorders are expected to ultimately affect the same cellular system [14]. Pathway-centric models are fundamental in figuring out the mechanisms of complicated diseases and recognition of candidate drug targets. Pathway-centric models represent pathways as graphs of circles or nodes, where larger nodes denote pathways with larger numbers of components, and edges between nodes symbolize interaction between the different pathway nodes [15].

Pathway analysis methods and databases

Differential expression (DE) of genes in experiments comparing two situations – such as two phenotypes, two drugs, two states (control vs disease; treated vs untreated) – and subsequent statistical analysis approaches such as ANOVA [16], t test [17], or Z scores [18] can help identify the genes or set of genes that contribute to the development of a particular phenotype. However, as genes are not expressed alone and are under the control of several regulatory elements, the identification of genes alone cannot elucidate the mechanisms of complex diseases; therefore, knowledge obtained from the DE of genes is studied in the context of information obtained from pathway databases. Pathway analysis coupled with data obtained from DE of genes helps to decipher the mechanisms underlying a particular condition and to identify which pathways are significantly affected. Several studies have reported the use of pathway databases to identify genetic markers, gene signatures, and mechanisms of complex diseases (Tab. I). Important pathway databases used in studying genetic, signalling, and metabolic pathways are presented in Table II. In addition to pathway analysis, network analysis is also carried out to see the interactions between various gene networks which are analyses collected from distinct populations, conditions, or groups [19].

Altered expression of specific genes as biomarkers and their exploitation as therapeutic targets

Biomarkers are biological molecules that act as indicators of normal or pathological processes or pharmacological responses to a directed therapeutic [41]. In addition, biomarkers are used in screening for disease, as diagnostic and prognostic factors, and for selecting patient-specific therapy. Biomarkers are also useful in evaluating the effect of drugs administered to patients or to cell lines for therapeutic and experimental purposes, respectively. Biomarkers must be reliable and reproducible because human health is at stake. Biomarkers can be discovered through gene expression analysis followed by feature selection methods that enable the discovery of a small subset of biomarkers that have the ability to discriminate between molecular subtypes of diseases [42]. Recent studies have evaluated gene expression in peripheral blood mononuclear cells (PBMCs) to identify biomarkers for disease [43], including Crohn's disease [44], Behcet's disease [45] and ulcerative colitis [44]. Relevant to the obesity epidemic, one study identified 9 genes that correlated with obesity indices in humans out of 19 genes differentially expressed in the PBMCs of high fat-fed rats [43]. Another study reported the identification of biomarkers of insulin resistance found in the expression profiles from adipocytes of subjects with insulin resistant obesity using Gene Expression Omnibus. The study identified 10 hub genes (genes with the most interactions with other genes) using various bioinformatic tools, such as GSEA, GO analysis, KEGG pathway analysis [46], and Cytohubba. Moreover, using these biomarkers, potential small molecular compounds that could treat insulin resistance were detected [23].

Tab. I. Examples of diseases identified by different methodologies of pathway analysis.

Disease	Software	References
Glioblastoma multiforme	TRED database, eQTL mapping	[15]
Alzheimer's disease	WebGestalt	[20]
Olfactory behaviour	R spider	[21]
Esophageal squamous cell carcinoma	ICSNPathway server with i-GSEA	[22]
White adipocyte insulin resistance	GO analysis; KEGG pathway analysis	[23]
Biliary cirrhosis	LRT and i-GSEA4GWAS	[24]
Bladder cancer	Combined outcomes of GSEA and ARTP	[25]
Bipolar disorder	IPA and GSEA-SNP	[26]
Major depressive disorder	GSEA and statistical analysis	[27]
Schizophrenia	ICSNPathway server with i-GSEA, MAGENTA, ALIGATOR, INRICH and Set Screen	[28, 29]
Coronary heart disease	VSEA in GWAS	[30]

TRED: Trascriptional Regulatory Element Database; eQTL: expression Quantitative Trait Loci; ICSNPathway: Identifiy candidate Causal SNPs and Pathways; i-GSEA: Gene Set Enrichment Analysis; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; LRT: Likelihood Ratio Test; GWAS: Genome-Wide Association Study; ARTP: Adaptive Rank Truncated Product; IPA: Ingenuity Pathways Analysis; SNP: Single Nucleotide Polymorphism; MAGENTA: Meta-Analysis Gene-set Enrichment of variaNT Associations; ALIGATOR: Association LIst Go AnnoTatOR; INRICH: INterval enRICHment analysis; VSEA: Variable Set Enrichment Analysis.

Bioinformatic tools for identification of drugs-disease-pathway interaction

In the past few decades, the pharmaceutical industry has successfully deployed its one drug, one target model,

Tab. II. Common pathway annotation databases.

Name	Database Description	References
KEGG	Genomic and pathway information in various organisms	[31]
PANTHER v.14	Evolutionary relationships data for protein analysis	[32]
Pathway Ontology	Contains several biological pathways, including altered and disease pathways, and the relationships between them	[33]
BioCarta	Defines gene sets for data analysis	[34]
SPIKE	Focuses on pathways describing cellular responses such as DNA damage response, cell cycle, apoptosis and hearing-related pathways	[35]
GeneOntology	Pioneered use of ontologies in computational biology	[36]
PID	Information about molecular and cellular signalling pathways	[37]
MetaCyc	Metabolic and enzymatic pathways from various organisms	[38]
REACTOME	A platform for annotating and visualising data from several databases	[39]
MSigDB	Collects gene sets by biological functions, GO, KEGG, positions, sequence regulation information	[40]

focusing on druggable genes, genes encoding proteins that can be modulated using experimental small molecule compounds. This model emphasizes only on a small subset of genes affected by the drugs, completely ignoring the mechanisms underlying the action of the drug on these genes and molecular pathways [47]. In addition, this paradigm ignores the function of synergistic molecules from different pathways and their effects on the same subset of genes. This means that although successfully deployed, this paradigm cannot fully explain the drug-target interaction. This is due to the fact that the onset of a disease cannot be reduced to a single change, but rather to a cascade of gene expression alterations under the influence of the physiological environment of the body. Furthermore, the drug itself does not only interact with a single target, but rather with pathways or metabolic patterns of the body [48]. In this scenario, systems bioinformatics holds promise in predicting drug-pathway interactions by elucidating the mechanisms underlying drug activity and its possible side effects. Identifying enrichment pathways or gene sets from drug-induced datasets can lead to the discovery of promising drug targets, with a focus on reducing side effects. In addition, unravelling drug-disease-pathway interactions can provide useful insights of the systemic drug efficacy. Some important pathway databases and networks used for drug-disease-pathway-interactions are presented in Table III.

Cell cultures as a model for studying drug-pathway interactions

Preclinical models, such as cell lines, have been successfully deployed in studying and predicting the response and mechanism of action of drugs on disease-related genes and dysregulated pathways. Cell lines provide a continuous source of biological material for experimentation. The field in which cell line models are most used is cancer research. Indeed, cell lines are used to study the effect of anticancer drugs, as well as to study genetic alterations found in specific types of tumors. In addition, cell line models are particularly useful in cases where it is difficult to obtain clinical samples or where the monetary or human cost of obtaining clinical samples is high [65].

In order to study the effects of anti-tumoral drugs on genetic variants involved in tumour formation, several cell line models are used [66]. Data can also be retrieved from the Cancer Genome Project (CGP) and from the Cancer Cell line Encyclopedia (CCLE) [67], which contain data regarding 36 cancer cell lines [68]. Immortalized cell lines are often used to test drug efficacy and toxicity, or to identify drug-specific biomarkers [69]. A typical example is the Epstein-Barr virus (EBV) transformed Human Lymphoblastoid Cell Lines (LCLs) [69]. The goal of this study is to demonstrate the importance of developing an experimental model to study the effects of natural molecules in cell lines.

Materials and Methods

Bioinformatic study for gene selection

Genes of interest, associated with a specific condition, disease, or process, were chosen by searching GeneCards with specific keywords, specifically, obesity, xenobiotics metabolism, endocannabinoid pathway, leukotriene B4 metabolism and signaling, inflammation, endocytosis, hypoxia, lifespan, and neurotrophins were considered and used as keywords. For each query, we identified a list of genes identified by a score to reflect the association of the records with the query: the genes above a specific score threshold were retained for further study. The resulting list of genes was analyzed in the KEGG database to define common metabolic, gene regulation or signal transduction pathways. Genes already studied in the MAGI laboratory in association with other conditions were also included in the final list. Reference material supporting the genes chosen was obtained from PubMed. Finally, using the free tool STRING the interrelationships between the products of the identified genes was highlighted.

Primer design

For each selected gene, pairs of primers were designed for evaluating gene expression through real time PCR

Tab. III. Databases for drug target discovery.

Database	Database Description	Applications	Reference
Drug-Path	Reports genes that can be upregulated or downregulated by drugs interactions	Retrieval of drug-induced pathway data. Highlights the dysregulated pathways of diseases.	[49]
DGIdb 4.0	Information on drug-gene interactions and druggable genes	Identification of drug targets and studying drug-gene interactions	[50]
PubChem	Information on chemicals and on their toxicity	Identification of chemicals that have potential to be used as drugs	[51]
NCBI dbGaP	Archived genetic data including the relation between phenotype and GWAS	Identification of genes involved in a disease with genotype-phenotype interaction studies	[52]
GWAS Catalog	Metadata of the most significant published results	Identification of disease genes, prioritization of candidate loci, prediction of disease risk and molecular disease mechanisms	[53]
ChEBI	Ontology of chemicals and molecular entities, especially small molecules	Supply of identifiers for unambiguously refer to chemical entities	[54]
DrugBank 5.0	Drug and Drug Target Info. Provides molecular information regarding drugs and their mechanisms of action, interactions with other drugs, and their targets	Study of pharmacological properties of drugs, drug-drug, drug-pathway, drug-food interaction elucidation	[55]
PharmGKB	Aggregated information of genetic variants-drug response interaction	Extraction of interactions between drugs-drugs/genes/pathways/SNP, diseases-pathway/gene-SNP	[56]
STITCH	Chemical interactions using information from molecular pathways, crystal structures and binding experiments	Identification of drug-pathway interactions	[57]
HMDB	Information about small human metabolites	Identification of drug-metabolome interactions	[58]
MetaboLights	Metabolomics experiments used for cross-platform and cross-species studies	Identification of metabolites' structure, biological roles, concentration, and localization in living systems.	[59]
eDGAR	Relationships among genes related to disease-gene associations	Identification of disease-gene association, gene-gene interaction. Detection of functional terms related to groups of genes	[60]
NPASS	Information on activity and sources of natural products	NP(Natural Product)-based drug discovery, mechanism elucidation of NP and in silico algorithms development	[61]
MetaCyc	Metabolic pathways and enzymatic reactions from organisms of all life's domains	Prediction of the metabolic pathways of an organism from its annotated genome	[39]
MassBank Japan	Mass spectral data of biological molecules	Identification of a chemical compound	[62]
HumanCyc	Metabolic pathways and enzymatic reactions	Analysis of omics data for metabolic pathways	[63]
CMap	Gene expression profiles of immortalized human cell lines after chemical treatment	Prediction of the effects and mode of action of drugs; drug repositioning	[64]

experiments. At first, real time PCR primers were retrieved from Harvard Medical School database (https://pga.mgh.harvard.edu/primerbank/index.html). Pairs of primers that produced amplicons with a length of ≤ 200 bp and with the melting temperature of closest to 60°C for both primers were selected.

