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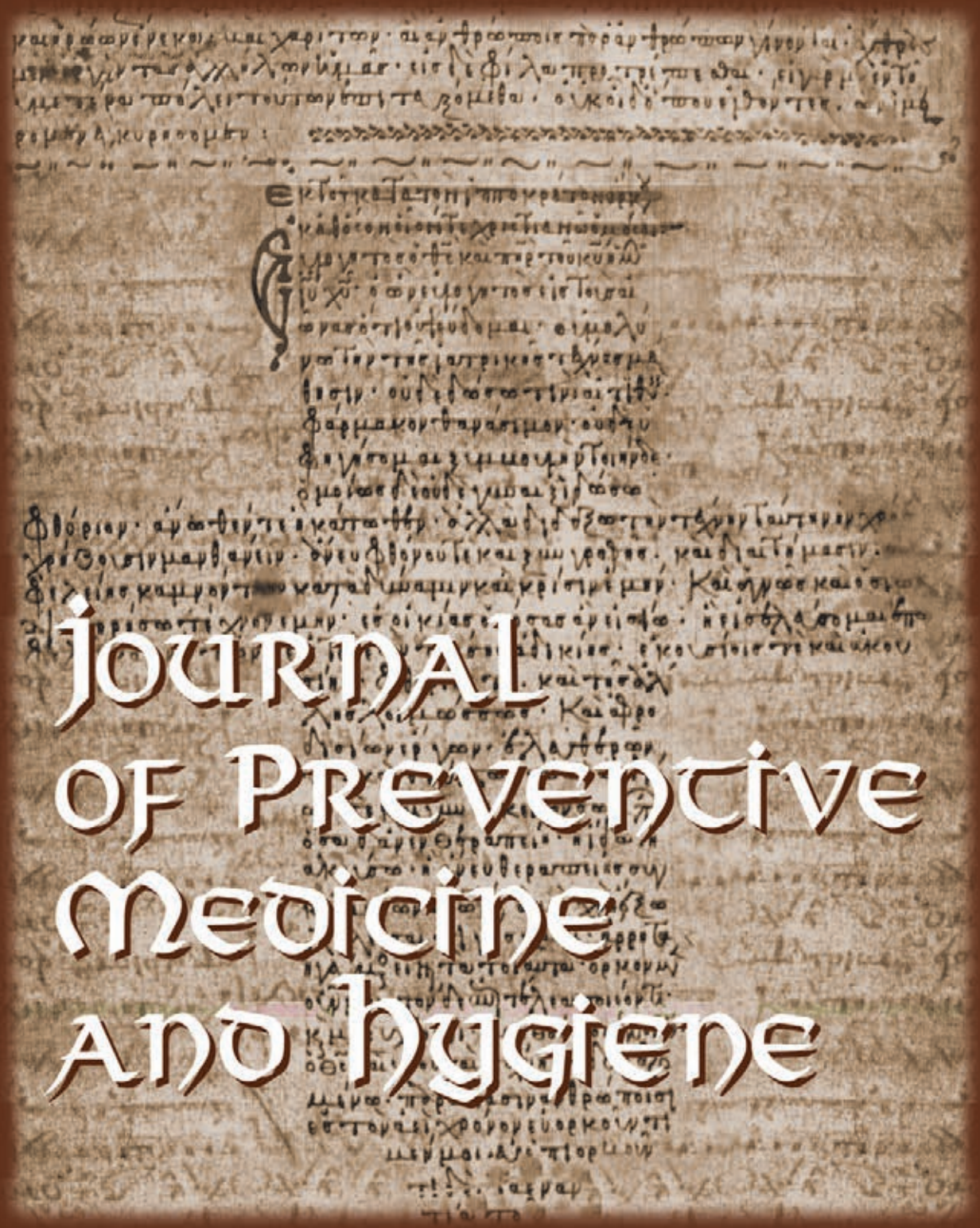

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REVIEW

Compounds with anti-influenza activity: present and future of strategies for the optimal treatment and management of influenza. Part I: influenza life-cycle and currently available drugs

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Influenza • Influenza life cycle • Antiviral • Licensed drugs • Amantadine • Rimantadine • Oseltamivir • Zanamivir • Peramivir • Laninamivir • Ribavirin • Arbidol

Summary

Influenza is a contagious respiratory acute viral disease characterized by a short incubation period, high fever and respiratory and systemic symptoms.
The burden of influenza is very heavy. Indeed, the World Health Organization (WHO) estimates that annual epidemics affect 5-15% of the world's population, causing up to 4-5 million severe cases and from 250,000 to 500,000 deaths.
In order to design anti-influenza molecules and compounds, it is important to understand the complex replication cycle of the influenza virus. Replication is achieved through various stages. First, the virus must engage the sialic acid receptors present on the free surface of the cells of the respiratory tract. The virus can then enter the cells by different routes (clathrin-mediated endocytosis or CME, caveolae-dependent endocytosis or CDE, clathrin-caveolae-independent endocytosis, or macropinocytosis). CME is the most usual pathway; the virus is internalized into an endosomal compartment, from which it must emerge in order to release its nucleic acid into the cytosol. The ribonucleoprotein must then reach the nucleus in order to begin the process of translation of its genes and to transcribe and replicate its nucleic acid. Subsequently, the RNA segments, surrounded by the nucleoproteins, must migrate to the cell membrane in order to enable viral assembly. Finally, the virus must be freed to invade other cells of the respiratory tract. All this is achieved through a synchronized action of molecules that perform multiple enzymatic and catalytic reactions, currently known only in part, and for which many inhibitory or competitive molecules have been studied. Some of these studies have led to the development of drugs that have been approved, such as Amantadine, Rimantadine, Oseltamivir, Zanamivir, Peramivir, Laninamivir, Ribavirin and Arbidol. This review focuses on the influenza life-cycle and on the currently available drugs, while potential antiviral compounds for the prevention and treatment of influenza are considered in the subsequent review.

Introduction

Influenza is a contagious acute respiratory viral disease characterized by a short incubation period, high fever, respiratory (e.g. runny and stuffy nose) and systemic symptoms (e.g. muscle or body aches) [1]. Most of the people affected by influenza recover in a few days or, at most, in 2 weeks. However, some patients may develop complications that may be very serious. The most common complications are bronchitis, pneumonia, ear infections, etc. People with underlying diseases, such as asthma, subjects with Chronic Obstructive Pulmonary Disease (COPD) or individuals with heart disease are at high risk of complications [2]. Related complications, such as myositis, acute encephalopathy or Reye's syndrome, are rare [3]. Reye's syndrome is classically characterized by rashes, vomiting and liver damage. It can typically occur during viral illness in children who have been taking aspirin for a long period [4].

Pneumonia can be caused by bacterial superinfection also called secondary pneumonia or viral pneumonitis [5, 6]. This is characterized by the complex interactions of host-co-infecting pathogens [7], and, in particularly frail and debilitated subjects, can result in the impairment of physical capabilities, dysregulation of immune responses and a delayed return to homeostasis [5, 6].
The burden of influenza is very heavy. Indeed, the World Health Organization (WHO) estimates that annual epidemics affect 5-15% of the world's population, causing up to 4-5 million severe cases and from 250,000 to 500,000 deaths [7]. The European Centre for Disease Prevention and Control (ECDC) estimates that approximately 10% of Europeans are infected each year [8]. Furthermore, the US government estimates that 5-20% of US residents catch influenza each year [9].
Influenza viruses belong to the *Ortomyxoviridae* family [10], with the other two genera being *Isavirus* and *Thogotovirus*, and have the ability to change their surface antigens relatively frequently [11]. When a major

variation occurs, if the virus adapts to humans during zoonotic spill-over, widespread diffusion of the virus is possible, resulting in a pandemic [12]. The most severe influenza pandemic was that of 1918, which caused 500 million cases and from 50 to 100 million deaths [13, 14]. During that devastating pandemic, the treatment of patients suffering from influenza was empirical and symptomatic, and was intended primarily to relieve fever and pain (e.g. aspirin administration), while epinephrine was used to treat forms of secondary pneumonia [15]. Only in the 1960s did the first antiviral drug against influenza, namely Amantadine, become available in the US [16, 17], while in 1993 another drug, Rimantadine, was authorized [18]. Later, in 1999, the anti-neuraminidase (NA) medications Zanamivir and Oseltamivir were both licensed in the US [19].

Since 1999, much knowledge concerning viral replication has been acquired, and new experimental hypotheses have been advanced for the development of new flu drugs and new protocols for both prevention and treatment. Anti-influenza drugs are an important complement to vaccination, which is the most efficacious weapon against the disease. In this review, it therefore seemed useful to deal with the issue of new/potential antiviral medications against influenza infections, especially in the light of the most recent scientific advances.

Biology of influenza viruses

Regarding the antigenic characteristics of the core proteins (nucleoproteins [NP] and Matrix proteins [M proteins]), three influenza virus types have been identified: A, B and C. Given the relevance of Influenza A Virus (IAV) to human pathology, we will provide a brief overview of its biology and life-cycle and underline the main differences among the three virus types in terms of structural and molecular biology.

The IAV particle varies in the range of 80-120 nm and is pleomorphic, being usually spherical, though cord-like forms with a diameter of 40-100 nm and a length in the range of 300 nm-20 μm can occur [11, 20, 21]. Transition from spherical to tubular form is not well understood: what is known so far is that M1 [22, 23], M2 [23, 24] and NP [25] could play a role in determining and modulating this process. Besides genetic traits, also the phenotype of the host cell, in terms of shape and polarization, seems to influence the viral form [21, 26]. Influenza B has a similar shape, being structurally indistinguishable from IAV [11], while Influenza C virus is usually filamentous and 500 nm long [11]. The IAV viral particle is an envelope made up of lipid rafts and spikes of two main types of glycoproteins: hemagglutinin (HA) accounts for about 80% (about 500 molecules) and NA for about 17% (about 100 molecules); M2 is the least abundant protein, with only 16-20 copies per virion [11, 27].

The particle of influenza B virus contains four proteins, namely HA, NA, NB, and BM2 [11, 28], while the particle of influenza C virus is made up of a major surface glycoprotein (HEF, hemagglutinin-esterase-fusion protein) and a minor surface glycoprotein (CM2). These surface glycoproteins form ordered hexagonal arrays [11, 29-32].

Underneath the viral membrane, M1 tightly binds the vRNPs, which consist of RNA strands (usually single strands but in certain cases double strands) [11] wrapped around NP and NEP, with a terminal polymerase ternary complex (PA, PB1, PB2). The genome is small (about 13-16 kilobases) and contains seven or eight pieces of segmented negative-sense RNA (eight segments for IAV and influenza B, seven for influenza C), each piece of RNA containing either one or two genes of 890 to 2,341 nucleotides each [11]. The genome codes for at least 11 proteins: hemagglutinin (HA) of about 76-77 kDa, NA of about 60 kDa, NP of about 60 kDa, M1 of about 28 kDa, M2 of about 15 kDa, non-structural protein type 1 (NS1) of about 26 kDa, non-structural protein type 2 (NS2) (also known as NEP: nuclear export protein) of about 11 kDa, PA of about 85 kDa, PB1 (polymerase basic type 1) of about 88 kDa, PB1-F2 of about 80 kDa [33-35] and PB2 (polymerase basic type 2) of about 91 kDa [36].

In particular, segments 1-3 code for the ternary polymerase complex (PB1, PB2 and PA, respectively), segment 4 for HA, segment 5 for NA, segment 6 for NP, segment 7 for the matrix proteins, and segment 8 for the non-structural proteins [11]. On the basis of the gene structures, segments can be divided into three classes: intronless, intron-containing and unspliced, and intron-containing and spliced. On the basis of the kinetics of the gene expression, they can be classified into "early" (segments 1-3, 5 and the unspliced segment 8 transcript) and "late" (segments 4, 6, 7 and spliced segment 8) classes. Usually, structural and functional/kinetic classifications do not correspond [37, 38].