When primers were not available in the database or did not meet the required criteria, we designed new primers by using the bioinformatics tool Primer3. The criteria for choosing the primer pairs are as follows:

- Primer length: 18-28 nucleotides;
- Resulting amplicon: ≤ 200 bp (optimal 80-120 bp);
- Melting temperature: 60°C (the two primers must not have more than one degree of melting temperature difference from each other);
- GC content: 20-80% (50% was optimal);
- Primers must not contain repeated nucleotide sequences and complementary regions;
- Primers must be designed preferably on different exons or across exon-exon junctions, to limit as much as possible, the amplification of non-specific regions.

The resulting primer pairs were analyzed by PRIMER BLAST to evaluate the specificity of the amplified region.

The ENSEMBL genome browser was used to confirm that the primers mapped at the exon-exon junction or on different exons. However, this was not an exclusion criterion of the primer pair, as for some genes it is not possible to satisfy this characteristic (e.g. monoexonic genes).

RNA extraction, retrotranscription and qPCR

Total RNA was extracted from selected cell lines and blood using the Tempus Spin RNA Isolation Kit, following the manufacturer's protocol. Cell lines were selected referring to GeneCards database "Expression" section, which shows the tissues that express most highly a gene of interest. Between the ones proposed, cell lines already present in MAGI laboratories were used. Blood was collected from patients used as negative control in previous projects [70]. The SuperScript VILO cDNA Synthesis Kit was used to generate first strand cDNA. Quantitative real-time polymerase chain reaction (qPCR) was performed by using the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania) on a QuantStudio 3 Real-Time PCR System, as reported [71].

Polymerase chain reaction (PCR) for the identification of cells expressing genes of interest

The PCR was performed with the aim of verifying that the primers selected for each gene-produced amplicons of the expected length, that there were no non-specific amplifications, and that the gene was expressed in the chosen cell lines.

qPCR primer efficiency evaluation

The evaluation of the efficiency of the primers is a fundamental step in qPCR, especially when studying gene expression, as it allows the correct analysis of data obtained. When the efficiency is calculated with the **ΔΔCt** method, it is assumed that the efficiency of the used primer is comparable to that of the primer for the housekeeping gene. If the efficiencies of the primers were dissimilar, the gene expression analysis could be affected by errors and misleading results would be obtained. The QuantStudio 3 Real-Time PCR System software calculated the efficiency of each primer pair. Primers whose efficiency was in the range between 90% and 110% were selected.

Fig. 1. Target genes evaluated in this work.

OBESITY
CD36, APOE, EP300, ACE, FTO, LIPE, MC4R, PPARGC1A, RETN, UCP1, ADIPOQ, HSD11B1, LEP, LEPR, CYP19A1, ESR1, IL6,GAPDH
XENOBIOTICS METABOLISM
FAAH, FAAH2, PPARA, LTB4R, LTB4R2, IGF1, AR, ADIPOQ, HSD11B1, LEP, LEPR, AHR, AHRR, AKR1C1, AKR1C2, AKR1C3, AKR1C4, ESR2, HPGD, LMNA, NCOA1, PLIN1, PRLR, VDR, CYP19A1, ESR1, ALOX5,GAPDH
ENDOCANNABINOID PATHWAY
CD36, CNR1, GPR18, GPR55, FAAH, FAAH2, PPARA, ALOX5,GAPDH
LEUKOTRIENE B4 METABOLISM AND SIGNALING
IL6, TNF, LTA4H, LTB4R, LTB4R2, ALOX5AP, GAPDH
INFIAMMATION
IL2, NOS1, NOS2, TLR2, TLR4, TLR7, IL6, TNF, APOE,GAPDH,RIPK1
ENDOCYTOSIS
CAV1, CLTB, ERLIN1, ERLIN2, RFTN1, SGMS1,GAPDH
HYPOXYA
ARNT, HIF1A, HIF1AN, HYOU1, VEGFC, VEGFR1(FLT1), VEGFR3(FLT4), EP300, VEGFA,GAPDH
LIFE-SPAN
IL6, TNF, APOE, ATG5, CERS2, COQ7, FCGR2A, SIRT1, SIRT3, SIRT6 , SOD1, TGFB1, IGF1, AR, GAPDH
NEUROTROPHINS
IGF1, BDNF, GDNF, HGF, IGF1R, NGF, NGFR, GAPDH

Results

In this work, a system for studying the expression of specific genes in selected cell lines was validated. 101 sets of primer pairs targeting specific genes (Fig. 1) were tested based on their efficiency values with the following results: 51 validated, 24 non-validated and 26 sets of primers targeting genes that were not expressed in the available cell lines requiring re-testing in other cell lines (Tab. IV).
The levels of expression were evaluated on the basis of the C_t. $C_t \leq 20$ = high expression; $20 < Ct \geq 23$ = high-medium expression; $23 < Ct \geq 26$ = low medium expres-

Tab. IV. List of primers from this work with qPCR efficiency between 90-110%.

Gene	Sequence (5'->3')	Tm	Cell Line/Tissue	Ct	Expression Level
AKR1C1	CCTAAAAGTAAAGCTTTAGAGGCCACC	60	Blood	27	Low
	GAAAATGAATAAGGTAGAGGTCAACATAAT				
AKR1C2	CCTAAAAGTAAAGCTCTAGAGGCCGT	60	Blood	32	Low
	GAAAATGAATAAGATAGAGGTCAACATAG				
AKR1C3	GAGAAGTAAAGCTTTGGAGGTCACA	60	Blood	26	Low-medium
	CAACCTGCTCCTCATTATTGTATAAATGA				
LTA4H	TCTGGGAGGACCGTATGTATG	60	Blood	27	Low
	ATTCCCTGTCCAGCTATGAGAT				
ALOX5	ACTGGCTGAATGACGACTGG	60	Blood	19	High
	CAGGGGAACTCGATGTAGTCC				
LEPR	TCCTCTTCCATCTTATTGCTTGG	60	Blood	24	Low-medium
	TCTTGGGGTTCGGAACATCT				
VEGFC	ATGTGTGTCCGTCTACAGATGT	60	Blood	26	Low-medium
	GGAAGTGTGATTGGCAAAACTGA				

Tab. IV. *Continues.*

Gene	Sequence (5'->3')	Tm	Cell Line/Tissue	Ct	Expression Level
FLT1(VEGFR1)	GAAAACGCATAATCTGGGACAGT	60	Blood	26	Low-medium
	GCGTGGTGTGCTTATTTGGA				
FLT4(VEGFR3)	TGCACGAGGTACATGCCAAC	60	HepG2	27	Low
	GCTGCTCAAAGTCTCTCACGAA				
APOE	GTTGCTGGTCACATTCCTGG	60	Caco2	21	High-medium
	GCAGGTAATCCCAAAAGCGAC				
BDNF	CTACGAGACCAAGTGCAATCC	60	SK-N-SH	27.5	Low
	AATCGCCAGCCAATTCTCTTT				
GAPDH	GGAGTCAACGGATTTGGTCG	60	ALL	17	High
	GACAAGCTTCCCGTTCTCAG				
SOD1	GGTGGGCCAAAGGATGAAGAG	60	Caco2	23	High-medium
	CCACAAGCCAAACGACTTCC				
TNF	GAGGCCAAGCCCTGGTATG	60	Blood	26	Low-medium
	CGGGCCGATTGATCTCAGC				
AHR	CTTAGGCTCAGCGTCAGTTAC	60	Caco2	25	Low-medium
	CGTTTCTTTCAGTAGGGGAGGAT				
ARNT	TGACTCCTGTTTTGAACCAGC	60	HaCaT	28	Low
	CTGCTCACGAAGTTTATCCACAT				
ATG5	AAAGATGTGCTTCGAGATGTGT	60	SK-N-SH	28	Low
	CACTTTGTCAGTTACCAACGTCA				
CERS2	GCTCTTCCTCATCGTTCGATAC	60	Caco2	22	High-medium
	CTTGCCACTGGTCAGGTAGA				
COQ7	GTTGATGGTTACGTTCAGGGT	60	MCF7	27	Low
	TTGTTGTAGTGATGTGCTATGCT				
FAAH	GTGACCTCCTATCTGGCTGAC	60	Caco2	28	Low
	CTCACAGGGACGCCATAGAG				
FAAH2	CATAGGCTTAGTAGGCCGAGC	60	Caco2	27	Low
	CTTTCTCTGTCGGATCAGCTTG				
HIF1A	GAACGTCGAAAAGAAAAGTCTCG	60	Caco2	24.5	Low-medium
	CCTTATCAAGATGCGAACTCACA				
HIF1AN	ACGAGAGGTTCCCTAATTTCCA	60	Caco2	23	High-medium
	ATGCCACCAGTACATTGGGAT				
IGF1R	AGGATATTGGGCTTTACAACCTG	60	Caco2	25	Low-medium
	GAGGTAACAGAGGTCAGCATTTT				
LMNA	AATGATCGCTTGGCGGTCTAC	60	Caco2	25	Low-medium
	CACCTCTTCAGACTCGGTGAT				
NCOA1	AGAGGCAACACGACGAAATAG	60	Caco2	24	Low-medium
	ACACTGCATTACTTCATAACGCT				
NGFR	CCTACGGCTACTACCAGGATG	60	Primary fibroblasts	33.5	Low
	CACACGGTGTTCTGCTTGT				
NOS2	TTCAGTATCACAACCTCAGCAAG	60	Caco2	24.5	Low-medium
	TGGACCTGCAAGTTAAAATCCC				
SIRT3	ACCCAGTGGCATTCCAGAC	60	Caco2	26	Low-medium
	GGCTTGGGGTTGTGAAAGAAG				
SIRT6	CCCACGGAGTCTGGACCAT	60	Caco2	27	Low
	CTCTGCCAGTTTGTCCCTG				
TGFB1	CTAATGGTGGAAACCCACAACG	60	Caco2	25	Low-medium
	TATCGCCAGGAATTGTTGCTG				
TLR2	ATCCTCCAATCAGGCTTCTCT	60	Caco2	28	Low
	GGACAGGTCAAGGCTTTTTACA				
TLR4	AGTTGATCTACCAAGCCTTGAGT	60	Primary fibroblasts	30	Low
	GCTGGTTGTCCCAAAATCACTTT				
VEGFA	AGGGCAGAATCATCACGAAGT	60	Caco2	26	Low-medium
	AGGGTCTCGATTGGATGGCA				
VEGFC	GAGGAGCAGTTACGGTCTGTG	60	Primary fibroblasts	25.5	Low-medium
	TCCTTTCCTTAGCTGACACTTGT				

Tab. IV. *Continues.*

Gene	Sequence (5'->3')	Tm	Cell Line/Tissue	Ct	Expression Level
HYOU1	GAGGAGGCGAGTCTGTTGG	60	Primary fibroblasts	26	Low-medium
	GCACTCCAGGTTTGACAATGG				
IL6	CCTGAACCTTCCAAAGATGGC	60	Primary fibroblasts	29.5	Low
	TTCACCAGGCAAGTCTCCTCA				
FTO	ACTTGGCTCCCTTATCTGACC	60	Caco2	23	High-medium
	TGTGCAGTGTGAGAAAGGCTT				
RETN	CTGTTGGTGTCTAGCAAGACC	60	HL60	27	Low
	CCAATGCTGCTTATTGCCCTAAA				
PPARGC1A	TCTGAGTCTGTATGGAGTGACAT	60	HepG2	28	Low
	CCAAGTCGTTCACATCTAGTTCA				
CYP19A1	TGGAAATGCTGAACCCGATAC	60	HepG2	27	Low
	AATTCCCATGCAGTAGCCAGG				
ESR1	CCCACTCAACAGCGTGTCTC	60	MCF7	26	Low-medium
	CGTCGATTATCTGAATTTGGCCT				
ADIPOR2	CTGGATGGTACACGAAGAGGT	60	Primary fibroblasts	24.5	Low-medium
	TGGGCTTGTAAGAGAGGGGAC				
EP300	AGCCAAGCGGCCTAAACTC	60	Primary fibroblasts	27	Low
	TCACCACCATTGGTTAGTCCC				
RFTN1	ATGGGTTGCGGATTGAACAAG	60	Primary fibroblasts	24	Low-medium
	AGCGGTATTCATAGGACACATCT				
SGMS1	TGTGCCGAGTCTCCTCTGA	60	Primary fibroblasts	24	Low-medium
	CCGTTCTTGTGTGCTTCCAAA				
CLTB	CGAGGAGGCTTTCGTGAAGG	60	Primary fibroblasts	24	Low-medium
	GCAGGCGGGACACATCTTT				
ERLIN1	TGGCTCCTTATGCAGTGTTTG	60	Primary fibroblasts	21	High-medium
	GGGCCATGAGGTTTAAGTCTTTC				
ERLIN2	TCCACCACGAACTGAACCAG	60	Primary fibroblasts	26	Low-medium
	AACAGCTCAATGTAGACCTCTTG				
ACE2	CAAGAGCAAACGGTTGAACAC	60	Caco2	31	Low
	CCAGAGCCTCTCATTGTAGTCT				
RIPK1	GGCATTGAAGAAAAATTTAGGC	60	Blood	22	High-medium
	TCACAACTGCATTTTCGTTTG				

sion; Ct ≥ 27 = low expression. T_m = melting temperature; C_t = cycle threshold; Caco2 = human colorectal adenocarcinoma; HepG2 = human hepatocyte carcinoma; MCF-7 = human breast cancer; SH-SY5Y, SK-N-SH = human neuroblastoma from bone marrow; HaCaT = human keratynocyte; HL60 = human promyelocytic leukemia.

Discussion

Identification of dysregulated gene expression pathways involved in human health and disease has significantly contributed to the testing of new compounds as potential drugs. The study of the genes involved in the conditions considered in this study, such as obesity and inflammation, are essential to learn more about the molecular pathways in these diseases and to potentially find new small molecule compounds that might help prevent or treat these diseases. Many primer pairs resulted in high or medium-high expression levels. Because of the elevated expression levels, these primer pairs can be exploited to evaluate dysregulated gene expression in vitro in various conditions. Following this method, it may also be possible to find and test in vitro new molecules with therapeutic potential that could be included in dietary supplements. Finally, diverse study models can be constructed based on these methods, focusing not only on a particular biochemical pathway-natural molecule interactivity, but also on a wider relationship.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; KD, GB, KA, KLH, STC, FB, PG: literature

search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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ORIGINAL ARTICLE

Ion mobility mass spectrometry with surface activated chemical ionisation as a method for studying the domain of water clusters

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Keywords

Water clusters • Water domain • Mass spectrometry • Quantum field theory

Summary

Water holds great relevance in various biological and biochemical systems. Water behaves as an excellent solvent, a reactant, a product and a catalyst of the reaction. The organisation of the water molecules, synergised by hydrogen bonds, builds up the structure of the water clusters. These water clusters significantly influence biological functions. To study the domain of water clusters using Ion mobility mass spectrometry with surface activated chemical ionisation. The experimental analysis was aimed to determine the water behaviour in terms of cluster formation before and after the application of a physical effect, namely low-frequency irradiation. A sanist platform-based spectrometer, manufactured by ISB srl with SACI version for protein analysis, was used as the equipment. Furthermore, for samples, we used pure de-ionised water, a part of which was used virgin, and another part was irradiated. Ion-mobility mass spectrometry (IM-MS) procedure was adopted as the experimental method. An electromagnetic frequency fields generator was used to subject the test samples to electromagnetic radiations between 7 Hz to 80 Hz. The presence of neutral water species was confirmed in the water samples. For the same m/z, water ion clusters in the untreated water were found to have a much higher intensity than the electromagnetically treated water. The presence of a water cluster near the $(M+H)^+$ in electromagnetically treated dilute arginine solution was also confirmed. It is possible to detect water ion clusters by using Ion mobility mass spectrometry and SACI with low surface potential (47 V). The water cluster formation and its characteristics were found to be different in the treated and non-treated water. The electromagnetic radiations of low frequency seem to affect the hydrogen bonds of the water molecules.

Introduction

Water holds great relevance in various biological and biochemical systems pertaining to human life [1]. Lack of water would jeopardise several chemical reactions paralysing biological functions, and the importance of water for the cells, organs and life forms can hardly be overemphasised [2]. It is observed that water presents itself as the matrix and medium for the genesis and sustenance of various life forms [3].

Inherently simple, water behaves as an excellent solvent, a reactant, a product of the reaction and also a catalyst. And though exhibiting reasonably normal behaviour, it is complex and anomalous in many instances [1]. Water exhibits quirky behaviour during experiments and scientific explorations, necessitating the deployment of more than one theory or model to understand the unusual aspects of water. A tighter association between the water molecules, as compared to other material molecules of the same size and shape, results in much higher cohesion between the molecules [4]. Consequently, this leads to higher surface tension, melting point and boiling point. Water also exhibits evident volumetric anomaly when its solid form (ice) floats on the liquid (water), which is opposite to the general observation of a solid sinking in its liquid form. Again this is attributed to the minimum volume of water at 4^0C or a temperature of maximum density (TMD) at 4^0C, which is in defiance of the increasing volume of simpler liquid monotonically [4]. Further, the negative slope of the water's pT equilibrium phase boundary between the solid and liquid results in liquification of ice due to pressure, against the customary solidification of liquid into solid when under pressure. In contrast to a maximum of one or two solid phases of other materials, water is known to have at least 17 distinct phases of solids. The quintessential hydrophobic effect of water represents its thermodynamic uniqueness while interacting with nonpolar molecules is another noteworthy feature of water.

The structure of water is more relevant and vital for scientific analysis rather than just focussing on the chemical composition of water. The organisation of the molecules, synergised by hydrogen bonds, builds up the structure of the water and, in the simplest form, is represented by $(H_2O)_2$, a dimer. These small clusters may associate with each other to form larger groups spread all over the water [5].

These water clusters significantly influence biological functions and can be studied experimentally or computationally [6].

Though water appears homogenous at the macroscopic level at the nanoscopic level, it is not. Two competing processes, namely the enthalpic and the entropic processes, are responsible for the structuring of liquid water [7].
The objective of this paper it to study the domains of water clusters in pure water samples and diluted arginine solutions using Ion mobility mass spectrometry with surface activated chemical ionisation and to determine the water behaviour in terms of cluster formation before and after the application of low-frequency irradiation.
And further, in the discussion section, we also intend to discuss the nature of such clusters formation in view of theories and knowledge accumulated in recent years.