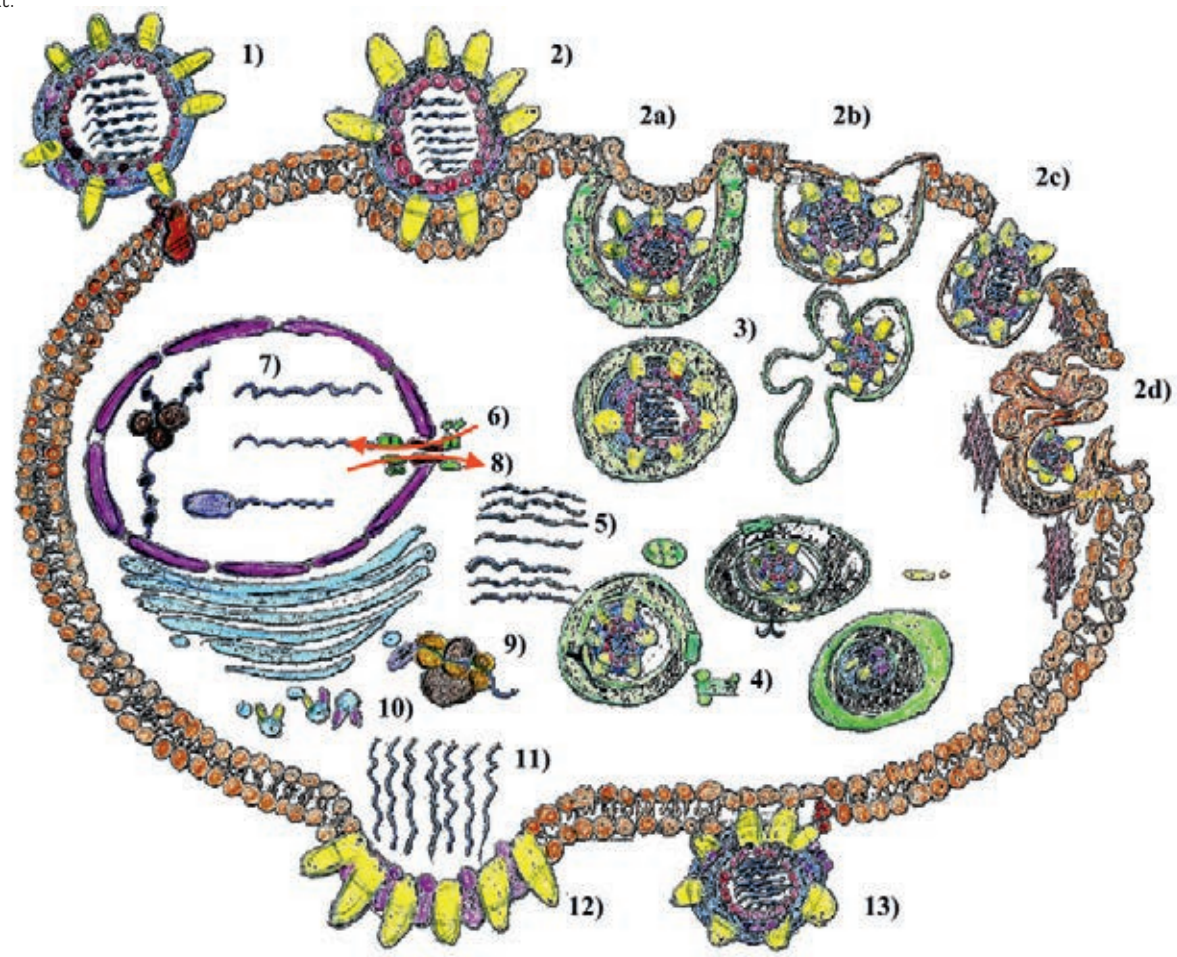
Moreover, the presence of overlapping genes and different splicing mechanisms may give rise to further accessory proteins, such as PB1-N40 [39], PA-X [39-40], PA-N155 [41], PA-N182 [42], M42 [43], and NS3 [43], which have been discovered and characterized only recently. Another accessory protein, NEG8 ORF, has been predicted [44].

The viral proteome thus reveals unexpected dynamism and complexity [43-44]; Matsuoka and coll. have designed a comprehensive map of influenza interactions, termed FluMap, which contains 960 factors and 456 reactions as of April 2012 [47].

Replication cycle of influenza viruses

The replication cycle of the virus (Fig. 1) is a complex, highly dynamic, biological process which consists of the following steps: 1) attachment of the virion to target cells and receptor binding (virus adsorption); 2) internalization into cellular regions by means of clathrin-mediated endocytosis (CME), caveolae-dependent endocytosis (CDE), clathrin-caveolae-independent endocytosis, and macropinocytosis; 3) endosomal trafficking via

Fig. 1. Schematic representation of the replication cycle of the influenza: 1) attachment of the virion to target cells and receptor binding (virus adsorption); 2) internalization into cellular regions by means of clathrin-mediated endocytosis (CME), caveolae-dependent endocytosis (CDE), clathrin-caveolae-independent endocytosis, and macropinocytosis; 3) endosomal trafficking via endosomes / caveosome / macropinosome / lysosomes to the perinuclear compartment; 4) pH-dependent fusion of viral and endosomal / organellar membranes; 5) uncoating; 6) nuclear importation; 7) transcription and replication; 8) nuclear exportation; 9) protein synthesis; 10) post-translational processing and trafficking; 11) viral progeny assembly and packaging; 12) budding; and 13) release. For further details, the reader is referred to the text.



endosomes / caveosome / macropinosome / lysosomes to the perinuclear compartment; 4) pH-dependent fusion of viral and endosomal / organellar membranes; 5) uncoating; 6) nuclear importation; 7) transcription and replication; 8) nuclear exportation; 9) protein synthesis; 10) post-translational processing and trafficking; 11) viral progeny assembly and packaging; 12) budding; and 13) release (modified from [48]).

The cells infected by the influenza virus are: alveolar and bronchial epithelial tissue (BET) cells, alveolar macrophages (AM), lung epithelial tissue (LET) cells and, in particular, type II pneumocytes, plasmacytoid dendritic cells (pDCs) and natural killer cells (NKs) [49, 50].

Influenza virus is able to activate Endoplasmic Reticulum (ER) stress, caspase pathway [51] or to finely tune host secreted molecules, such as lung mucins [52], in order to avoid being trapped and subsequently eliminated. Moreover, it recruits host factors and misuses them [53]. Although the mechanisms of influenza virus replication are not fully understood, scientific projects for new drugs against influenza cannot ignore the biological cycle of this virus.

Influenza virus entry

The first important event during infection in humans is the attachment of influenza virions to the apical cell surface (event known also as virus adsorption). Indeed, the entry of the Influenza virus into target cells is an essential process whereby viral genomes are delivered from extracellular virions to sites of transcription/replication in the cell nucleus [54]. During this phase, thanks to the surface glycoprotein HA, the virus interacts with (-2,3)- or (-2,6)-linked sialic acid receptors [55]. The physicochemical conformation of these receptors is not identical in different species of animals – humans, seals, birds, pigs, horses, etc., which are the natural reservoir of the virus. The vast majority of human receptors are located in the upper respiratory tract, but man also possesses receptors typical of birds, which are located deep in the respiratory tract [56].

HA is a homotrimeric integral type 1 membrane cylinder-like glycoprotein, approximately 13.5 nanometres long. HA monomers are synthesized as precursors (HA0) containing a hydrophobic signal sequence. After being translated, they are glycosylated and cleaved into two smaller subunits: namely, HA1 of 50 kDa and HA2 of 27 kDa, which are linked by a disulfide bridge. Each subunit is characterized by a long, central, α-helix connected to the membrane by HA2 and surmounted by HA1, a spherical head containing the sialic acid binding sites (receptor binding sites or RBSs, also known as receptor binding pocket or RBP) [57]. The apolar domain of HA2, near the cleavage site, is known as the "fusion peptide" or HAfp23 [58, 59], since it is characterized by a domain of highly conserved N-terminal 23 residues. HAfp23 has a helical-hairpin structure consisting of two tightly-packed helices, which are fundamental to inducing the negative curvature ("fusogenic conformation" of HA) [60].

There are at least eighteen HA subtypes [61]. These are further subdivided into two groups: group 1 comprises H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18 (from waterfowl, the last two having been recently isolated from bats in Guatemala and Peru) [61, 62]; group 2 comprises H3, H4, H7, H10, H14, and H15 [62]. However, a recent experiment has proved that the virus can also enter into cells whose surface has been completely depleted of sialic acid-based glycoproteins and glycolipids [63]. This seems to suggest alternative entry routes. Indeed, H17 and H18 do not bind to sialic acid and their receptor is still unknown [64].

Entry is essentially through receptor-mediated endocytosis [65], though an alternative uptake pathway, namely macropinocytosis, has quite recently been discovered [66-68]. Receptor-mediated endocytosis can be CME or mediated by lipid rafts: CDE or non-clathrin non-caveolae endocytosis [69-71].

CME is the most common pathway through which the virion is internalized. The clathrin triskelion is made up of three heavy chains, which constitute the backbone of the polyhedral structure, and of three light chains, which finely tune the assembly / disassembly of the triskelion [72]. Adaptor proteins, such as AP-2 [73], epsin-1 [74], epsin-15, recognize specific internalization signals located on cargo receptors, and take part in the formation of clathrin-coated pits (CCPs).

Caveolae are small flask-like infoldings of the membrane, with high levels of cholesterol and glycosphingolipids and with caveolin as the integral membrane scaffolding structure. The remaining entry routes have been investigated less. In clathrin caveolae-independent endocytosis, the Ras-phosphoinositide 3-kinase (PI3K) signalling pathway may play a major role [75].

The PI3K inactive complex (PI3K p110-p85 heterodimer) moves to the plasma membrane, where the SH2 (Src Homology 2) domains of p85 engage the phosphotyrosine residues present in receptor-associated proteins, causing a molecular rearrangement of the complex, in such a way that p110 is now enzymatically active and can recruit / produce intracellular second messenger, such as PtdIns(3,4,5)P3 (phosphatidylinositol-(3,4,5)-trisphosphate), from PtdIns(4,5)P2 (phosphatidylinositol-(4,5)-bisphosphate). Subsequently, several effector proteins with pleckstrin homology (PH) domains mediate an array of different signalling cascades: namely, activation of Akt, mTOR (mammalian Target Of Rapamycin), PKC (Protein Kinase C), PTEN (Phosphatase and tensin homolog) pathways [76].

Macropinocytosis can be activated by cell growth factors such as the Epidermal Growth Factor (EGF) or Nerve Growth Factor (NGF) or can be induced by phorbol esters. Macropinocytosis is Rac1-, PAK1- (p21-activated kinase type 1), cholesterol- and actin-dependent, since it extensively reorganizes the plasma membrane and implies energy mechanisms, such as Na^+/H^+ exchanger (NHE) activity [77, 78]. Also other pathogens such as Ebolavirus [79, 80], arenavirus [81], adenovirus [82, 83], HPV (Human Papillomavirus) [84], HIV (Human Immunodeficiency Virus) [85], Escherichia coli [86], Trypanosoma cruzi [87], vaccinia virus [88], African Swine Fever virus [89], RSV (Respiratory Syncytial Virus) [90] and HCV (Hepatitis C Virus) [91], among the others, use macropinocytosis as additional entry pathways.

These different routes (CME, CDE, non-clathrin non-caveolae endocytosis and macropinocytosis) result in different internalization yields and may be related to the different degrees of virulence and pathogenicity of influenza strains [92].

Endosomal trafficking

After attaching to the respiratory cell surface, the influenza virus, as already mentioned, can penetrate into the cell through more than one mechanism, and the final result is that the virions are internalized in an endosomal compartment [93]. According to the previous routes, virions can be packaged into CCPs and clathrin-coated vesicles (CCVs) or into caveosomes / macropinosomes. Subsequently, they are further processed into early endosomes (EEs) and into late endosomes (LEs) [94].

Endosomal trafficking usually follows influenza virus uptake. Virions are taken up into Rab-5/Vps4 and Rab-7/LAMP (lysosomal-associated membrane protein) positive endosomes. Rab-5/Vps4 and Rab-7 are Rab guanosine triphosphatases (GTPases) that are responsible for the processes of trafficking and fusion for EEs and LEs, respectively [95-97].

LAMPs are a series of proteins – 3 isoforms are currently known, namely, LAMP1 (known also as CD107a), LAMP2, and LAMP3 (DC-LAMP, TSC403 or CD208) – that are involved in endosomal maturation [98]. Moreover, the expression of LAMP3 has been found to be predictive of the host's response to influenza [99].

Cullin-3 (Cul3) belongs to the Cullin-RING-ligase family (CRL). It functions as a scaffolding protein in the Bric-a-brac, Tramtrack, Broad-complex (BTB)-Cul3-Rbx1 ubiquitin E3 ligase complex and is involved in LE maturation and cargo trafficking [100, 101].

Endosomal trafficking is a complex multifaceted process that can be divided into three stages: stage I, which

is actin-dependent, stage II, which is dynein- and microtubule-dependent, and stage III, which is microtubule-dependent [102].

Influenza envelope fusion

In order for the virus to emerge from the endosome and for ribonucleoprotein (RNP) to be released into the cytosol, the envelope must fuse with the endosome membrane. This part of the life-cycle of the virus requires the acidity of the lumen of the endosome to decrease to a pH value of about 5 [103, 104]. In this process, a crucial role is played by M2, which not only acts on ion channels, thereby allowing acidification of the interior of the virus [105], but also alters its conformation, resulting in changes of the curvature of the viral envelope [106, 107]. This leads to two important events, namely: the dissociation of the M1 protein from RNP and a dramatic change in the conformation of HA [108], which can expose its fusion peptide. This peptide may allow fusion of the viral envelope and the endosome membrane and the release of RNP into the cytoplasm. The cleavage of HA occurs through a proteasic enzymatic action [109]. Proteases that can cause the cleavage of HA are widely distributed throughout the human body, and the precise role that each plays in cutting the HA is not exactly known. Nevertheless, proteases are known to belong to two main classes, namely trypsin-like enzymes or furin-like serine enzymes [109]. These enzymes are produced by the cells, particularly those of the respiratory system, in the presence of inflammation (proinflammatory cytokines/chemokines, neutrophils, etc.) [110].

The release of RNP into the cytosol is also enabled by the host's aggresome processing machinery (made up of dynein, dynactin and myosin II) [111]. Further molecules and pathways could take part in this process.

M2 is more than just a simple ion channel. Indeed, it plays a multifaceted role in the entire viral life-cycle, as the mechanism of proton permeation, conductance and acidification is crucial to each different step and activity of the virus. After endocytosis, the low endosomal pH (in the range 5-6) activates the M2 channel prior to HA-mediated fusion, and favours dissociation of the M1 protein from the vRNPs, thereby facilitating the entry of RNPs into the nucleus. M2 is fundamental to genome unpacking and the release of the viral RNA during viral uncoating [112]. Moreover, M2 enables the viral RNA to package and facilitates virion scission and budding during viral maturation and assembly, replication and infection: during transport to the cell surface, in the trans-Golgi network (TGN) membrane, M2 acts as a proton-leak channel and prevents the activation of *de novo* synthesized HA by regulating the pH of the Golgi apparatus of the host cell and equilibrating its pH with the pH of the viral interior. Indeed, if it were not elevated in this way, the low pH would cause a conformational rearrangement of HA, whose intracellular cleavage would prematurely inactivate the new influenza viral progeny [113, 114]. M2, together with other proteins such as NS1 and HA, could play an additional role of fine-tuning the apoptosis of infected cells, thus favouring viral replication [115].