Materials and Methods

SACI ionisation technology is considered to be a very soft ionisation technique that does not modify the analyte-solvent coordination significantly [8]. This is substantiated by the fact that it has been employed to monitor the peptide-cation adduct in solution [9]. The SACI-CIMS is an evolved version of SACI technology that enables solvent cloud ion species by deploying mass spectrometry.
Ion-mobility mass spectrometry (IM-MS) synergises ion mobility with mass spectrometry enabling compounds of a predetermined (m/z) range in an analyte solution to be characterised together. In an IM-MS, an IM chamber is inserted after the ionisation source of the mass spectrometer (an electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI) source, for example) but before the mass spectrometer optics and analyser. Such placement of the IM chamber creates an additional analyte separation step which helps reduce the spectral noise [10].
During the IM-MS procedure, the analyte ions, generated in the ionisation chamber, enter the ion-mobility section and move through the chamber according to their charge, which gets created as a result of an electrostatic or electrodynamic force generated by an electric field. The Ion-mobility chamber contains the gas at almost the atmospheric pressure, and the moving analyte collides with it. Within the chamber, the collision cross-section and the charge help separate them. While characterising proteins using Mass spectroscopy, the inclusion of an IM step has been shown to be useful. The characterisation of different spatial isoforms is enabled by including an IM step due to the difference in their collisional cross-sections with the gas molecules generating different time-dependent isoform drifts.
Further, during the ionisation process, as a consequence of substantial solvent-ion clustering, the ESI and APCI sources generate noise. Dynamic IM-MS, e.g., high-field asymmetric waveform ion mobility spectrometry (FAIMS), has been employed to filter analyte ions by their m/z values before they enter the MS analyser and to retain ion-solvent clusters within the ionisation source, thereby simplifying the spectrum of an analyte sample by producing multiple spectra which individually report a subset of the analyte components. However, notwithstanding the ability of FAIMS to optimise the separation of analyte components, the choice of appropriate gas is vital for optimal performance.
The low-voltage ionisation source employed by a Surface-activated chemical ionisation (SACI) source increases the spectral sensitivity by ionising mainly analyte solute molecules (10). SACI is a polarisation effect induced at a surface inserted into an APCI chamber that is subjected to a smaller potential (50-300 V) than is employed by a classic atmospheric pressure ionisation source (~3000-6000 V). It has been observed that under SACI conditions, there is a considerable reduction in the number of solvent-ion clusters while the analyte ion population is noticeably enhanced. Under low voltage SACI conditions, a highly interesting and uniquely unreported phenomenon was observed, which we call the “in-source Cloud Ion-mobility Mass Spectrometry” (CIMS) effect. This effect is observed singularly in the SACI mode when the surface potential is fixed and the entrance capillary voltage of the MS is varied. However, it is essential to note that ESI and APCI operate at high voltage ionisation conditions, unlike the SACI, which operates at low voltage.
Consequently, the observations confirm that with increased internal capillary voltage ions with larger m/z values gain focus, while those with smaller ratios of m/z remain defocussed. It is further observed that the ion-cloud spatial distribution in the low-voltage SACI source depends on the rate of the in-source gas circulation. The scope and utilisation of the SACI-type MS are greatly enhanced due to the newly discovered CIMS effect as the additional ion filter located before the analyser substantially improves the spectral quality and definition of the target molecule [10].
The experiment was conducted using the following equipment and chemicals (Tab. I).

Sample preparation

SACI-CIMS was utilised to analyse two different types of water solution and a dilute solution of Arginine:

a. 100 µL Pure water without treatment, by direct infusion;
b. 900 µL of water electromagnetically irradiated at 7 Hz to 80 Hz for 5 minutes, using an electromagnetic frequency fields generator;
c. A dilute solution of Arginine was made with a concentration of 100 ng/mL. And this solution was exposed to radiated and non-radiated water, respectively.

Mass spectrometry

The data was acquired by means of the SANIST platform provided with a Surface Activated Chemical Ionization- Cloud Ion Mobility Mass Spectrometry (SACI-CIMS) [10]. The surface potential was regulated at 47 V for the experiment. The nebulising gas pressure was set at 75 Psi, and the flow rate of the dry gas parameter was regulated at 1.0 L/min. The experiment was performed with the nebuliser temperature equal to

Tab. I. Equipment and chemicals using in the experiment.

Equipment	Model	Manufacturer	Revision date	Revision
Mass Spectometer	SANIST platform	ISB srl	13/09/2021	06/2022
SACI	SACI version for protein analysis	ISB srl	02/02/2021	06/2022
Component	**Manufacturer**	**Expire**	**Lot nbr**	**Product code**
Deionized water	VWR	07/09/2023	21J014008	83645.320
Arginine	Sigma-Aldrich	08/09/2022	1374555341408312	2008111

300°C while the dry gas was at a temperature of 320° C. The voltage at the entrance ion lens was set at 550 V. The charged water clouds between 100 and 800 intensity were focused for analysis by the mass analyser.

An electromagnetic frequency fields generator was used to emit EM radiations between 7 Hz to 80 Hz. The low-frequency radio waves at 7 Hz to 80 Hz are able to deposit their energy more profound into the material because of better penetration but at the same time are non-ionising. These waves are able to agitate polar molecules leading to their vibration, and hence increase in temperature is achieved.

Results

Figure 1 depicts the MS result of the charged water cloud m/z ion species. As prominently highlighted in the chart, the mass difference of 18 Da between the peaks unambiguously corresponds to the H_2O molecular weight. The peak of 220 corresponds to the water cluster $(H_20)_{12}$. While the peak at 238 represents $(H_2O)_{13}$. And further, when one more molecule of water gets added, it results in m/z of 256, which is $(H_2O)_{14}$. Similarly, adding one molecule of water to the peak of 222.9 takes it 240.9 and finally to 258.9 with more additional H_2O.

To further verify and confirm the presence of water clusters, the ion at m/z 256.8 [corresponding to $(H_2O)_{14}$] was isolated and fragmented further. The corresponding result was as depicted in Figure 1b. And in this particular case, a neutral loss of 18 Da emphatically vouches for the presence of neutral water species.

The experiment had two samples of water, out of which one was pure and not exposed to any radiations or physical effect, and the other was subjected to electromagnetic radiations between 7 Hz and 80 Hz for 5 minutes. The results of the MS analysis of both the samples are represented in the Figure 2a and 2b respectively.

Interestingly in both the samples, two water ion clusters are observed at average m/z 250 and 650, respectively. And these are $(H_2O)_{14}$ and $(H_2O)_{36}$, respectively.

Further, it can be observed that the water ion cluster at m/z 250 of the untreated water registers an intensity of 1250 counts/s while the water ion cluster at m/z 250 in the case of irradiated water has an intensity of 400 Counts/s. Thus, intensity in the first case is more than double that of the second case.

Moreover, the abundance of the lower m/z cluster as compared to the higher ones in electromagnetically treated water is lower against the same parameter of the untreated water.

However, the intensity of about m/z 650 in the case of treated water seems to be lower in absolute values compared to untreated water. And this indicates the lesser formation of larger clusters in treated water than in untreated water for the same value of m/z.

As the experiment progressed further, some water cluster species seemed to be localised near the analyte species, as observed in the full scan spectrum. A solution of Arginine at a high diluted concentration of 100 ng/mL was infused and exposed to treated and untreated water,

Fig. 1. a) Water cloud ion species, b) Fragmentation spectrum of the water cluster species at m/z 256.8.

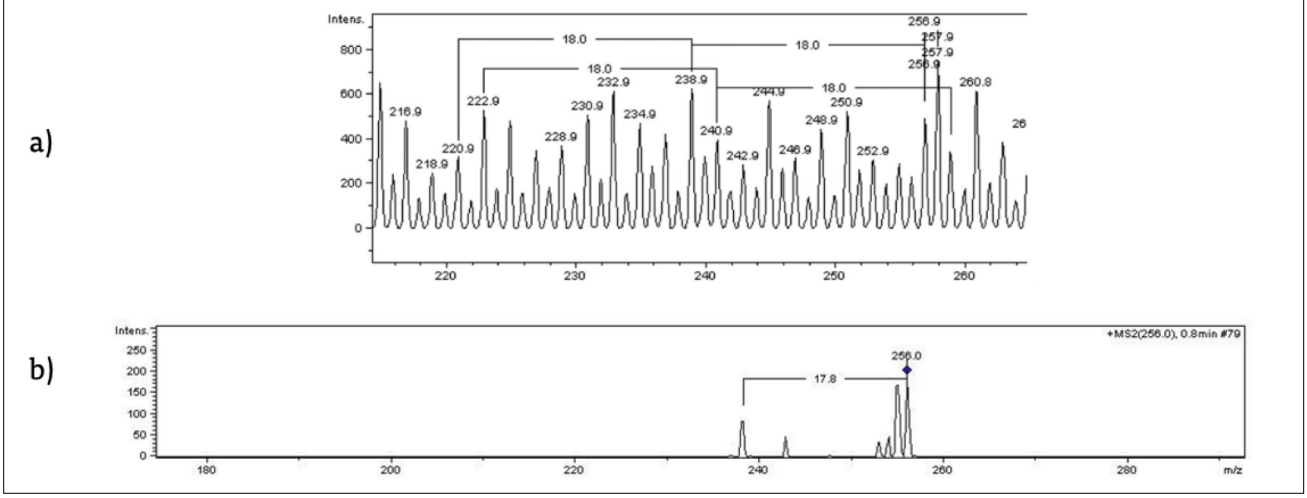
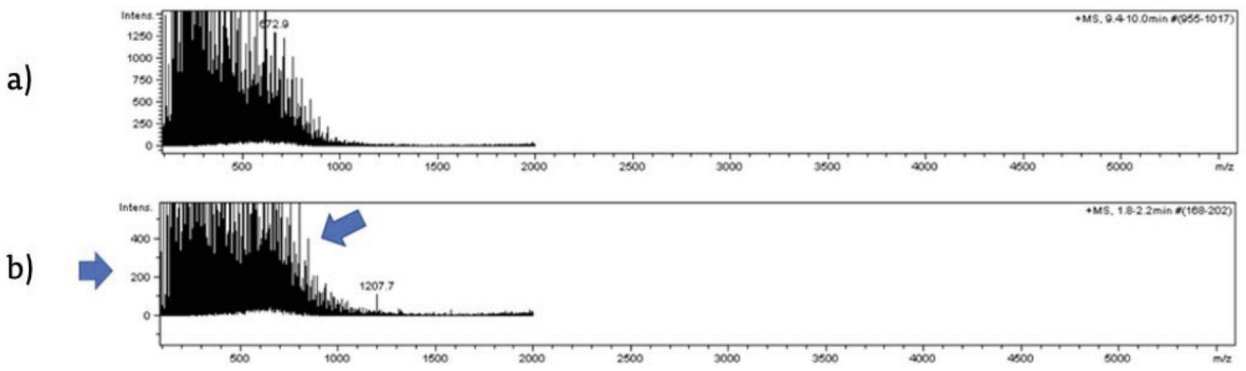


Fig. 2. Water ion cluster distribution obtained in a) not irradiated and b) treated water (7-80 Hz).



respectively. These two samples were further analysed, and Figure 3a shows the result of untreated arginine water solution while Figure 3b shows the result of treated arginine water solution.