NS1 is involved in several biological tasks, such as mRNA splicing and translation, cell survival, and immune defence. In particular, as far as the type I interferon (IFN-I)-mediated response is concerned, it interacts with PACT/PRKRA (Protein activator of the interferon-induced protein kinase / Protein kinase, interferon-inducible double stranded RNA dependent activator), which is an important cofactor for the IFN-I response elicited by the viral RNA-sensor RIG-I (Retinoic acid-Inducible Gene I). Therefore, it blocks PACT/RIG-I-mediated activation of IFN-I [116, 117]. Moreover, it binds latent protein kinase PKR (Protein Kinase R, also known as Protein kinase RNA-activated or interferon-induced, double-stranded RNA-activated protein kinase, or eukaryotic translation initiation factor 2-alpha kinase 2 – EIF2AK2), whose activation would inhibit viral protein translation and synthesis [118], and also TRIM25 (tripartite motif-containing protein 2) [119, 120]. Recently, it has been shown to interact with an array of host proteins, such as interleukin-6 receptor (IL-6R), MHC class I HLA-B, cathepsin B, ubiquitin, and adenosine deaminase acting on RNA (ADAR1) [121].

With regard to M2 ion channel activity, the heart of this mechanism is the HxxxW motif of the inner transmembrane (TM) residues [122-125]. In this HxxxW motif, Histidine 37 putatively acts as the pH sensor and, when the pH is low, the protonation of the imidazolic ring destabilizes TM packing because of electrostatic repulsion. Tryptophan 41, which acts as a primary gate, rotates, becomes unlocked from Aspartic acid 44 ("the channel lock") and, being now parallel to the axis of the pore, makes the protons flow. By contrast, Valine 27 acts as a secondary gate (the so-called "Valine 27 valve"); its importance has been confirmed only recently by the multi-scale simulation carried out by Liang and coll. [126]. On the basis of the exact role of the Histidine 37 tetrad, two models have been proposed: the shutter model, in which the biprotonated charge of Histidine 37 does not change during the proton flux (proton diffusion is coupled with water wire via the Grotthuss mechanism), and the shuttle model, in which the protonation status of Histidine 37 is subject to changes during excess proton transfer [127]. However, the exact mechanism of highly selective transport of protons is not known.

Acidification is enhanced by the viral activation of the PI3K cascade [76].

Nuclear importation

The next event in replication is the importation of RNP into the nucleus. The trafficking of RNP into the cytoplasm is achieved extremely rapidly, by means of a mechanism of diffusion, without the intervention of microtubules, intermediate filaments or actin filaments, through the nuclear pore complex (NPC). Some important components of the NPC are the nucleoporins Nup37, Nup43, Nup45 [128], Nup50 [129], Nup54 [130], Nup58 [128], Nup62 [131], Nup75, Nup88, Nup93, Nup98, Nup107, Nup133 [128], Nup153 [132-135], Nup160 [128], Nup214 [130], NuTF2 (Nuclear Transport Factor 2) and Nup358/RanBP2 [128].

Nup37, Nup88, Nup96, Nup107, Nup133 and Nup160 belong to the so-called Nup107 subcomplex [136], while NupL1 (Nup45/Nup58), Nup54, and Nup62 belong to the Nup62 subcomplex [137]. Other components are Magoh, ALADIN, Tpr (Translocated promoter region), EJC (exon junction complex), NLP1/CG1 (Nucleoporin-Like Protein type 1), Seh1, Rae1/Gle2 and POM121 (nuclear envelope pore membrane protein type 121) [128].

When RNPs reach the nuclear membrane, nuclear importation is mediated by the binding of the nucleoprotein with the alpha importins, which then bind importin-β [129]. Simultaneously, the importins must interact with PA, PB1 and PB2 [130], and this affects the interaction of the RNP with the same importins [131]. Specifically, subunits PB1 and PA are imported by RanBP5 or karyopherin beta3 (also known as importin beta3, or importin 5), whilst subunit PB2 is imported by importin alpha-3 or importin alpha-7. NPs are imported by importin alpha-1 [132-141]. Other molecules that take part in nuclear importation are Hsp70 and Hsp90 [142-144].

In the nucleus, the importins detach from RNP. Although it is not clear, nor is the mechanism known at the molecular level, dissociation of the RNP should occur after separation from the importins. Thus, after the spreading of RNP in the nucleoplasm, transcription and replication can initiate.

Transcription and replication

The transcription and replication of viral nucleic acid are not fully understood. However, the phenomena involve coordinated and differentiated RNA, NP and RdRp activities. The package that consists of the genomic segments (sRNA), the unit of trimeric polymerase and the nucleoprotein is the elementary replication unit of the influenza virus (vRNP*)*. Therefore, in the nucleoplasm, vRNP needs to trigger the first round of its replication cycle, i.e. copying its genomic information onto mRNAs. Subsequently, mRNA exportation occurs in the cytoplasm.

The influenza polymerase is a heterotrimeric ~250 kD complex. It plays central roles in the viral life-cycle and is directly responsible for RNA synthesis for both viral replication and transcription. Moreover, it recruits host factors such as DnaJA1/Hsp40 [145].

The PA subunit interacts with host factors such as the mini-chromosome maintenance complex (MCM, a putative DNA helicase) and hCLE/CGI-99 [146, 147]. The PB2 subunit binds to the host RNA cap (7-methylguanosine triphosphate (m(7)GTP)) and supports the endonuclease activity of PA in order to "snatch" the cap from host pre-mRNAs [148, 149]. Moreover, PB2 interacts with the acetyl-CoA found in eukaryotic histone acetyltransferases (HATs) [150].

Viral mRNA synthesis is initiated by a *cis*-acting viral RNA polymerase, which is part of the vRNP structure and is bound to the vRNA promoter. However, mRNAs are not able to translate the genetic message efficiently; indeed, they need to be capped. Specifically, the virus must use the pre-mRNA of the cell and, for this purpose, a coordinated process mediated by PB2, PA and the cellular Polymerase II (Pol II) is necessary. Very briefly, PB2 binds mRNAs with cellular Pol II and PA, which, by means of an endonuclease mechanism, generates capped-mRNAs, which are translatable at the ribosomal level.

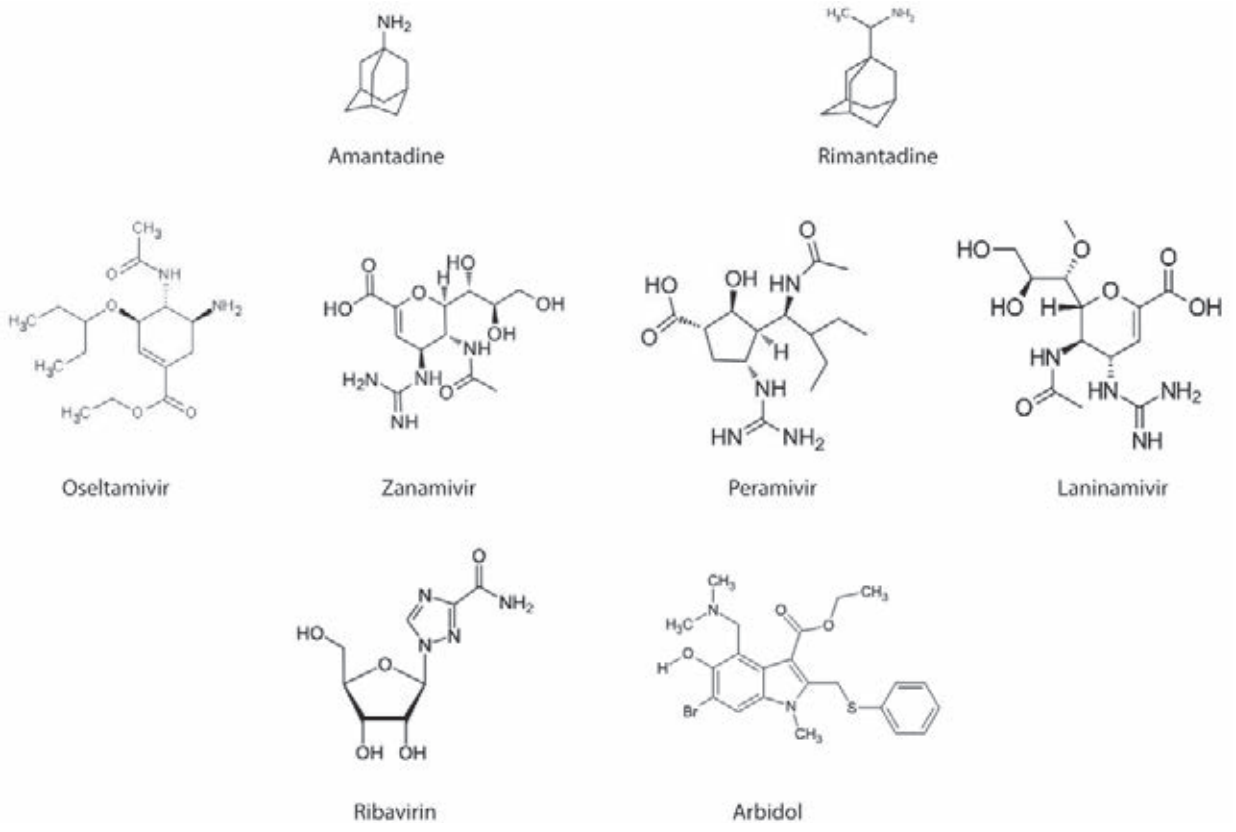
According to a model proposed by Fodor [151], a *trans*-acting polymerase activity is necessary for vRNA replication. Because of the negative polarity of vRNA, positive RNA (cRNA) must be synthesized from the mRNA templates. The replication of vRNA could then be performed by a *trans*-acting RNA polymerase, which may be distinct from the polymerase in the packages of RNPs. Various hypotheses have been formulated regarding the proteins, for instance NPs, that trigger *trans*-acting polymerase [152, 153]. Although this assumption is contradicted by other studies, which have shown viral replication in the absence of NP [154], the presence of newly synthesised NPs appears to be important in stabilizing the length of the segments of viral RNA. Furthermore, other studies have suggested that NEP could regulate viral RNA synthesis [155]. The importation of newly synthesised nucleoproteins and polymerases is also very important in order to assemble nucleoprotein with the negative-sense viral RNA. This event is not completely understood and requires coordinated interaction between Nuclear Localisation Signals (NLSs), PA, PB1, PB2, NP and importins [156-158]. In addition, as recent research has demonstrated [159, 160], NP plays a major role in the assembly of vRNP by interacting with polymerase subunits and is involved in the nuclear and cytoplasmatic transportation of vRNP. Specifically, NPs are oligomerized in ring structures, which interact with viral RNA segments and also with polymerase trimers. This latter interaction allows the viral RNA to twist into a double helix with the polymerase complex at one termination, and with a loop of RNA at another termination [161-164].

To progress, the infection requires a coordinated two-way flow between proteins synthesized in the cytoplasm (for instance M1 protein and NP) and the viral RNA that has been replicated in the nucleus. In this way, the RNP, which is full of vRNA, NP, M1, PA, PB1, PB2, and the non-structural proteins can reconstitute in the nucleus.

The transcription factory is made up of components such as SFPQ/PSF (splicing factor proline-glutamine rich [164], though its entire molecular anatomy is not known. As already mentioned, splicing mechanisms are crucial to increasing the dynamism and complexity of the influenza proteome. Specifically, the RED-SMU1 spliceosomal complex interacts with PB1 and PB2 and is responsible for the splicing of NS1. Other components of the spliceosomal complex are: EJC, SR (serine/arginine-rich proteins, such as SRp40, SRp55 and pre-mRNA Splicing Factor type 2 / Alternative Splicing Factor – SF2/ASF), NS1-BP and hnRNP K (heterogeneous nuclear ribonucleoproteins K) [165-168].

Transcription and replication are enhanced by activation of the PI3K cascade via NS1 [76].

Fig. 2. Chemical structure of the available licensed anti-influenza drugs: M2 blockers (Amantadine, Rimantadine), Neuraminidase inhibitors (NAIs; Oseltamivir, Zanamivir, Peramivir, Laninamivir), Ribavirin and Arbidol.



Nuclear exportation

Subsequently, through more than one mechanism, such as that of small proteins capable of passing through the nuclear pores easily, NS1 and NEP/NS2, the RNP complexes can be exported into the cytoplasm.

The export machinery is a factory made up of several components: E1B/AP5, Rae1/mRNP41 [132], NXF1-TAP (Nuclear RNA export factor type 1) [133], CBC (nuclear cap-binding complex, in particular the 80 kDa subunit), REF/Aly, TREX, P15-INXT, YB-1 (Y-box-binding protein type 1), CRM1/XPO1 (exportin type 1) [169], Rcc1 (Regulator of chromosome condensation type 1), CHD3 (chromodomain helicase DNA-binding protein type 3), RMB15B [170], RanBP3 [171], DDX19B, an ATP-dependent RNA helicase [172], and Hsc70 (heat shock cognate protein 70) [173] among others.

Briefly, two main export routes can be described: the NXF1-dependent and the CRM1-dependent pathways. RNPs are exported via CRM1, whilst HA, NA are transported by NXF1.

CRM1 pathway includes: Nup88, Nup214, Rcc1, RanBP3, CHD3, Hsc70, NS2, among others [174, 175].

Post-translational processing and trafficking

After being translated, proteins are transported to the Golgi network, where they are modified. Modifications, such as glycosylation of HA and NA, palmitoylation/S-acylation of HA and M2, phosphorylation of NS1, and SUMOylation of M1, NS1, NP, PB1 and NEP by SUMO-1/2/3 (Small Ubiquitin-like Modifier type 1/2/3) are essential steps in the production of functionally active viral proteins [176]. These tasks are performed by kinases, such as Cyclin-Dependent Kinases (CDKs) and extracellular signal-regulated kinases (ERKs) [177].

Proteins are retrogradely transported by COPI and Rab8 from the *trans*-Golgi apparatus to the *cis*-Golgi and ER [178].

The quality of protein folding and the correctness of post-translational processing are checked by proteins such as malectin, calnexin, calreticulin and ERp57 [179-183].

Apoptosis pathway

The exportation of RNPs is favoured as the infection progresses, which results in activation of the mechanism of spontaneous cell death (apoptosis) through the activation of caspase 3 [184]. By altering the nuclear membrane, the activation of caspase 3 increases perviousness through the nuclear pore [185, 186]. Indeed, caspase signalling pathways play an important role in the activation, replication, propagation and pathogenicity of the influenza virus, and are therefore related to the severity of influenza symptoms and its clinical burden [187]. The virus finely tunes and modulates the host cellular pro-

teins involved in the processes of regulation and control of the induction of apoptosis [188].
The units of RNP released from the nucleus are concentrated in the perinuclear cytoplasm [189], particularly in the region of the centre for organizing microtubules (MTOC) [190] and, subsequently, in the area of recycling endosomes (REs) [191]. Interaction with the REs allows the RNPs to interact better with the network of microtubules, and thus to orient themselves and to travel towards the cell membrane [192]. The exportation of RNPs is a complicated mechanism that requires well-synchronized timing, and results in an accumulation of RNPs on the apical surface of the cell in the late stages of viral multiplication.
In the late stages of viral replication, the accumulation of HA molecules on the cell membrane, probably by activating the mitogen-activated protein kinase (MAPK) [193], increases the exportation of RNPs, which, through a still unknown mechanism, is oriented towards the apical surface of the cell.
Subsequently, as each unit of RNP contains only one of the eight segments of the viral genome, it is particularly important that the different segments be assembled properly. The viral RNAs themselves mediate this process through a "hierarchical assembly" signalling mechanism [194].

Budding and release

As the infection progresses, the apical membrane becomes rich in viral proteins, which together initiate the budding of the virus around the complex of RNPs, at the regions of the membrane where the extruded HA, the NA, and M1 and M2 proteins are concentrated [195]. Viral proteins are then delivered to the plasma membrane and assembled. Here, Rab11a [196] and HRB (HIV Rev-binding protein) [197] play an important role. HA is able to initiate the process of budding, but not to complete it. This requires the mediation of NA, M1 and M2 proteins [195]. It is also important to consider that, during the formation of the positive curvature of the cell membrane, the suitably assembled units of RNPs move toward the distal part of the viral bud, so that they can be properly wrapped by the viral envelope. As the budding process progresses, a stalk is generated which holds the virion to the cell. The viral envelope must then detach itself from the cell membrane. The M2 protein appears to be crucial to this process. Indeed, it is capable of generating a positive curvature of the membrane), which is necessary in order to enable the spherical virions to split off [195]. However, the virus is not yet free; it is bound to the cell by the binding of HA molecules with the units of sialic acid of the membrane surface. NA molecules must therefore detach the sialic acid from the cell surface in order to accomplish viral budding.
The enzymatic mechanism of influenza virus sialidase has been studied by Taylor, who showed that the enzyme catalysis process is particularly complex and consists of four steps. During the first step the α-sialoside is distorted from a chair conformation to a pseudoboat conformation when the sialoside binds to the sialidase. The second step leads to sialosyl cation, an oxocarbocation intermediate. The third step is the formation of Neu5Ac, as α-anomer. The fourth step involves its mutarotation and the subsequent release of the thermodynamically-stable β-Neu5Ac [198]. Finally, these steps lead to sialic acid hydrolysis.
Currently, 11 isoforms of NA are known; NA10 and NA11 have recently been isolated from bats and are not able to cleave sialic acid. Their precise role and mechanism are still unknown [54].
NA is further classified into two groups: group 1 (N1, N4, N5 and N8) and group 2 (N2, N3, N6, N7 and N9), based upon primary sequence [199]. Group 1 NAs contain a 150-cavity (formed by amino acids 147–151 of the 150-loop), an exposed pocket near the active catalytic site, whereas group 2 NAs lack this cavity [200].
Budding occurs via a VPS4 and VPS28 independent pathway [201, 202].

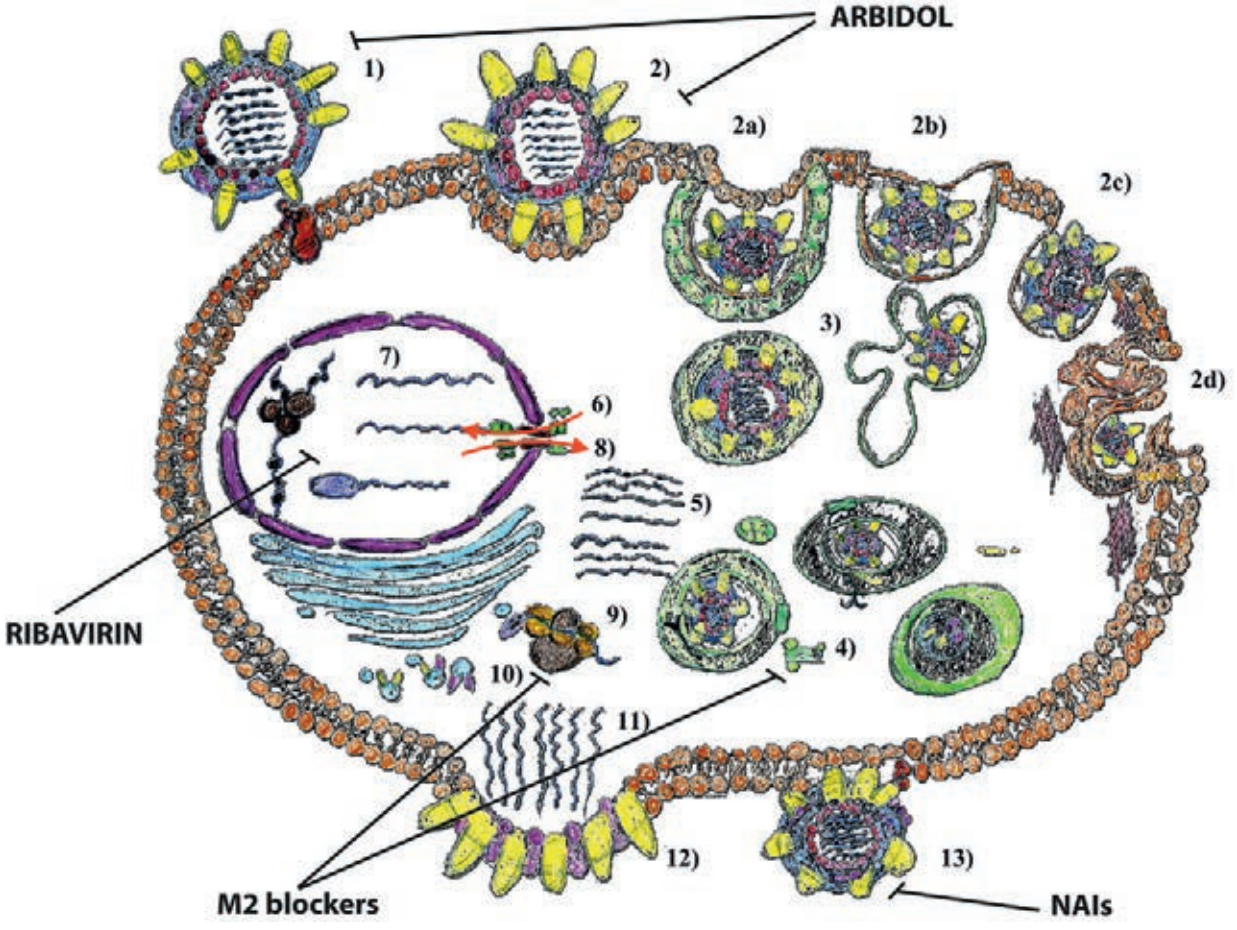
Currently available drugs

M2 inhibitors

Amantadine and Rimantadine (Figs. 2, 3) were the first generation of influenza antiviral agents [203]. Amantadine (1-aminoadamantane) is a derivative of the hydrocarbon tricyclo[3.3.1.1.3,7]decane. Amantadine can be administered either as a hydrochloride derivative (Symmetrel), as 100 mg tablets or syrup, or as its effective derivative Rimantadine (α-methyl-1-adamantanemethylamine hydrochloride, Flumadine). At high concentrations, Amantadine and Rimantadine non-specifically raise the pH within cellular endosomes, thus inhibiting or retarding the acid-induced conformational change in viral HA. At low concentrations, Amantadine and Rimantadine specifically inhibit the ion-channel activity of the M2 protein [204].
Crossing the brain-blood barrier and being present in the cerebrospinal fluid (CSF) with a concentration around 75% of serum level, Amantadine can also be used to treat Parkinson's disease [205], depression and obsessive-compulsive disorder (OCD) [206], Huntington's disease [207], attention deficit hyperactivity disorder (ADHD) and other neuropsychiatric diseases [208], cocaine abuse and dependence [209], HCV [210], Creutzfeldt-Jakob's disease [211], Borna's disease [212], herpes and post herpes zoster neuralgia (PHN) [213].
This variety of uses seems to suggest that Amantadine, besides blocking the M2 channel, acts on an array of receptors, from the dopaminergic receptors to noradrenergic, serotoninergic, cholinergic, and N-Methyl-D-aspartate (NMDA) receptors [214, 215].
After being rapidly adsorbed, with an excellent bioavailability profile (usually in the range 86-94%) [216], the drug reaches peak plasma levels within 4 hours [216]. The plasma elimination half-life is about 11–15 h in patients with normal renal function. It has a plasma protein binding of about 67% [216].

The drug, after being poorly metabolized and being widely distributed, is almost completely excreted via glomerular filtration and tubular secretion: this implies a dose adjustment when administered to patients with renal failure or to the elderly, such as reducing the daily dose of 100 mg, instead of 200 mg.
Acting on muscarinic receptors, some patients may experience anti-muscarinic adverse effects such as orthostatic hypotension, gastrointestinal discomfort (nausea, vomiting, anorexia), congestive heart failure [216]. Moreover, because Amantadine has some Central Nervous System (CNS) stimulatory properties, adults may complain of confusion, disorientation, jitteriness, anxiety, mood disorders, slurred speech, insomnia, ataxia, tremors, and, rarely, nightmares, oculogyric episodes. These symptoms are usually more common (up to 15-30%) when the drug is used for different weeks for prophylactic purpose. When instead used for treatment (less than a week), it is better tolerated. In rare cases, seizures, hallucinosis/hallucinations, coma, acute psychosis and cardiac arrhythmia may occur, usually in patients with underlying psychiatric comorbidities [216, 217]. Adult Respiratory Distress Syndrome (ARDS) has been rarely and anecdotally reported [218].
Crossing the placenta and being present in breast milk, it is teratogenic at least in animals, even though safety has not been established in pregnant women: for precaution sake, it belongs to class C. Pregnancy is recognized as one of the risk factors for catching influenza and untoward outcomes (higher morbidity, hospitalization and mortality rates), but cannot benefit from adamantanes.
Also in children and in the elders, the effectiveness in preventing, treating and shortening the duration of influenza A appears to be limited, according to a recent systematic review [219].
Rimantadine can be administered as 100 mg tablets. It reaches peak plasma concentration after 3-6 hours. The plasma half-life is long (24-36 hours). Rimantadine is more metabolized than Amantadine: only 25% is secreted unchanged [216]. It has a plasma protein binding of about 40%.

Fig. 3. Schematic representation of the sites of action of anti-influenza licensed drugs. The steps of the replication cycle of the influenza virus are the following: 1) attachment of the virion to target cells and receptor binding (virus adsorption); 2) internalization into cellular regions by means of clathrin-mediated endocytosis (CME), caveolae-dependent endocytosis (CDE), clathrin-caveolae-independent endocytosis, and macropinocytosis; 3) endosomal trafficking via endosomes / caveosome / macropinosome / lysosomes to the perinuclear compartment; 4) pH-dependent fusion of viral and endosomal / organellar membranes; 5) uncoating; 6) nuclear importation; 7) transcription and replication; 8) nuclear exportation; 9) protein synthesis; 10) post-translational processing and trafficking; 11) viral progeny assembly and packaging; 12) budding; and 13) release. Abbreviations: NAIs (neuraminidase inhibitors).



Rimantadine is characterized by lower rates of ADRs [220]: compared to Amantadine, it is better tolerated by children and the elderly.
Unfortunately, the use of M2 inhibitors has been limited by the emergence of drug-resistant strains of influenza viruses [221], such as the mutations of pore-facing residues (V27A, A30T, S31N, G43E), mutations of close interhelical residues located at the N-terminal half of the channel (L26F), and mutations of far interhelical residues far located at the C-terminal half of the channel (L38F) [221-222].