The First mass spectrum (Fig. 1b) does not show the presence of water clusters.

However, in the second one, the treated Arginine solution (Fig. 3b), a clear ten-molecules water cluster peak at m/z 181 was observed (red arrow in Fig. 3b). It is interesting to note that water clusters exists in the presence of Arginine in water.

Figure 4a below shows the Total ion current mass chromatogram of arginine solution 100 ng/ml non-radiated (Red Line) and irradiated (Green Line), which is an aggregation of hundreds or even more of the mass to charge units revealed in the mass spectrums. It can be observed that the intensity of the radiated dilute arginine solution is lower than that of the untreated arginine solution. The radiated solution (green line) has comparatively lesser peaks and is relatively smoother as compared to the non-radiated sample of Arginine (red line).

The mass spectra of the two arginine solutions also indicate lower intensity values for the radiated sample compared to the non-radiated ones. Also, the number of visible peaks in the radiated sample is lower than that available for the non-radiated sample.

As indicated in Figure 4b, Arginine is prominently present at m/z 175.4; however, its intensity in the case of untreated Arginine solution seems higher than in the treated solution. Around m/z values of 193, the Arginine with water molecule can be detected. In general, the number of peaks in the irradiated samples is lesser than in the non-radiated samples. The continuance of the study could perhaps lead to a better understanding of the relation between the duration of irradiation and the formation of water clusters. Further research and studies can be carried out to determine if the effect of irradiation is long-lasting or even permanent. In fact, the literature shares some hypotheses, wherein it is believed that the effects of the electromagnetic field treatment can be long lasting [11].

Discussion

The results of the current study deploying the SA-CI-CIMS method also evidenced a presence of water

Fig. 3. a) Mass spectrum of arginine solution in untreated water, b) Mass spectrum acquired with Arginine in acqueos solution in full scan mode.

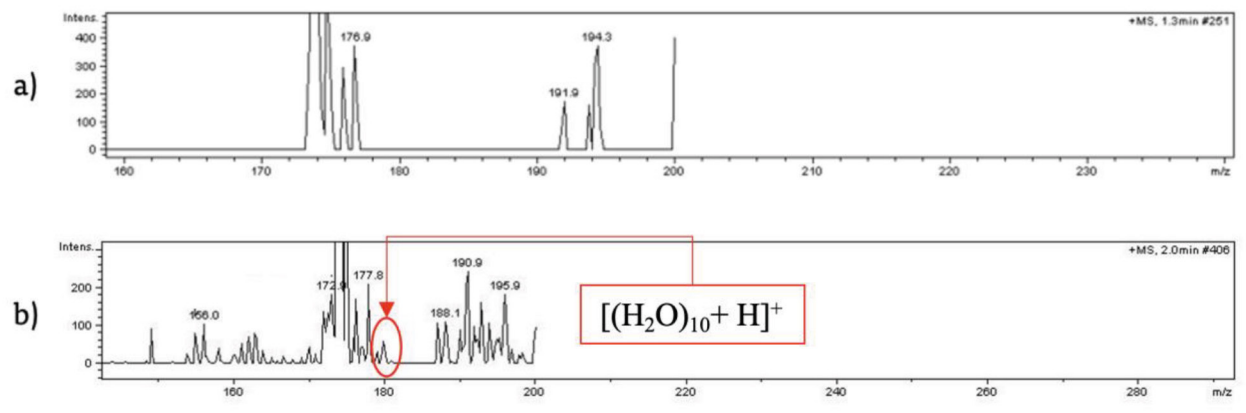
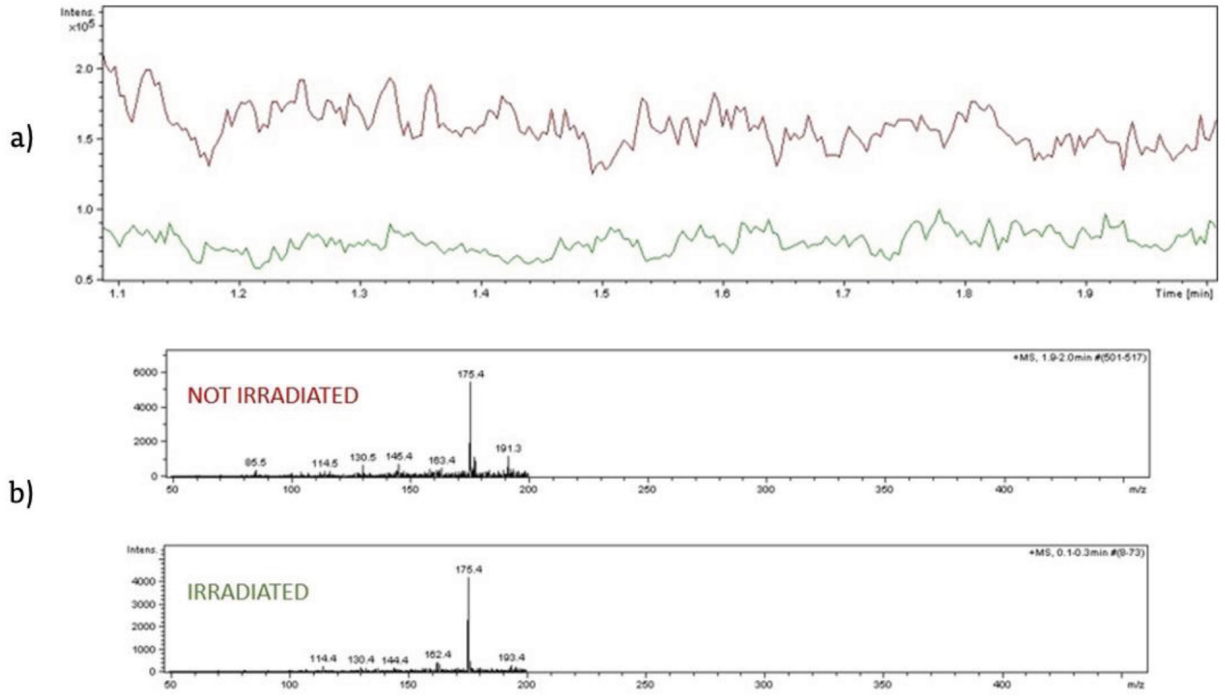


Fig. 4. a) Total ion current mass chromatogram of arginine solution 100 ng/ml non-irradiated (RED LINE) and irradiated (GREEN LINE), b) Mass spectra of arginine solution 100 ng/ml non-irradiated (RED) and irradiated (GREEN).



clusters similarly to other experimental methods such as the ones conducted by Simone Konig and Henry Fales of National Institutes of Health, Bethesda, Maryland, USA [12] and also in line with the results of another experiment conducted by Frank N. Keutsch and Richard J. Saykally, Department of Chemistry, University of California [13].

Cluster formation is one of the peculiar properties of a water molecule. And this is due to its molecular structure, which is responsible for the critical role of water in chemistry, biology and life.

Molecular Structure and Spectral Analysis Of Water

Based on the recent theories, analysis of the behaviour of water molecules resultant from its biphasic structure describes it as a dynamic dissipating system. A significant molecule on earth, water, is composed of one atom of oxygen and two of hydrogen. A covalent bond between the oxygen and the two hydrogen atoms builds the water molecule. The two hydrogen atoms share a pair of electrons with the oxygen atom. However, this arrangement renders the oxygen with an electronegative charge while the hydrogen atoms are electropositive. Consequently, the water molecule is a dipolar molecule. And the presence of this electric dipole enables water to orient itself in the presence of external electric fields.

The spectral analysis of the water molecule highlights the asymmetric nature of its structure, which explains many unusual properties of water. The two O-H covalent bonds are symmetrical with a length of 0.97 Angstroms) and an angle of 104.5° between them [14]. Due to the dipole property, a water molecule can build *hydrogen bonds* with four neighboring water molecules forming pentamers, as well as to form bigger aggregates (e.g. octa-, icosa- or higher eders) in various configurations, which are, however, highly dynamic [11]. The excellent solvent capabilities and properties of water can be attributed to its unique structure.

The Water structure

It has been shown that at the molecular level, water does not have a homogeneous structure but, instead, is in dynamic equilibrium between the changing percentages of assemblies of different kinds of oligomers and polymers. The structure of these 'clusters' or the units themselves is dependent on temperature, pressure and composition [15]. Recent studies indicate the ability of the water molecules to rearrange themselves into different clusters depending on the electromagnetic fields that it is subjected to, rather than being just a homogenous fluid.

Frank and Wen introduced the concept of the flickering cluster model, resulting from water-water interaction wherein the hydrogen bond resonance between electrostatic and covalent bonding lead to the formation and destruction of aggregates of water molecules tetrahedrally linked by dipolar bonds [16]. Essentially the surrounding water is responsible for the cluster of water molecules created.

The model proposed by Emilio Del Giudice and Giuliano Preparata (1988) is characterised by a larger scale of clusters [16]. Based on quantum field theory, they de-

scribe sub-micron-sized coherence domains of water, each of which can contain many millions of molecules. The bonds between water molecules within these domains can be thought of as antennae receiving electromagnetic energy from outside. And with this received energy, the water molecules can release electrons, making them available for chemical reactions. The size of the coherence domains corresponds to the wavelength of the coherent field and, in the case of water, corresponds to about 1/10 micron.

Though it has been established in the last decade that magnetic and electromagnetic stimulations alter the physicochemical properties of water, the exact mechanism of the phenomena eludes the scientific community [17, 18].

Quantum Field Theory

This approach encouraged Emilio Del Giudice, Giuliano Preparata and many other researchers to study water and its characteristics from different perspectives.