NA Inhibitors

In recent times, the most widely used antivirals against influenza have been the inhibitors of Neuroaminidase: namely Oseltamivir and Zanamivir (Figs. 2, 3). From a chemical point of view, NA inhibitors can be classified into: sialic acid derivatives (or 5,6-dihydro-4H-pyran derivatives), benzoic acid derivatives, cyclohexene derivatives, cyclopentane derivatives, pyrrolidine derivatives and natural products. The first NA inhibitors were representatives of the first chemical class, being unsaturated syalic acid analogs, such as DANA and FANA, which were initially described by Meindl and Tuppy in 1969 [223].
NA is a homotetrameric enzyme of about 220-240 kDa that is essential to the reproduction of the influenza virus. Indeed, it exerts at least three crucial actions. First, neuraminidase frees the virus from the respiratory mucus and allows it to reach the cells of the respiratory mucosa more easily. The coordinated action of HA, NA, M1 and M2 is required during the phase of viral budding. Finally, NA is required in order to release the virus from the cell surface by cutting the molecules of sialic acid that still anchor the virus to the cell surface by means of HA after completion of the replication cycle. This action also facilitates separation of the self-aggregated virions of the viral progeny. Burnet and coll. [224] first had the idea that an inhibitor of NA could be an effective antiviral agent, but only when the crystal structure of NA and its complex with neuroaminic acid were defined by Coleman in 1993 was it possible for von Itzstein [225] to synthesize a neuroaminic acid derivative with an enhanced affinity for influenza NA. This compound is Zanamivir (4-guanidino-Neu5Ac2en, or 4-GU-DANA). Its mechanism of action, which is identical to that of Oseltamivir, is characterized by the fact that the molecule mimics sialic acid; thus, it enters into competition with the acid and reversibly binds the molecules of viral NA. Zanamivir was designed to concentrate locally in the respiratory tract, while Oseltamivir (GS4104) was designed to have a high bioavalability (80%) concentration after oral administration. Indeed, Oseltamivir is very well absorbed from the gastrointestinal tract and is rapidly metabolised to active Oseltamivir carboxylate (GS4071) ([3R,4R,5S]-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate phosphate) in the liver by hepatic carboxyl-esterase 1. The active metabolite is distributed throughout the body, including the upper and lower respiratory tract, middle ear, tracheal lining, nasal mucosa and lungs [226]. Plasma half-life varies from 1-2 to 3-4 hours, being 6-10 hours for the carboxylate form [226]. Oseltalmivir is extensively metabolized (more than 95%).
Oseltamivir and Zanamivir result comparable in terms of clinical profile and superior to the adamantanes [227, 228]. Regarding the clinical effectiveness of the two drugs, a recent meta-analysis by Jefferson et al. [229] revealed that both drugs had a modest therapeutic effect in healthy adults and that prophylactic treatment with either Zanamivir or Oseltamivir was effective in preventing the disease.
Zanamivir is supplied in "Rotadisks" with four blisters containing 5 mg of powder each. Five Rotadisks are packaged with a Diskhaler inhalation device. Oseltamivir is available as 75 mg capsules or as an oral suspension containing 12 mg/mL (Oseltamivir phosphate) [217].
ADRs are usually nausea, vomiting. Recently, CNS toxicity in infants younger than one year of age has been reported [217].
While adjustments are necessary in subjects suffering from renal failure, no adjustments are needed in patients with hepatic failure or mild obesity. Oseltamivir can be administered to pregnant women [230].
While the majority of currently circulating influenza viral strains are resistant to amantadanes, the resistance of both H1N1 and H3N2 strains and of type B viruses to Oseltamivir and Zanamivir is very low. For this reason, the US CDC and the WHO recommend their use for the treatment and prevention of seasonal and pandemic influenza [231, 232].

Other licensed drugs

Other NAIs

Two analogues of Zanamivir and Oseltamivir, which are currently licensed in Japan and other Asian countries, are Peramivir and Laninamivir octanoate hydrate (CS-8958) (Figs. 2, 3).
The former is only administered intravenously because of its very poor bioavailability [233]. It has been licensed in Japan and in South Korea, but was only temporarily approved for emergency use in the USA during the H1N1 pandemic [234].
The latter drug is very promising because of its long-acting inhibitory effect [235]. Inavir was launched in Japan in October 2010 as a 20-mg dry powder inhaler. It is a prodrug that is converted in the airway to laninamivir (R-125489), the active NAI and is retained at high concentrations for at least 10 days after a single inhalation of 40 mg. Only 15% of the drug is orally bioavailable [223]. Commonly reported ADRs were psychiatric, gastrointestinal and CNS disorders [223]. A single inhalation dose makes Inavir a quite convenient drug, even though children and young adolescents could not inhale it properly [232].

Ribavirin

Considering the compounds targeted against the transcription and replication of vRNA, one of the first de-

veloped drug is Ribavirin (Figs. 2, 3). Ribavirin, also known as the trade name of Virazole, is the guanosine nucleoside analog: 1β-D-ribofuranosyl-1,2,4,-triazole-3-carboxamide. Its mechanism of action is not completely known. However, Inosine 5'-monoposphate dehydrogenase (IMPDH) appears to be the principal target of the molecule [234]. This inhibition diminishes the intracellular concentration of GTP (Guanosine-5'-triphosphate), and this would stop viral protein synthesis and limit the vRNA replication. Crotty et al also demonstrated that Ribavirin is a vRNA lethal mutagen, resembling guanosine or adenosine and causing mutations in RNA replication [235]. However, the need of high doses of the drug to have good clinical results have limited the use of Ribavirin as anti-influenza drug, and a recent revision of the literature by Chan-Tack et al. suggests that there are not conclusive results about the beneficial use of Virazole for treatment of influenza [236].
Ribavirin can be administered orally, by aerosolization, rarely by intravenous route [237]. ADR is dependent on the administration route, being extravascular haemolytic anemia if the drug is delivered intravenously, a bronchospasm if aerosolized [216].

Arbidol

There are several potentially effective drugs, which act as HA inhibitors. However, only one medication, the small indole derivative Arbidol (ARB) or Umifenovir (Figs. 2, 3), or ethyl-6-bromo-4-[(dimethylamino)methyl]-5-hydroxy-1-methyl-2[(phenylthio)methyl]-indole-3-carboxylatehydrochloride monohydrate, has been licensed [232-248].
ARB was created by the Center for Drug Chemistry in Moscow [246], has been licensed in Russia 20 years ago and since 2006 has been used in China for the prophylaxis and treatment of pneumonia caused by influenza viruses A and B [234]. ARB probably exerts a multiple antiviral action: a direct virucidal effect, a block of the virus at the level of cell-entry (attachment and internalization), and impairment of viral replication, because of its ability to bind with proteins and lipids [232-248]. Several studies have demonstrated that ARB is also effective against other enveloped and non-enveloped viruses, such as Hepatitis B Virus (HBV), HCV, RSV, some Picornavirus (such as rhinovirus 14), Poliovirus 1, Coxsackievirus B5), parainfluenza type 3 (PIV3), as well as the avian coronavirus, infectious bronchitis virus, Chikungunya virus, Reovirus, Hantaan virus, Vesicular stomatitis virus (VSV) and Marek disease virus, an avian oncogenic herpesvirus [234].
It is metabolized in the liver and redistributed in the body tissues.
The principal biotransformation pathways include sulfoxidation, dimethylamine N-demethylation, glucuronidation, and sulfate conjugation. The major metabolite is sulfinylarbidol, followed by unmetabolized arbidol, N-demethylsulfinylarbidol, and sulfonylarbidol. CYP3A4 is the major isoform involved in ARB metabolism, whereas the other P450s and flavin-containing monooxygenases (FMOs) play minor roles. Plasma half-life is long (up to 25 hours) [243].

Conclusions

In recent decades, few antiviral drugs against influenza virus infections have been available. This has limited their use in human and animal outbreaks. Indeed, antiviral drugs used during seasonal and pandemic outbreaks have usually been administered as mono-therapy and, sometimes, in an uncontrolled manner in farm animals. This has led to the emergence of viral strains that are resistant, especially to the compounds of the amantadane family. For this reason, it is particularly important to develop new antiviral drugs against influenza viruses. Indeed, although vaccination is currently the most effective means of mitigating the effects of influenza epidemics and can delay the spread of new pandemic viruses, as maintained by the Advisory Committee on Immunization Practice (ACIP), antiviral drugs can be very useful in allowing manufacturers to prepare large quantities of pandemic vaccines. In addition, antiviral drugs are particularly valuable in complicated cases of influenza, particularly in hospitalized patients and in individuals at risk, such as the elderly or patients with chronic respiratory diseases. In such cases, it would be particularly desirable to have more antivirals and to administer them in an appropriate manner [249, 250].

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Abbreviations

A: Alanine; ACIP: Advisory Committee on Immunization Practice; ADAR1: adenosine deaminase acting on RNA type 1; ADHD: Attention Deficit and Hyperactivity Disorder; ADR: Adverse Drug Reactions; AM: alveolar macrophage; AP-2: Adaptor Protein 2; ARB: Arbidol; ARDS: Adult Respiratory Distress Syndrome; ASF: Alternative Splicing Factor; BET: Bronchial Epithelial Tissue; BM2: Type B Influenza Virus Matrix Protein 2; BTB: Bric-a-brac, Tramtrack, Broad-complex; CBC: cap-binding complex; CCP: Clathrin-Coated Pit; CCV: Clathrin-Coated Vesicle; CD: Cluster of Differentiation; CDC: Center for Disease Control and Prevention; CDE: Caveole-Dependent Endocytosis; CDK: Cyclin-Dependent Kinase; CHD3: chromodomain-helicase-DNA-binding protein type 3; CME: Clathrin-Mediated Endocytosis; CNS: Central Nervous System; COPD: Chronic Obstructive Pulmonary Disease; CRL: Cullin-RING-Ligases; CRM1: Chromosome Region Maintenance type 1; cRNA: complementary RNA (positive RNA necessary as template for viral RNA replication, or template RNA); CSF: Cerebrospinal Fluid; DANA: 2,3-didehydro-2-deoxy-N-acetylneuraminic acid; E: Glutamic acid; ECDC: European Centre for Disease Prevention and Control; EE: Early Endosome; EGF: Epidermal Growth Factor; EJC: Exon Junction Complex; ER: Endoplasmic Reticulum; ERK: extracellular signal-regulated kinase; F: phenylalanine; FANA: 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid; FMO: flavin-containing monooxygenase; G: Glycine; GTP: Guanosine-5'-triphosphate; GTPase: guanosine triphosphatase; HA: Hemagglutinin; HAfp23: Hemagglutinin fusion peptide 23; HAT: histone acetyltransferase; HBV: Hepatitis B Virus; HCV: Hepatitis C Virus; HEF: Hemagglutinin-Esterase-Fusion protein; HIV: Human Immunodeficiency Virus; hnRNP K: heterogeneous nuclear ribonucleoproteins K; HPV: Human Papillomavirus; HRB: HIV Rev-binding protein; Hsc: Heat shock cognate protein; HSP: Heat Shock Protein; IAV: Influenza A Virus; IFN-I: type I interferon; IL: interleukin; IL6R: interleukin 6 receptor; IMPDH: Inosine 5'-monoposphate dehydrogenase; kD: kilodalton; L: Leucine; LAMP: lysosomal-associated membrane protein; LE: Late Endosome; LET: Lung Epithelial Tissue; M protein: Matrix protein; M1: Matrix protein 1; M2: Matrix Protein 2; m(7)GTP: RNA cap 7-methylguanosine triphosphate; MAPK: Mitogen-Activated Protein Kinase; MCM: mini-chromosome maintenance complex; MHC: Major Histocompatibility Complex; mRNA: messenger RNA; mRNAs: messenger RNA segment; mRNP: messenger ribonucleoprotein; MTOC: MicroTubules-Organizing Centre; mTOR: mammalian Target Of Rapamycin; N: Asparagine; NA: Neuroaminidase; NAIs: Neuroaminidase inhibitors; NEP: Nuclear Export Protein; NES: Nuclear Export Signal; NGF: Nerve Growth Factor; NHE: Na^+/H^+ exchanger; NK: Natural Killer cell; NLP1: Nucleoporin-Like Protein type 1; NLSs: Nuclear Localization Signals; NMDA: N-Methyl-D-aspartate; NP: Nucleoprotein; NPC: Nuclear Pore Complex; NS1: Non-Structural protein type 1; NS1-BP: NS1 Binding Protein; NS2: Non-Structural protein type 2; NS3: Non-Structural protein type 3; Nup: Nucleoporin; NuTF2: Nuclear Transport Factor 2; NXF1: Nuclear RNA export factor type 1; OCD: Obsessive-Compulsive Disorder; ORF: Open Reading Frame; PA: Acidic Polymerase; PACT: Protein ACTivator of the interferon-induced protein kinase; PAK: p21-activated Kinase; PB1: Basic Polymerase 1; PB2: Basic Polymerase 2; pDC: plasmacytoid dendritic cell; PH: pleckstrin homology; PHN: Post-Herpetic Neuralgia; PI3K: phosphoinositide 3-kinase; PIV: Para-Infliuenza Virus; PKC: Protein Kinase C; PKR: Protein Kinase R (also known as Protein kinase RNA-activated, or interferon-induced, double-stranded RNA-activated protein kinase, or eukaryotic translation initiation factor 2-alpha kinase 2 – EIF2AK2); Pol II: Polymerase II; POM121: nuclear envelope pore membrane protein type 121; PRKRA: Protein kinase, interferon-inducible double stranded RNA dependent activator; PtdIns(3,4,5)P3: phosphatidylinositol-(3,4,5)-trisphosphate; PtdIns(4,5)P2: phosphatidylinositol-(4,5)-bisphosphate; PTEN: Phosphatase and tensin homolog; qPCR: quantitative Polymerase Chain Reaction; RanBP: Ran Binding Protein; RBP: Receptor Binding Pocket; RBS: Receptor Binding Site; Rcc1: Regulator of chromosome condensation type 1; RdRp: RNA-dependent RNA polymerase complex; REs: Recycling Endosomes.; RIG-I: Retinoic acid-Inducible Gene 1; RNA: Ribonucleic Acid; RNP: Ribonucleoprotein; RSV: Respiratory Syncytial Virus; S: Serine; SF: pre-mRNA Splicing Factor; SFPQ/PSF: splicing factor proline-glutamine rich; SFRS: Serine/arginine-rich splicing factor; SH2: Src Homology 2; siRNA: short interfering RNA; SR: Serine/arginine-Rich protein; sRNA: genomic segments of RNA; SUMO: Small Ubiquitin-like Modifier; T: Threonine; TGN: Trans-Golgi Network; TM: transmembrane; Tpr: Translocated promoter region; TRIM: tripartite motif-containing protein; US: United States of America; V: Valine; Vps: Vacuolar protein sorting; vRNP: viral Ribonucleoprotein; VSV: Vesicular stomatitis virus; WHO: World Health Organization; XPO1: exportin 1; YB-1: Y-box-binding protein type 1.