The generally accepted model assumes that water molecules are connected to each other through *'hydrogen bridges'* that arise from their hydrogen atoms, which are supposed to be abandoned by the electrons migrated into the molecule's electron cloud. Though the term hydrogen bridge is used in chemistry and biology as well, their conceptual meaning is different in either case.

According to the classical model, the abandoned hydrogen atoms become a source of attraction for the electronic clouds of the adjacent molecules, which, swelling outwards, form a bridge with the hydrogen nucleus. This is why quantum physics interprets hydrogen bridges as the consequence of the dynamics of electrodynamic quantum coherence that leads the gas to collapse into a liquid and not as the cause of the cohesion of the liquid. However, in biological processes, molecules follow chemical codes (e.g. DNA, Krebs cycle, Respiration) which lead a given molecule to interact only with a predefined one. Del Giudice and Preparata, therefore, wondered what the physical dynamic is that gives rise to this ordering, i.e. that causes the molecule to interact only with a given one and not with the others. The answer to these questions comes from applying quantum field physics to describe the molecular model of water and its behaviour. In the light of quantum physics, water takes the form of a complex autopoietic system characterised by a structure consisting of two phases: coherent and incoherent [19].

At this point, it is worth introducing the theorem postulated by Giuliano Preparata, which encapsulates years of experimentation and the work of many scientists [20].

"*Given an extended electromagnetic field with frequency F0 and two molecules with frequencies F1 and F2 respectively, if the frequencies of these molecules are different, nothing 'happens', i.e. short-range interactions can take place. If the frequencies of these molecules and the field are equal, then the molecules attract each other with a tremendous force. This can happen even at a distance, depending on the size of the field. There is, therefore, a principle of selection that can take place even at great distances, thus providing an explanation for the rapidity of biological reactions*".

On the basis of this theorem, the group led by Del Giudice hypothesised the existence of a background electromagnetic field with a given frequency that is able to attract molecules vibrating at the same frequency.

According to Giuliano Preparata, there is a threshold of matter density beyond which a 'super-radiative phase transition' occurs: the system reorganises itself and reaches a new stable configuration, in which the matter field and the electromagnetic field coherently oscillate at a common frequency [20].

This leads to a significant energy gain proportional to the density. Hence, the density increases enormously up to the limit value determined by the repulsive forces linked to the Pauli principle, as well as those linked to the Coulomb repulsion [21].

The cohesive part, highly structured in tetrahedral form, which simulates the so-called hydrogen bond, generates magnetic patterns capable of interacting in principle with weak electromagnetic signals and storing the information they carry [22].

The coherent network of Water Coherence Domains then becomes a candidate for being a dissipative system, as described by Prigogine.

The interaction between water-protein has been accepted as a significant influencer of chain folding, internal dynamics, conformational stability, binding specificity and catalysis for a substantial period of time [23].

The standard quantum theory fails to propose quantum coherence for liquid water because of its inability to recognise quantum fluctuations and interaction between light and water. Despite the fact that conventional quantum electrodynamics field (QED) theory applies only to gases, Giuliano Preparata, Emilio Del Giudice, and others broadened its scope to include the condensed phase of liquids. It was subsequently observed that large and coherent domains (CDs) form when the vacuum electromagnetic field interacts with water. In such CDs, the water molecule constantly oscillates between the stable ground state and the excited state close to the ionising potential of water. Present in water at ambient temperature and pressure, these CDs offer possible explanations for the bizarre behaviour of water and are responsible for the sustenance of life on the planet [24, 25].

The primary focus is observing the interaction between the Quantum Elementary Particles (atoms/molecules) with its radiative electromagnetic field. Several observations and research studies support the fact that under predetermined conditions, all the particles transit to a coherent self-consistent physical state oscillating in unison with the same phase between the ground state and an excited state just short of the first ionisation comprising of about a million of almost untethered electrons. Consequentially, the inner periphery of the CD sphere is most likely negatively charged with complementary positively charged protons just external to the coherent domain. Thus, the surface of the Coherent Domain develops into a "redox pile". This phenomenon of the stimulated CD lays the foundation for all the oxidation-reduction

energy metabolism that drives and sustains all the life processes [26].
The CD becomes a cavity for the electromagnetic field because the dynamics give the photon an imaginary mass, according to the Anderson-Higgs-Kibble mechanism. All the molecules within a CD show a larger volume than their ground state since they oscillate in unison between the ground state of the individual molecule and an excited state. The electrodynamic attraction is neutralised by thermal collisions that push the molecules out of phase. Therefore, at a non-zero temperature T, as in the case of Landau liquid helium, each liquid becomes a two-phase system, where a fraction Fc (T) of the constituent particles behaves consistently, while a fraction Fnc (T) = 1- Fc (T) forms a dense gas trapped between the CDs. Since coherent molecules are larger than incoherent ones, the density of the coherent fraction is lower than that of the incoherent one; the density of the coherent fraction of liquid water was estimated to be 0.92 – the same value as the density of ice. According to thermal dynamics, inside the liquid, there is a continuous crossing of molecules between the two fractions (coherent and incoherent) so that, while the total number of coherent molecules remains constant at a given temperature T, their spatial distribution keeps changing.
The same phenomenon, which has its roots in Coherent QED, maybe at the origin of the formation of water supramolecular structures, which can be obtained through various kinds of purely physical low-energy triggers, and at the same time, explain their permanence in time [27]. This characteristic explains why experiments such as neutron scattering, which have a greater resolution time than the typical time of change of coherent structures in space, find the liquid as homogeneous. Only experiments with a reasonably short resolution time (of the order of collision time, 10^{-10} s) could detect the actual uneven structure of liquid water. However, there are other kinds of experimental considerations that may indicate the presence of larger aggregates in aqueous solutions that could be traced to QED predictions on coherent domains [28].

Conclusion

In line with the successfully conducted experiment, the authors could further study the water clusters using Ion-mobility mass spectrometry and detect the presence of water clusters in the tested samples. The spectrometry results of the diluted arginine solution infused with treated water also confirm the presence of the water cluster near the analyte molecule ion in agreement with the observation of Elia et al. (2014) as mentioned above [27]. The analyte ion is able to surround itself with rearranged water clusters when the water is treated electromagnetically. This helps confirm the ionic/polar concept explaining the excellent solvent properties of water.
It can also be concluded that electromagnetic radiations have the ability to reorient the structure of the water clusters. Water treated with low-frequency electromagnetic radiations water tends to form less of smaller clusters as compared to untreated water. It can also be concluded that exposure to electromagnetic radiations strengthens the hydrogen bonds of the water cluster resulting in a smaller number of low-intensity water clusters. However, this opinion is highly contested.
Moreover, the presented experimental results are supported by the detailed survey analysis of current theories based on the Quantum Field Theory of Coherent Domains presented in the discussion part.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; SC, ML, SM, IZ, KD, SP: literature search, editing and critical revision of the manuscript.
All authors have read and approved the final manuscript.

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Review

Gene variants in eating disorders. Focus on anorexia nervosa, bulimia nervosa, and binge-eating disorder

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Keywords

Eating disorders • Anorexia nervosa • Bulimia nervosa • Binge-eating disorder • Genetic variants • Genome-wide association studies

Summary

Eating disorders such as anorexia nervosa, bulimia nervosa and binge-eating disorder, have a deep social impact, concluding with death in cases of severe disease. Eating disorders affect up to 5% of the population in the industrialized countries, but probably the phenomenon is under-detection and under-diagnosis. Eating disorders are multifactorial disorders, resulting from the interaction between environmental triggers, psychological factors, but there is also a strong genetic component. In fact, genetic factors predispose for approximately 33-84% to anorexia nervosa, 28-83% to bulimia nervosa, and 41-57% to binge eating disorder. Twins and family studies have provided an unassailable proof on the heritability of these disorders. Other types of genetic studies, including genome-wide association studies, whole genome sequencing and linkage analysis, allowed to identify the genes and their variants associated with eating disorders and moreover global collaborative efforts have led to delineate the etiology of these disorders. Next Generation Sequencing technologies can be considered as an ideal diagnostic approach to identify not only the common variants, such as single nucleotide polymorphism, but also rare variants. Here we summarize the present knowledge on the molecular etiology and genetic determinants of eating disorders including serotonergic genes, dopaminergic genes, opioid genes, appetite regulation genes, endocannabinoid genes and vitamin D3.

Introduction

Eating disorders (EDs) are disorders characterized by an alteration in eating habits and by excessive worry and distorted vision of one's own body weight, associated with a high rate of morbility and mortality [1]. The main EDs recognized by the Diagnostic and Statistical Manual of Mental Disorders are anorexia nervosa (AN), that is characterized by distorted body image and weight loss, bulimia nervosa (BN) and binge-eating disorder (BED) characterized by consume of large quantities of food in a short time with loss of control, which in the case of BN can be followed by compensatory behaviors such as abuse of diuretics and laxatives and self-induced vomiting [2]. About 70 million people across the world of every gender, age and ethnic group have been reported an eating disorder. The etiology of eating disorders is influenced by developmental, social, and biological processes [3].

By twin and family studies, it was extensively demonstrated that eating disorders are heritable [4]. These studies had also demonstrated a high level of shared genetics between AN and BN [5-6]. Subsequent genome-wide association studies (GWAS) were fundamental to understand the molecular mechanisms involved in eating disorders. In particular, genetic risk score from GWAS results can be very useful in clinical practice [7]. Currently, there are especially GWAS results on anorexia nervosa with multiple genetic loci identification, suggesting that several genetic variants are associated with AN disease risk [8].

The role of rare and structural variants in eating disorders it was explored by studies of whole-exome and whole-genome analyses. Instead, gene expression studies had offer insight into the genes and molecular mechanisms that influence phenotypes [4].