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ORIGINAL ARTICLE

Relationship of Internet addiction with self-esteem and depression in university students

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Key words

Internet addiction • Self-esteem • Depression

Summary

Background. *The aim of the study was to investigate the relationship of self-esteem and depression with Internet addiction in university students.*
Methods. *The present descriptive-analytic correlation study involved 408 students (150 female and 258 male) who had been selected by means of a cluster sampling method from among all the students studying in Birjand Islamic Azad University. Students were evaluated through the Beck Depression Inventory (BDI), Cooper Smith Self-Esteem Inventory (CSEI) and Internet Addiction Test (IAT).*
Results. *The results indicated that 40.7% of the students had Internet addiction. A significant correlation emerged between depression, self-esteem and internet addiction. Regression analysis indicated that depression and self-esteem were able to predict the variance of Internet addiction to some extent.*
Conclusions. *It may be important to evaluate self-esteem and depression in people with Internet addiction. These variables should be targeted for effective cognitive behavioral therapy in people with Internet addiction.*

Introduction

Using modern technologies is a common feature of today's world. As one of the most widely used of these technologies in the modern world, the Internet is playing an increasingly significant role in revolutionizing peoples' lives [1]. Indeed, it is frequently used for on-line purchasing, data collection, chatting, communicating with others and so on. Internet use has increased enormously in the last 50 years and now it seems that every aspect of people's lives has been affected by the "Global Village". Although the Internet offers many advantages in the era of global communication, its improper or excessive use can produce many negative consequences [2].
Excessive Internet use, which is also called uncontrolled use of the Internet, pathological Internet use, net addiction or Internet addiction, causes problems at work and in social life [3, 4]. Internet addiction is generally defined as an uncontrollable desire to use the Internet, the devaluation of time spent without connecting to the Internet, intense nervousness and aggression in the event of deprivation, and progressive deterioration of social and family life [5]. The growing number of studies conducted on Internet addiction reveals that Internet Addiction Disorder is a psychosocial disorder, the features of which include lack of patience, symptoms of isolation and emotional disorders and interruption of social relations [6].
Recent research has placed increasing emphasis on Internet misuse and its consequences, both psychological and behavioral, among young people [7-10]. Such consequences include the emergence of possible behavioral alterations, loss of control, school failure, social isolation and an increase in family conflict [6, 11]. Several studies have reported correlations between Internet Addiction Disorder (IAD) and depression [12-17].
The relationship between Internet addiction and self-esteem has been investigated in several studies. These studies have revealed that personality traits, self-esteem and psychiatric disorders are associated with Internet addiction [18]. Young (1998) reported that the vast majority of Internet addicts have a history of depression and anxiety. Low self-esteem has also been reported [19]. In some other studies, self-esteem has emerged as a factor associated with Internet use and problematic Internet use. In addition, research on self-esteem and use of the Internet includes studies examining adolescents' use of some social networking sites and its association with their self-esteem. These studies have shown that adolescents with low self-esteem tend to spend more time on social networking sites than those with higher self-esteem [20-22].
In the present study, our objective was to determine the prevalence of Internet addiction and its relationship with depression and self-esteem among students. To this end, we investigated depression and self-esteem and their relationship with the Internet addiction among students.

Methods

The statistical population of the present study comprised all the male and female students studying at the Islamic Azad University of Birjand. To select the statistical sample of the study, we applied a cluster sampling method, through which 408 students were chosen from among all the students at Birjand Azad University.

Young's Internet Addiction Test (IAT)

The IAT has 20 items associated with Internet use, including psychological dependence, compulsive use, withdrawal symptoms and related problems of school, sleep, family and time management. For each item, a graded response can be selected (1 = "not at all" to 5 = "always"). The minimum score is 20, while the maximum is 100; the higher the score, the greater the level of Internet addiction. As suggested by Young, cut-off scores for the IAT were used to classify Internet users on the basis of the severity of their addictive behavior (Young, 1998b). In the present study, the same cut-off scores were used: Minimal users (scores 20 to 39) – average online users who have complete control over their Internet use, Moderate users (scores 40 to 69) – those experiencing occasional or frequent problems due to Internet use, Excessive users (scores 70 to 100) – those who have significant problems caused by Internet use.
IAT is the most famous measurement in the Internet addiction field and has been used by many researchers [23-25]. This instrument has exhibited good psychometric properties in previous studies. For example, in Yang et al.'s study, the internal consistency (Cronbach's alpha) of IAT was found to be 0.92, and its test-retest reliability proved satisfactory [25]. Moreover, Widyanto and McMurran (2004) reported that "the IAT has high face validity"[24].

Cooper Smith Self-Esteem Scale (CSEC)

The Cooper Smith Self-Esteem Scale has 58 items, 8 of which – numbers 6, 13, 20, 27, 34, 41, 48, 55 – are lie detectors. The remaining 50 items are divided into four subscales: general self-esteem, social self-esteem (peers), family self-esteem (parents) and educational self-esteem (school). Items are scored zero or one. Consequently, the minimum total score possible is zero and the maximum is 50. If the respondent scores more than 4 out of the 8 "lie detector" items, it means that the validity of the test is low, and that the subject has tried to portray himself to be better than he is. Bahrampour ascertained that the reliability of this questionnaire was 0.90 and 0.92 for male and female students, respectively [26].

Beck Depression Inventory

The Beck Depression Inventory was used to assess depression in the study group. This scale was developed in 1961 by Beck and colleagues. The scale consists of 21 Likert-type questions. Each question contains four options, which are scored from 0 (none) to 3 (severe). The total score ranges from 0 to 63 points. Students with scores of 17 points or more are suspected of having depression. The validity and reliability of the Farsi version of BDI have been demonstrated in Iran [27].

Results

Of the students enrolled, 36.8% were female and 63.2% male. Six students (2.2%) had severe levels of Internet addiction, 38.5% had medium Internet addiction, and (59.3%) had no Internet addiction. On comparing Internet addiction between male (16.41 ± 23.44) and female (18 ± 29.76) students, the average difference was 3.54 and the mean Internet addiction score was significantly lower in females than in males ($p \leq 0.01$). Multivariate regression analysis revealed that depression and self-esteem scores could significantly predict Internet addiction scores (Tabs. I, II).
According to the above table, the correlation coefficient of Internet addiction and depression score was 0.31, and the depression score was able to predict about 10% of the Internet addiction score. In addition, the depression score could significantly predict the Internet addiction score.
On adding the self-esteem variable to the regression equation, the determination coefficient is 11%. This means that the self-esteem variable increases the predictive power of the Internet addiction score by 1%, and the correlation of these variables is 0.33 with the Internet addiction score. On the basis of the above table, it can be

Tab. I. Regression model, variance analysis and regression statistical characteristics (depression).

Model	Sum of Squares	Df	Mean Square	F	Sig	R	R Square	Adjusted R Square	Std. Error of theEstimate
Regression	12377.56	1	12377.56	43.72	0.001	0.31	0.09	0.098	16.82
Residual	114936.51	406	283.09						
Total	127314.08	407							

Tab. II. Regression model, variance analysis and regression statistical characteristics (depression self-esteem)

Model	Sum of Squares	Df	Mean Square	F	Sig	R	R Square	Adjusted R Square	Std. Error of the Estimate
Regression	13936.98	2	6968.49	24.89	0.001	0.33	0.118	0.115	16.73
Residual	113377.09	405	279.94						
Total	127314.08	407							

Tab. III. Results of Multiple Regression Analysis Predicting Internet Addiction

Predictor Variables	B	Std. Error	T	Sig.
depression	0.31	0.12	6.61	0.001
self-esteem	0.45	0.91	-2.36	0.001

concluded that the ratio of regression variance to error variance is significant, which means that the self-esteem variable is significantly involved in the regression line. The Table above shows that the calculated t is significant at a level of $p < 0.001$. Thus, it can be concluded that the ratio of the slope, which is determined by the depression factor, to the standard error is significant. In addition, it can be concluded that the ratio of the slope, which is determined by the self-esteem factor, to the standard error is significant, while the Internet addiction score has an inverse relationship with self-esteem.

Discussion

The results indicated that 40.7% of the students had Internet addiction. Of these, 2.2% had severe addiction and 38.5% had moderate Internet addiction. Globally, an average ratio of 2-5 million Internet addicts per 50 million regular users has been estimated. In other words, about 5 to 10 per cent of Internet users have IA [28] In a study on Turkish college students, 9.7% of respondents were Internet addicted [29]. The same result was reported in an Iranian study [30]. Another study in Iran also reported that 10.8% of medical students were Internet addicted, 2.8% and 8% of whom had severe and moderate IA, respectively [31]. The statistics yielded by different studies are very similar and slight differences may be attributed to differences in sample size and instruments. The present study showed that the rate of IA was significantly higher in male than in female students. Although a few studies have reported higher rates in female students [32], the results of the present study are consistent with those of most of the previous studies, suggesting that male gender is a predictor of IA [33-35]; indeed, in one study, the risk of IA was 3.5 times higher among male students than female students [31]. In a review of IA, Chou et al. concluded that male internet users were more at risk of IA owing to a stereotyped use of sexual contents; however, female users may be asymptomatic or may present limited symptoms [32]. It also seems that male students are more likely to become Internet dependent because they are more experienced in using the Internet, receive less parental supervision and use the Internet for entertainment purposes more than females do [36] The results of the present study revealed that self-esteem was significantly and negatively correlated with Internet addiction among students. Furthermore, self-esteem was found to be a significant predictor of Internet addiction. In the literature, many studies have examined the association between self-esteem and pathological Internet use [37-39]. Based on the results of these studies, we can conclude that a negative relationship exists between these two variables. Griffith's studies provide important findings in disclosing this relationship. He states that the participants' use of Internet is highly associated with its perception as a coping style and a way of compensating some deficiencies, such as low self-esteem. According to him, it makes users feel better, as it allows them to assume a different personality and social identity. In other words, these users derive great satisfaction from Internet use. As can be seen, when individuals have low self-esteem, they may perceive the Internet as a way of making up for these shortcomings; increased Internet use may, however, turn into a dependent relationship. As expected, depression positively predicted Internet addiction. Recent studies on Internet addiction showed that Internet addiction was related positively to a decrease in social interactions, depression, loneliness and lower self-esteem [40, 41]. Thus, it can be said that this finding is consistent with those of other studies that have found a positive relationship between depression and internet addiction [41-44]. In addition, supportive data can be found in the studies of depressed individuals, who are more likely to engage in Internet use. Therefore, it appears that if individuals can reduce their internet addiction, they may reduce their depression level [45]. The present study also has limitations. First, because it was conducted at the University of Birjand, its results cannot be generalized to other regions and universities without further research. Second, because some students manifested fatigue and impatience in answering the large number of questions in the questionnaire, their answers might have been false or distorted.

Conclusion

The present study investigated the prevalence of Internet addiction and its relationship with depression and self-esteem among students. It may be important to evaluate self-esteem and depression in people with Internet addiction. These variables should be targeted for effective cognitive behavioral therapy in people with Internet addiction.

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ORIGINAL ARTICLE

Redefining the technical and organizational competences of children vaccination clinics in order to improve performance. A practical experience at the ULSS 12 Venetian Public Health and Hygiene Service

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Key words

Vaccination • ULSS • Children vaccination clinics

Summary

Introduction. *Since Regione Veneto suspended compulsory vaccination for children in 2008, and because of an increasing disaffection of parents to the vaccine practice, the vaccination rates have been slowly but steadily decreasing. The aim of this study was to analyze internal and external factors of immunization reduction and to implement potential solutions of the problem.*
Methods. *Servizio Igiene e Sanità Pubblica of ULSS 12 Veneziana (SISP – Hygiene and Public Health Service) analyzed and addressed both, the reasons of parents who do not vaccinate their children and the internal problems regarding vaccination clinics management, information to families, procedures and guidelines and, in general, the communication skills of the vaccination staff.*
Results. *A positive trend in vaccination rates was observed, especially in Venice historical centre. Moreover the staff reported a better working atmosphere and benefit from sharing common goals and procedures, even though the workforce was reduced of about 30% in terms of equivalent unit (EU).*
Discussion. *The continuous quality improvement method followed in this experience led to a steady increase in vaccination coverage in all territorial clinics, to a better adhesion of guidelines and standard operating procedures and to a general professional empowerment of SISP staff. The service now offered to the population is better and more efficient, since the workforce has been reduced. Future goals are to improve information about vaccinations among the population.*

Introduction

Given the current historical and cultural contingency that sees people wanting more and more to participate actively in decisions regarding their children's health (empowerment), which sometimes makes them question even the strongest scientific evidence and clinical experience – as in the case of vaccination – an analysis was conducted at the ULSS 12 Venetian Public Health and Hygiene Service (*Servizio Igiene e Sanità Pubblica* - SISP) on the reasons for the growing disaffection for vaccination with a view to containing the consequent small but steady reduction in vaccination coverage [1-7].
In a nutshell, the problem has two main causes: one concerns vaccination clinic accessibility and service management; the other relates to the socio-cultural sphere of people who call the whole vaccination system into question, in terms of their scientific authoritativeness, transparency, uniformity of action, and openness to confrontation [3, 4].
The aim of this study was to test some solutions for containing the reduction in vaccination coverage in the field, adapting the approach during the process if necessary. Both, internal service management issues and external causes of opposition to vaccination due to misinformation or unjustified misgivings were approached in this study.

Methods

Situational analysis

External factors

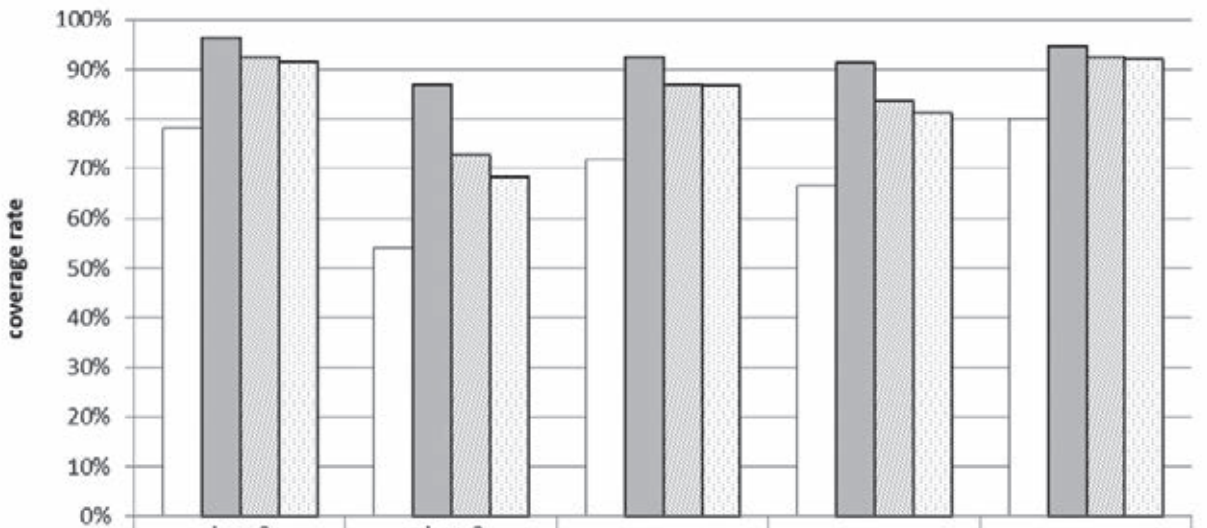
Scientific research on this topic has pinpointed some characteristics of the personal and socio-cultural background of people who do not have their children vaccinated.
The parents, and especially mothers, who do not have their children vaccinated can be defined as: vaccine-hesitant (they fear vaccination but tend to accept it in the end); late vaccinators (who accept some but not all the proposed vaccines, and tend to have vaccinations done later than at the age recommended by the health system); and rejecters (who refuse any kind of vaccination). Unlike the rejecters, the late vaccinators and vaccine-hesitant categories are might be influenced in their decisions concerning vaccinations [8].

In the Veneto Region (north-east Italy), non-vaccinators have a relatively high cultural and economic level by comparison with vaccinators, they are of Italian nationality, older, and have more than one child. It is interesting that health workers (especially mothers) tend to vaccinate less than the general population, thus confirming a widespread pseudo-knowledge and inadequate awareness about vaccination even among health professionals [3, 4].
In Italy, 80% of parents with children of immunization age use the internet, and the majority of them consult health sites [9]. It is easy to find false information about vaccines on the internet, and even for a person trying to use several different search engines and YouTube, the first sites to be listed are more likely to be anti-vaccination [10-13].
In the Veneto, the sources of information used by non-vaccinators are mainly web sites and blogs, but also various types of organization specializing in children's health and wellbeing, or word of mouth coming from parents whose children were presumed to have suffered vaccine-related damage [3, 4].
Generally speaking, family pediatricians rarely address this topic when routinely examining children who are well (and even if they do, this kind of intervention seems to be of scarcely influential in terms of changing or orienting parents' attitudes to vaccination) [3, 4].
A commonly-held opinion among non-vaccinators is that health personnel at vaccination clinics are neither very competent as regards vaccine-preventable diseases and vaccine safety, nor very willing to discuss these matters [3, 4].
In the latter's defense, it has to be said that it takes time to establish a constructive exchange of opinions, for which these operators would need to have very solid scientific grounds and, even more difficult, very good listening and counseling skills - such expertise demands constant updating and the acquisition of a working method that is still little used in public health services.

Internal factors

The organizational problems emerging from our survey can be summarized as follows.
In the Veneto Region, most of the local public health services organize children's vaccination services centrally, under the direct authority of a Public Health and Prevention department [14, 15].
At the Venetian ULSS 12, however, this activity has been shared for historical and organizational reasons between territorial branches of the health service dedicated to primary health care called districts, or *Distretti Socio Sanitari* [16, 17]. The SISP interfaces directly with the Veneto Regional Authority, reporting on vaccination coverage in the population and providing technical and scientific support for the various vaccination clinics, but it does not organize and control their activities directly.
Information for families differed from one territorial clinic to another: some of the personnel either suggested or at least did not oppose parents' requests for personalized vaccination schedules instead of those recommended in the official Vaccination Plan issued by the Regional Authorities [18, 19]. In particular, some vaccinations that are usually administered together were separated and administered at subsequent visits, thus delaying the protection of the child concerned and of the population as a whole. Some vaccines were not promoted, or even openly discouraged, by some health professionals because the disease was considered relatively benign (e.g. chicken pox), or because the vaccine was considered unsafe (e.g. measles). In some circumstances, little attention was paid to the guidelines on the contraindications to vaccination (e.g. the need to delay administering vaccines after even mild diseases, to reject children for vaccination if not been examined recently by the family pediatrician, etc.).
Organizing training and refresher courses for the personnel proved difficult and time-consuming because of the need to have their participation authorized by the complex hierarchical management of the territorial public health unit. Also, because the public health department did not have direct control over the vaccination clinics' activities, putting into practice any new recommendations was usually delayed and little encouraged.
The various territorial clinics had different opening hours and accessibility issues. There were too many local branches, with an excessive distribution of the personnel that did not meet the population's real needs. At most of the clinics there were no activities designed to invite 5- to 14-year-old children to be vaccinated.
The collection of the children's medical histories was redundant and not always the same at the various vaccination clinics.
There was no standardization of the administrative procedures handled by the clinics, e.g. the electronic vaccination register and the telephone booking system.
Now that vaccination is no longer mandatory, new vaccines have been included in the vaccination schedule and a wider population could benefit from them, some diseases are reappearing as a result of lower vaccination rates, and the population is increasingly distrustful of the health authorities, the vaccination services have important challenges to meet that demand rapid, unambiguous, evidence-based responses [3-5, 20-22].
To meet the demands of a changing world, it has therefore become necessary to reshape the organizational model of vaccination clinics to make them more effective and efficient in programming and modulating their activities.
All these considerations are supported by a look at the very different vaccination rates seen in various areas of the ULSS 12 considered here: while the Distretto 2 (Lido di Venezia, Cavallino Treporti and lagoon islands) had very high rates, the Distretti 3 and 4 (Mestre, Marcon and Quarto d'Altino) had intermediate rates, and the Distretto 1 (Venice historical town) had low rates, especially for some vaccinations (pneumococcus, chicken pox and measles). This difference was not without its consequences: in the winter of 2010-11 there was a small epidemic of measles in Venice city center, that involved 11 unvaccinated children aged 0-14 (Fig. 1).

Fig. 1. Vaccination rates in children born in July – December 2009 (survey conducted in March 2011).



	dose 3 hexavalent	dose 3 Pneumococcal	dose 1 Measles	dose 1 Varicella	dose 1 Men C
district 1	78,2%	54,1%	71,8%	66,4%	80,0%
district 2	96,4%	86,9%	92,4%	91,2%	94,7%
district 3	92,5%	72,6%	86,9%	83,6%	92,5%
district 4	91,5%	68,2%	86,7%	81,0%	92,1%

Aim of the experimental project and operational steps

To improve the manageability and flexibility of the local vaccination clinics and increase the population's adherence to vaccination programs, we designed an experimental project that involved centralizing the management of about half of the clinics under the SISP (as a first step preparatory to the transfer of all the vaccination clinics under the SISP).

Some practical goals were defined to change the way vaccination is promoted and to implement a continuing improvement of the organization:

- to establish targets shared by the SISP and the territorial vaccination clinics;
- to create a working group with frequent email exchanges between the SISP, general practitioners, family pediatricians and doctors at the vaccination clinics to promote the circulation of information and discuss common strategies to make the population aware of the importance of vaccination;
- to set up a regular information flow on the vaccination rates between the ULSS vaccination coordinator and the local doctors in the field in order to react promptly in the event of problems occurring in a given area;
- to extend the existing '*Vaccinare Informati*' (Vaccinating well informed) freephone service (activated in 2009 to deal with an outbreak of influenza A [H1N1] virus) to extend the provision of support and information to the population about vaccination generally, health precautions and prophylaxis for international travelers, preventive recommendations in the event of outbreaks of infectious diseases (meningitis, flu, measles, scabies, etc.);
- to organize training meetings and refresher courses for all personnel;
- to define standard procedures for managing vaccination clinics (inviting parents to attend, collecting children's medical histories, coadministering vaccines, adverse reactions, informative leaflets for parents, vaccine storage, etc.);
- to have vaccines supplied directly to the local clinics, instead of through the SISP;
- to centralize at the SISP the planning and management of some vaccination programs, e.g. for HPV (human papilloma virus) in adolescent girls, or booster doses at 14 years of age, or catch-up doses for those not vaccinated against meningococcus C, and second doses of MMR (measles mumps rubella) vaccine between age 5 and 14.

Results

The reorganization of the vaccination clinics for children was based on a continuous quality improvement approach, introducing the changes gradually. It began in March 2011 in the two districts with the worst performance (4 of the 14 vaccination clinics in districts D1 and D4). Results were assessed as the work proceeded,

Tab. I. Vaccination rates (%) in the 4 districts considered from March to September 2011 for children born in 2009.

Vaccine	District 1			District 2			District 3			District 4		
	March (CI 95%)	September (CI 95%)	Difference (CI 95%)	March (CI 95%)	September (CI 95%)	Difference (CI 95%)	March (CI 95%)	September (CI 95%)	Difference (CI 95%)	March (CI 95%)	September (CI 95%)	Difference (CI 95%)
Hexavalent	75.5 (69.8-81.2)	85.8 (81.2-90.4)	10.3 (6.3-14.3)	94.7 (91.3-98.1)	97.0 (94.4-99.6)	2.3 (0-4.6)	91.8 (89.5-94.1)	92.5 (90.3-94.7)	0.7 (0.0-1.4)	89.2 (86.2-92.2)	93.4 (90.9-95.9)	4.2 (2.2-6.2)
Pneumococcus	50.9 (44.3-57.5)	70.8 (64.8-76.8)	19.9 (14.6-25.2)	84.7 (79.3-90.1)	92.2 (88.1-96.3)	7.5 (3.5-11.5)	67.7 (63.7-71.7)	86.1 (83.2-89)	18.4 (15.1-21.7)	62.1 (57.3-66.9)	82.2 (78.4-86)	20.1 (16.2-24)
Measles	69.1 (63-75.2)	81.3 (76.1-86.5)	12.2 (7.9-16.5)	92.4 (88.4-96.4)	97 (94.4-99.6)	4.6 (1.4-7.8)	84.0 (80.9-87.1)	90.3 (87.8-92.8)	6.3 (4.3-8.3)	82.7 (79-86.4)	91.6 (88.9-94.3)	8.9 (6.1-11.7)
Varicella	64.1 (57.8-70.4)	76.3 (70.7-81.9)	12.2 (7.9-16.5)	91.2 (86.9-95.5)	96.4 (93.6-99.2)	5.2 (1.8-8.6)	80.6 (77.3-83.9)	86.3 (83.4-89.2)	5.7 (3.7-7.7)	76.9 (72.8-81)	85.2 (81.7-88.7)	8.3 (5.6-11)
Meningo C	79.1 (73.7-84.5)	84.9 (80.2-89.6)	5.8 (2.7-8.9)	94.1 (90.6-97.6)	97.0 (94.4-99.6)	2.9 (0.4-5.4)	91.6 (89.3-93.9)	92.7 (90.5-94.9)	1.1 (0.2-2.0)	89.4 (86.4-92.4)	93.1 (90.6-95.6)	3.7 (1.8-5.6)

adapting the solutions to each territorial clinic. By May 2012, all the clinics were being run by the SISP.