Several neuropeptides, neurotransmitters and hormones are involved in eating disorders. The complex brain homeostatic control of feeding involves neural circuits located in the hypothalamus (hunger signals, initiating feeding behavior) and the brainstem (satiation signals, limiting meal size) [9]. Hypothalamic NPY/AgRP neurons produce Neuropeptide Y and Agouti-related peptide responsible for an orexigenic signal increasing the ACTH, cortisol and prolactin release and involved in appetite regulation. These neuropeptides are associated to high food intake with up-regulation in AN [10]. Instead, orexins are orexigenic neuropeptides involved in endocrine system regulation, with an important function in insulin, glucagon, and leptin secretion in response to

glucose [11]. Another neuropeptide involved in eating disorders is Proopiomelanocortin (POMC) is an anorexigenic peptide at the hypothalamic ARC regulated by leptin [12]. Several research connect also the peptidic hormone oxytocin signaling and eating disorders. Specific oxytocin receptor genes polymorphisms have been found [13].

Neurobiological mechanisms underlying eating disorders might involve an overreaction of the immune system, generating, in turn, a dysfunction of neuropeptide signaling. Also, the brain- gut-microbiota axis allows a bidirectional communication between gut microbes and the brain through endocrine, neural, immune, and metabolic pathways [9].

Although determining the cause of eating disorders is complex because there are both genetic and environment factors that can contribute to their development, there is growing scientific interest to identifying causal genes of eating disorders.

Genetics research can improve knowledge about the heritability of eating disorders thanks to new molecular technologies such as *Next Generation Sequencing* [4].

Here, we focus on genetic knowledge summarized in order of the type of eating disorder considering all the most recent genetic results.

Anorexia nervosa

The genetic mechanisms underlying AN have been the most investigated and studies related to this disorder are those that have provided the most results. AN is a multifactorial disorder with a strong genetic component. The familial nature of AN has already been demonstrated for several decades, with a heritability range of 33-84% [14-15].

Several genetic studies have made it possible to identify many genetic loci involved in molecular pathways that might lead to anorexia [16].

- *Serotonergic genes*: the serotonin or 5-hydroxytryptamine (5-HT) system is involved in food intake, mood, and body weight regulation [17]. It has been hypothesized that 5-HT activity is altered in the acute illness state of AN. Most positron emission tomography studies of AN patients have targeted the 5HT1A and 5HT2A receptors and 5HTT [18]. In particular, important AN targets could be the 5-HT2A receptor gene and the 5HT-transporter-linked polymorphic region (5-HTTLPR) [19]. An increase in 5-HT reuptake occurs following the administration of estrogens which alter the mRNA and protein levels of some markers of serotonin [20]. Moreover, AN patients may have *HTR1D* gene variations [21]. Recently, it has been identified a positive relationship between the serotonin transporter gene *SLC6A4* methylation levels and resting-state functional connectivity between the dorsolateral prefrontal cortex and the salience network in AN patients [22]. Anyway, it is unlikely that this pathway is the only one involved in the onset of AN in a subject because it is associated with numerous psychiatric disorders and therefore cannot be considered a specific vulnerability factor for AN [16-19].
- *Dopaminergic genes*: The dopaminergic system modulates thinking processes, reward, emotional behavior, substance dependence, feeding and motor activity. Dopamine (DA) is a catecholamine that acts primly through of two G protein-coupled DA, D1 (D1R) and D2 (D2R) receptors. DA has been implicated in the pathophysiology of AN by preclinical and clinical evidence. A gene that plays an important role in the dopamine system is DAT1 that encodes a transmembrane protein that regulates dopamine reuptake from synapses and possesses variable number of tandem repeats in its 3'-untranslated region. Polymorphisms in the number of repeats influence *DAT1* expression (VNTR 10R/9R). The TaqIA restriction endonuclease site in *DRD2* (rs1800497) has been shown to reduce the density of D2 autoreceptors in the striatum. Moreover, the rs6280 variant in *DRD3* increases the affinity for endogenous dopamine. Recently, it has been demonstrated that AN patients carrying the homozygous variant Gly9Gly genotype in the dopamine D3 receptor have worse symptomatology [23].
- *Opioid genes*: Opioid receptors are involved in food intake, reward sensitivity, pain, and vulnerability to addictive disorders. Several *OPRD1* polymorphisms were associated with AN. In particular, *OPRD1* variants were associated with AN restricting type [21].
- *Appetite Regulation Genes:* The communication between gut and hypothalamus involves a huge numbers of appetite hormones. After stimulation, anorexigenic peptides are released while the levels of the orexigenic peptide ghrelin reduce. Ghrelin is an appetite stimulating hormone produced in the stomach and pancreatic cells that is inversely associated with body mass index (BMI) [24]. In response to prolonged starvation the level of ghrelin in the plasma increases [25]. Leptin is a hormone produced by adipocytes and involved in the food intake and regulation of energy balance [26]. In AN patients the level of plasma circulating leptin in cerebrospinal fluid is reduced (hypoleptinemia) [27]. The serum level of leptin is significantly decreased in AN patients but only moderately increased in obese patients [28]. An increased concentration of NPY, which mediates leptin receptors, is associated to body mass deficiency with high concentrations of leptin, suggesting defects in the regulatory axis [29].

  The pancreatic polypeptide (PP) peptide tyrosine-tyrosine (PYY) belongs to NPY family and is postprandially secreted in ileum and colon with an anorexigenic role [30]. Its peripheral administration decreases appetite along with weight loss through inhibition of the arcuate hypothalamic nucleus expression of NPY/AGRP [31]. Anyway, serum levels of PYY hormone are less diminished in AN as comparated to BN/BED [6].

  Cholecystokinin (CCK) is a peptidic hormone of the gastrointestinal system that promotes satiety but has

been also associated with anxiety [32]. CCK plasma levels in AN patients and control group are similar both prior to and after a fooding suggesting a hormonal adaptation. However, in older analysis, in AN patients CKK plasma showed a postprandial increase [33-34].

GLP-1 is a brain-gut peptide that exerts a hormone-neurotransmitter action increasing satiety and inhibiting food intake, energetic expenditure, and insulin levels [35]. GLP-1 level decreases in AN patients, while insulin and glucagon levels increase, indicating an alteration in glucose homeostasis [36]. Oxyntomodulin (OXM), which acts through GLP-1 receptor, inhibits food intake, and reduces plasma levels of ghrelin [24].

- *Endocannabinoid genes*: endocannabinoid system controls food intake through both central and peripheral mechanisms. CB1 and CB2, the cannabinoid receptors, are expressed in multiple brain regions that control food intake [37]. Genetic variants in *CNR1* and *CNR2* genes, influence food intake and body weight and they have been associated to AN [38]. Systemic and local administrations in animals of both exogenous cannabinoids (i.e. THC) and endocannabinoids (i.e. AEA, 2-AG) increase food intake [39]. CB1 receptor antagonists are hypophagic and reduce body weight [40]. Cannabidiol, quite the opposite, can prevent the hyperphagic effect induced by the CB1 receptor agonist [41-42]. Genetic variants in *CNR1*, which encodes the CB1 receptor, are related to the susceptibility to AN. The basis of the non-Mendelian inheritance of AN could be associated with *CNR1* (AAT)n trinucleotide repeats, but functional studies are needed to prove the differential effect [43].

  Anandamide, also known as arachidonoylethanolamine (AEA) plays a key role in feeding behaviour generating pleasure after food consumption [44]. Plasma levels of this lipid mediator are downregulated in AN patients [45].

  In fact, anandamide binds to CB1R and inhibits neuronal differentiation [46].

  Palmitoylethanolamide (PEA) binds the cannabinoid-like G-coupled receptors GPR55 and GPR119. The anorectic action of exogenous PEA is mediated by transcription factor PPARα in the small intestine [47]. After a high-fat feeding in mouse the concentration of PEA decreases [48]. Plasma PEA concentration increases in AN patients after exposure to a non-favorite meal [49].
- *Vitamin D3:* Vitamin D3 is a steroid hormone whose deficiency, that leads to defects in bone mineralization, has been associated to AN [50-51]. Vitamin D3 modulates peroxisome proliferator-activated receptor gamma (PPARγ), involved in inflammation related to the diet [52]. The role of vitamin D3 in AN might be associated to its ability to regulate neurotrophic factors, guaranteeing neuroprotection and neurotransmission control [53].

By linkage and association studies on AN, chromosomes 1, 2, 4, and 13 were identified as possible regions associated to AN. The analyzed genes were associated to neural signaling, either by neurotransmitters or by hormones affecting the satiety regulatory system in subcortical structures of the brain, such as the hypothalamus. However, the small sample size of these type of studies was a limit and meta-analyses gived disaccord evidence [5].

Several GWAS for the identification of genetic variations related to the disorder were conducted on AN. Until 2019 a single genome-wide locus on chromosome 12 (lead SNP: rs4622308) related to AN was identified in a region that regard also diabetes mellitus type 1 and autoimmune disorders. Interestingly, successively the Anorexia Nervosa Genetics Initiative (ANGI) the Genetic Consortium for Anorexia Nervosa (GCAN), and the Wellcome Trust Case Control Consortium-3 (WTCCC-3) along with UK Biobank have detected eight chromosomal regions, comprising 120 genes, significantly associated with AN. Analyses in silico and research by available large-scale in vitro data have revealed that four of the genes of these chromosome regions might be more likely to be associated to the AN etiology: *CADM*1, *MGMT*, *FOXP1*, and *PTBP2* [8, 16, 17]. Interestingly, Hinney et al. (2017) [53] described three significantly altered loci correlating AN risk with increased BMI. The genes associated to those loci are *CTBP2*, *CCNE1*, *CARF* and *NBEAL1* [54]. In a large screening of 152 candidate genes by GWAS rare variants associated to AN were identified in *EPHX2* that encodes a protein involved in cholesterol metabolism. Moreover, variants in *ESR2*, encoding the estrogen receptor 2, can be associated with AN in female [55].However, at the base of the limits of these studies there are several factors, such as the winner's curse, small sample size, moderator variables explaining and lack of heterogeneity of the cohorts [4, 8]. Anyway, the results of these GWAS showed that AN is highly polygenic.

By whole genome sequencing and linkage analysis to analyze two families with recurrence of eating disorders, were detected a missense variant cosegregating with the affected family members in *ESRRA*, and a potentially damaging variant in *HDAC4* (histone deacetylase 4) that play a significant role in the estrogen system. transcriptional studies revealed that expression of the *HDAC*4 deacetylase repressed the transcription of ESRRA- induced target genes, whereas *ESRRA* and *HDAC4* exhibited interaction in both in vivo and in vitro studies. For which variants in *ESRRA* and *HDAC4* cause a decrease in the activity of ESRRA and an increase in the likelihood of AN onset [56].

By familial whole-exome analysis, were been identified variants of *NNAT* in two male AN probands: one nonsense variant (p.Trp33*) and one rare variant in the 5′UTR. Moreover, by a large screening were identified 11 *NNAT* variants in AN patients (40% male and 6% female) [57].

In an another whole-exome sequencing study were identified genes carrying damaging variants belonged to

three pathways: (a) neuropeptide hormone signaling, (b) inflammatory pathway, and (c) cholinergic neurotransmission [58].
Recently, have been sequenced the whole exome of one family and found three ultra-rare deleterious variants of *DRD4*, *NMS*, and *CCKAR*, linked with the reward pathway, in three affected members. In the other study, the authors identified de novo variants in *CSMD1*, *CREB3*, *PTPRD* and *GAB1* involved in the dopamine pathway and neuron differentiation [59, 60].
Epigenetic mechanism may help initiate and maintain AN. Frieling et al. described higher levels of methylation in the promotors of DAT1 (dopamine active transporter 1) and DRD2 (dopamine receptor D2) in AN patients. Other study linked AN weight loss to hypermethylation and reduced expression of *POMC* [61].

Bulimia nervosa

Twin studies have yielded heritability estimates for BN ranging from 28% to 83% [14, 15]. As for AN, specific biological systems are involved in BN.

- *Serotonergic genes*: As with AN, BN patients develop an egosyntonic personality and is associated with other psychiatric diseases, moreover medications acting over 5-HT pathways are efficacy over BN patients, so it is conceivable role of serotonergic system dysfunction in eating disorders onset and progression [62]. Abnormalities in peripheral 5-HT uptake have been observed in BN patients [63]. As with AN, most genetic studies of the 5-HT system in BN have focused on the *5-HTTLPR* transporter gene and the *5-HT2A* receptor gene [64]. However, there are conflicting results on the involvement of this route on the BN [19].
- *Dopaminergic genes:* The dopaminergic system has also been of interest in the pathophysiology of BN, in fact abnormalities in DA system have been observed through neuroimaging investigations [65]. Mesocorticolimbic dopaminergic alterations correlate with an high physical activity in BN patients and can trigger a dopamine-dependent stress response. The dopaminergic system modulates thinking processes, reward, emotional behavior, substance dependence, feeding and motor activity. Dopamine (DA) is a catecholamine that acts primly through of two G protein-coupled DA, D1 (D1R) and D2 (D2R) receptors. DA has been implicated in the pathophysiology of AN by preclinical and clinical evidence. A gene that plays an important role in the dopamine system is *DAT1* that encodes a transmembrane protein that regulates dopamine reuptake from synapses and possesses variable number of tandem repeats (VNTRs) in its 3'-untranslated region (3'-UTR). Polymorphisms in the number of repeats influence *DAT*1 expression (VNTR 10R/9R). The TaqIA restriction endonuclease site in *DRD2* (rs1800497) has been shown to reduce the density of D2 autoreceptors in the striatum. Moreover, the rs6280 variant in *DRD3* increases the affinity for endogenous dopamine. Recently, it has been demonstrated that AN patients carrying the homozygous variant Gly9Gly genotype in the dopamine D3 receptor have worse symptomatology [66].
- *Opioid genes:* opioid peptides play a key role in feeding behaviour generating motivation and pleasure in food consumption, so it is likely to believe that opioid genes also play a decisive role in BN [43]. In fact, some reward-related brain dysfunctions have been described also on rodent animal models of BN by affecting also opioid levels [67]. The bulimia treatment with naloxone that is an opioid receptor blocker is very effective [68].
- *Appetite Regulation Genes*: Although appetite regulation genes are genetic candidates to BN, studies thus far have been limited as compared to AN studies. Polymorphisms in ghrelin and its receptor *GHS-R1a* are not associated with bulimia [69].

Although leptin modulate reward-related behavior that has a relationship with BN, currently conflicting results were obtained on the association of reward learning and plasma leptin levels in BN. However, a positive correlation of plasma leptin levels and BMI in subjects with BN has been described. The plasma leptin levels are restored in remitted BN patients and are a relevant factor for remission [70]. Finally, serum levels of PYY hormone are decreased in BN patients compared with AN patients [71]. Anyway, basal plasma PYY levels increase in the phases of abstinence from binge eating and vomiting to return to control levels after recovery in BN. However, fasting plasma PYY levels during symptomatic phase of BN were unchanged. BN patients have impaired secretion of CKK that is a satiety factor and PYY secretion inductor. Hence, depressed PYY levels may result from reduced CCK secretion. Moreover, there is a negative correlation between PYY increase and ghrelin decrease. For which a pathway involving peripheral hormonal signals, such as ghrelin and PYY, may be related to BN [72]. GLP-1 secretory decrease was detected in BN patients being this concurrence limited to binge-eating and vomiting episodes [73].

- *Endocannabinoid genes*: the endocannabinoid system plays an important role in the control
- of BN by acting via central and peripheral mechanisms. *FAAH* and *CNR1* polymorphisms have been associated to BN, but not found a synergistic effect of the two polymorphisms in BN. An association of a *CNR2* polymorphism with BN has also been identified [74].
- *Vitamin D3*: BN patients can show severe hypovitaminosis D3 responsible for lack of inflammatory response and reduction in mood in patients with long-term eating disorders.

Many linkage and candidate gene studies of BN have been conducted but have provided few definitive conclusions. Interestingly, in a linkage study of 308 families with BN a suggestive peak of linkage on chromosome 10p13-12 already associated to obesity was been identified. This linkage value increased in families with BN and self-induced vomiting [75].

By a GWAS three polymorphism for *HTR2A* have been associated with poor treatment response in BN patients [76].

Binge-eating disorder

Twins' studies have showed that the heritability of BED is 41-57% [15]. BED, obesity, and weight-related co-morbidities are genetically correlated [77].
Specific biological systems are involved also in BED.

- *Serotonergic Genes*: Polymorphisms of three serotonergic genes, *5-HTT, 5-HT2C* and *5-HT2A* have been investigated in BED [78]. In particular, important results were found by Monteleone et al., who analyzed the *5-HTTLPR* polymorphism of the *5-HTT* gene more frequent in obese people with BED [69, 78].
- *Dopaminergic genes*: Several studies have been examined the role of polymorphisms of 6 dopaminergic genes in BED: *DRD2, ANKK1, OPRM1, COMT, DAT1*, and *DRD3* [76]. In particular, the polymorphism Taq1A of *DRD2* in BED patients is associated to higher reward sensitivity and obesity [78-80].
- *Opioid genes*: The opioid antagonists treatment decrease intake of fat and sucrose diets and suppress palatable food intake [81]. Interestingly, in rat BED model, memantine treatment blocks the compulsivity associated with the intake of the highly palatable food [82].
- *Appetite Regulation Genes*: Serum levels of PYY hormone are decreased in BED compared with AN [71].
- *Other Genes*: Several polymorphisms of 8 other genes in BED have been investigated: *GR, MC4R, BDNF, prepro-NPY, prepro-GHRL, FAAH, FTO* and *CLOCK*. A significant association was found between the polymorphism rs6198 within exon 9 beta of *NR3C1*and BED [78].

In a GWAS 15 polymorphisms in *HTR2A*, a gene implicated in appetite process and satiety, have been analyzed in BED and the polymorphism rs2296972 in *HTR2A* has been associated with trend level of less likelihood of BED [76].
In conclusion, several genetic factors contribute to the etiology of eating disorders. The genetic studies show that while there are great genetic similarities between AN, BN and BED, there are also notable differences. Anyway, it is important a correct diagnosis also genetic because eating-disorders have a deep social impact and an enormous cost to public healthcare systems [83]. An important goal for clinical care of eating disorders is obtaining individual-level genetic information for improving management of the patients. Indeed, a precise genetic diagnosis of eating disorders is complicated by the fact that a complex and dynamic interplay between environmental factors, epigenetic marks, and genetic predisposition is involved in the development of these disorders. Gene variants identification in known genes and novel candidate genes identification are necessary. The limited success of genetic studies so far may also result from a focus on symptoms of the disorders, rather than the causes of them. Currently, it's possible integrating basic scientific understanding of the role of genetic risk in eating disorders into clinical practice and the ability to identify genetic markers of risk could allow for early screening in those at high risk. By genetic analysis can be obtained results to pursue to examine treatment response. Findings can also be included in multifactorial risk estimation algorithms that account for other genetic factors, such as rare variants, and environmental risk factors to improve prediction. Moreover, the use of animal models to determine genetic influences on feeding can improve understanding of genes that might represent novel pharmacotherapeutic targets for eating disorders. It is clear that there is a need for a specific and comprehensive molecular diagnostic test with optimal performance for the diagnosis of eating disorders. In this scenario, the usefulness of next-generation sequencing technology arises. It would be useful to use a molecular test that may include genes that have been found to carry polymorphisms conferring a higher susceptibility to the onset of eating disorders, genes that have been found to carry rare variants in eating disorders patients for whom segregation analysis has been performed, genes associated with syndromes presenting eating disorders among their main features and genes that have been found to carry polymorphisms conferring a higher susceptibility to the onset of disorders of food intake [16]. The UK Biobank (ukbiobank.ac.uk) is a unique epidemiological resource to improve prevention, diagnosis, and treatment of psychiatric and somatic illnesses [84]. Recently, it has been processed the Eating Disorders Genetic Initiative (EDGI) designed to expand genomic discovery across the three major eating disorders, AN, BN and BED, that represents the largest genetic study of eating disorders ever to be conducted and it builds on the Eating Disorders Working Group of the Psychiatric Genomics Consortium (PGC-ED and ANGI) studies. The data that EDGI regard the genetic factors influencing stability versus fluctuation of eating disorder clinical presentation and precision-medicine questions regarding identification of optimal interventions informed by genotype [85]. Therefore, ever new scientific efforts are underway to identify the genetic basis of eating disorders and to develop better eating disorders patient management.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

Matteo B: study conception, editing and critical revision of the manuscript; Kevin D, MRC, Kristjana D, GB, MCM, GM, VP, SX, Marsida B, DB, TB: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

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