Contact between the local vaccination clinics, the SISP coordinating group, and family pediatricians and general practitioners increased, especially in the more complex situations (involving children with rare diseases, immigrants, high-risk vaccinations, etc.).

Standardized working procedures and materials at the vaccination clinics, and shared protocols and guidelines were introduced in the spring of 2011 as part of an institutional quality accreditation process. This included aspects of vaccine storage, the letters of invitation for vaccination, informative leaflets, and the forms used to record children's medical history, which were concise and the same for all the clinics. Opening hours were also standardized (8:30-12:30) at all the vaccination clinics. The vaccination management software (ONVAC) introduced at our ULSS as a pilot project in 2010 eased the process of standardizing working procedures.

Training meetings with the personnel were organized once a month and concerned the definition and acquisition of standard working procedures covering all aspects of their work, from vaccine storage to vaccine administration, contraindications and adverse reactions to vaccination, counseling for parents opposing vaccination, immunization recommendations for people traveling to high-risk countries, and the management of vaccination schedules for immigrant children. This facilitated the personnel's adhesion to the new working procedures, protocols and guidelines, their acquisition of expertise and their self-confidence in managing precautions, contraindications, adverse reactions and parental counseling.

The supply of vaccines directly to the local clinics (without the SISP's intermediation) began in spring 2011; it sustained the personnel's empowerment concerning the proper planning of their activities and vaccine storage.

The freephone service and telephone contact was reorganized: two call centers were created, one for the mainland area and one for Venice city center and its lagoon area. The availability of the service was extended to 25 hours/week instead of the few hours a week under the previous arrangements, and its scope was expanded to provide general recommendations on health and vaccine-related issues. It also became possible to better coordinate activities in the field by assigning vaccination appointments to the various clinics according to need.

The centralized personnel management led to a greater uniformity of the procedures and meant that operators were interchangeable among the various clinics in the event of vacations or sick leave.

The vaccination rates were monitored from the baseline when the reorganization process began, revealing a positive trend for all vaccines in all the districts involved. As expected, the greatest improvement was seen in the districts where the reorganization process began (D1 and D4) (Tab. I).

The reorganization process also gave the personnel some spare time to contact families whose children had yet to be vaccinated, and to invest in their continuing education and training activities. Members of staff were also able to take part in some health promotion schemes such as the promotion of breastfeeding and vaccination in antenatal classes.

By May 1st 2012, all 14 vaccination clinics were under SISP management. This demanded the transfer of 28 of the 37 health professionals involved (70% of all the personnel – measured in terms of equivalent units [EU]), while the other 30% remained on the district's payroll involved in other territorial activities (postnatal support for new parents, in-school activities for children with chronic diseases, infant care). Specifically, 3 doctors and 6 nurses previously employed in vaccination activities were deployed to such other activities in the districts. It is worth noting that there was a kind of 'natural selection' amongst the personnel, that led the more motivated to work with the SISP, thus promoting a virtuous process of personal empowerment amongst the staff.

We saw in increase in the vaccination activities for the population aged 0-17, as shown in Table II, with 2,124 more vaccines administered in one year while the reorganization process was underway (2011-2012), by comparison with the previous year. Once all the vaccination clinics had come under the SISP management (2012-2013), we recorded a further slight increase in the number of vaccines administered (+ 718), despite the 30% reduction in the workforce involved (Tab. II).

Tab. II. Number of vaccinations performed in children before, during and after reorganization the vaccination clinics.

	01.03.2010 – 28.02.2011		01.03.2011 – 28.02.2012		01.03.2012 – 28.02.2013	
	Vaccinations in 0-13 year-olds	Vaccinations in 14-17 year-olds	Vaccinations in 0-13 year-olds	Vaccinations in 14-17 year-olds	Vaccinations in 0-13 year-olds	Vaccinations in 14-17 year-olds
Venice and lagoon islands	7,446	493	8,042	568	7,163	491
Mestre and mainland area	17,992	3,118	19,272	2,197	20,626	1,737
SISP – central clinic	1,409	1,122	2,261	1,364	1,881	2,524
Total	**31,580**		**33,704**		**34,422**	
Difference	**+2,124**				**+718**	

Discussion and conclusions

During the reorganization process, the personnel showed a better compliance with the guidelines and a greater sense of responsibility in their various activities.

The basic goal that has been reached is a greater uniformity and a continuous improvement in interfacing with the population that has improved the credibility of the SISP and, as a consequence, people's adhesion to vaccination programs. This result is confirmed by the higher vaccination rates and the better relations seen between the population and the vaccination clinic personnel and other health professionals.

An important consequence of the reorganization was an improvement in the working atmosphere between members of staff, who now share targets and protocols more and better, speaking the same language with the population and with other health professionals such as family practitioners and hospital staff.

Another important outcome is a greater efficiency, demonstrated by an increase in the number of vaccines being administered by a much reduced workforce (70% of the operators previously involved). This goes to show that standardized work processes and a centralized management of activities can strongly influence results, even more than the size of the labor force involved.

The centralized organization has also enabled the population to access any vaccination clinic, making the service offered more flexible to cope with parents' needs, as well as giving the personnel from different areas a chance to share their experiences.

We believe that the organizational changes implemented will also enable the SISP to provide the population with a better-quality information and improved communications in the field of vaccination. This better communication between the SISP and family pediatricians, standardized working procedures and guidelines, more overall training, and the acquisition of counseling skills will enable health professionals to deal more effectively with the late vaccinators and vaccine-hesitant.

As of 2014, as a next step, the SISP will start organizing group meetings between the parents of newborn, SISP medical staff and family pediatricians to deal with the population's fears and uncertainties concerning vaccination.

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ORIGINAL ARTICLE

Women and alcohol. A survey in the city of Barletta

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Key words

Women • Binge drinking • Alcohol problems

Summary

Introduction. *The aim of this survey was to evaluate the qualitative and quantitative relationship among women from Barletta - a national renowned wine center - and their alcohol consumption.*
Methods. *The AUDIT questionnaire was used to assess the prevalence of alcohol hazardous consumption among women. Questionnaires were submitted from March to November 2012. The sample was composed of 150 women older than 13 years of age, selected by stratified sampling based on age group.*
Results. *107 women were enrolled with a total response rate of 71.3%. 62% of enrolled women consumes alcoholic beverages with a frequency that goes from 2-3 times a week to less than once a month, usually 1 or 2 alcoholic units. The binge-drinking was reported by 5% of women. Women who reported alcohol-related risk behaviors were less than 3%, they were single and between 18 and 60 years old and such behaviors occur less than once a month. The final score, calculated for all the women from their questionnaire answers, was not higher than 8, with an average score of 1.3 (SD = 1.5; range: 0 to 2.8). The comparison of the average scores of the three age groups showed a statistically significant difference (F = 5.8, p = 0.004).*
Discussion. *Data from literature showed a change in the habits of alcohol intake by the global, European and also Italian population. These changes also affect and involve female. Our study found a quite moderate alcohol consumption among women from Barletta, with only 1% who consumes 3 or more alcohol units and drink more than four times a week and 3% who had hazardous behavior related to their alcohol consumption. Statistical significance was found for the age and the lack of stable relationships. The analysis of characteristics of at risk women (old age and single-status) suggests that much attention should be paid to them and they should represent the main subject of future social interventions to prevent alcohol related problems in the city of Barletta.*

Introduction

Alcohol is probably the drug that humanity has been long consuming and, together with tobacco, it is the most widespread, too. The alcohol consumption, as well as cigarette smoking, is an unnecessary habit accepted and allowed in all countries (except Islamic ones); it often has a social meaning in joyful occasions and social gatherings because it helps having good and friendly relations or gives importance to special events. As communicated by WHO, the pattern of alcohol consumption has changed during the last decades compared to the past and has shown an increase in the proportion of heavy drinkers (people who drink more than 150 mls of alcohol per day) and those who drink spirits (aperitifs, bitters, liqueurs, etc.), in addition to wine and beer, from 39.5% to 41.1% over the 1998-2005 years [1, 2]. What worries most is that 50% of the under-14-year-old population consumes several kinds of alcoholic beverages and it is gradually raising the number of adolescents and young women who drink alcohol, often between meals: this proportion amount to over 21% among males aged 11-15 years, to almost 59% of 16-17 years and to more than 75% of 18-19 years; the amount among females aged 11-15 years is 17%, more than 42% among 16-17 years and 61% of 18-19 years. As a consequence, the alcohol related risks involving the working setting (drop in school performance and attendance) or being exposed to car accidents, use of violence, etc., are increasing in the younger groups [3]. Furthermore, alcohol is the third factor causing disability, critical illnesses such as cancer, vascular and liver diseases, premature mortality in the world (the second in Europe) [1, 2]. 2.5 million deaths per year (3.8% of all deaths) were caused by alcohol, 320,000 of which occur in people from 15 to 29 years old. In Europe, the percentages are doubled since the European Region has one of the highest alcohol consumption per capita [4, 5].
In Italy a change from a Mediterranean to a Nordic pattern of alcohol consumption was observed. Between 2000 and 2010 it was reported an increase both of frequency and quantity of alcohol consumption, often in one single occasion. A change in the kind of alcoholic beverage was found, too, especially in younger people, who prefer to drink aperitifs, cocktails and spirits, instead of wine and beer.
The quantity increase of alcohol consumption was registered both for males and females, these ones drinking

as much as men do [6]. The proportion of people aged 18-24 years who use to drink between meals increased from 33.7% to 41.9% and of people aged 14-17 years from 14.5% to 16.9%, the female part tripled in the last fifteen years. The binge drinking increased, too, with no genre difference since 2003 and it involved the 13.4% of men and the 3.5% of women in the 2010 [7]. Considering the relationship between woman and alcohol, in the past, women drank less, less frequently and in different situations than men. Nowadays, women still drink less than men, but this difference is gradually decreasing (in some European countries it is even reversed) [5, 8]. Moreover drinking habits and patterns are changed, especially in younger people: once women drank alone in the house, because of an old sense of privacy or for other reasons, today it frequently happens to see women consuming different kinds of alcoholic beverages in public places [9, 10]. The origins of this change are still not well defined: changes of social balances and the most frequent involvement of women in the business world certainly have played a role, because they push women to drink alcohol in different ways than in the past, as a sort of social and sexual claim. In some countries (especially in northern Europe) someone begins to talk about "drunkorexia", an eating disorder which implies the alcohol is the main food in a person's diet, and this could cause some related conditions, such as anorexia and hypovitaminosis. In Italy about 170,000 girls under 16 years consume alcohol even though abstinence is strictly recommended [11-13]. The relation between woman and alcohol is different from the relation that men have with it, either for the way of alcohol intake or the consequences it produces: beyond the genetic aspects and the different weight, women have a lower percentage of water in their body composition than men and this implies that, when women drink, alcohol produces the same effects as in males, because of the high solubility of alcohol, but with a lower alcohol amount (the liquid volume where alcohol will dissolve is lower, it will rise higher concentrations in women than men with the consumption of the same amount). Also the alcohol dehydrogenase hepatic enzyme (which metabolizes alcohol) has a lower concentration in female organism: therefore, the blood alcohol level stays high for a longer time and it also reduces the incubation period of alcohol-related pathologies, such as steatosis, cirrhosis and liver cancer.
The data from the PASSI Survey 2010 [14] showed that in Puglia the proportion of subjects who consumes alcohol is the same as the whole nation, while the hazardous drinkers proportion is lower. A hazardous drinker is characterized by at least one of the following behavior:

- regularly drinking between meals;
- heavy drinker: according to the definition of the threshold value for the heavy alcohol consumption, defined by the National Institute of Research on Food and Nutrition in accordance with the most recent scientific evidence, heavy drinkers are males who drink more than 2 alcohol units per day and females who drink more than 1 (the alcohol unit corresponds to a can of beer or a glass of wine or a shot of alcohol);
- binge drinking: is defined as the consumption of five or more alcohol units for males, four or more units for females on a single occasion, at least once a month.

In Puglia, 13% of people enrolled in the survey can be classified as "at-risk drinker" (in Italy, 19%). This estimate corresponds to about 365,000 people aged 18-69 years, 80,000 (21.9%) of which in the range 18-24 years old age [14].
The risky alcohol consumption is more frequent:

- among respondents aged 18-24;
- for males;
- among people with a middle/high educational attainment who are in financial difficulties.

As regard females, the survey showed that the distribution of at risk drinking women is different in the different local health services of the region (range: 3% Lecce - Taranto 12%), with lower and higher percentages than the regional averages, like for the ASL Lecce and Taranto.
The survey performed in the city of Barletta is aimed to evaluate the qualitative and quantitative relationship between women from this town and their alcohol consumption.
Barletta is a city in the Puglia Region and it belongs to the ASL BAT. It has a population of about 94,904 inhabitants, with an average age of 39.7 years, the birth rate is 9.8% and the death rate is 7.4%. It is a national renowned wine center, thanks to the presence of a big winery and other wine factories.

Methods

To assess the prevalence of alcohol hazardous to the health consumption among women of the city the AUDIT questionnaire (Alcohol Use Disorders Identification Test) was used, which is a questionnaire developed by the World Health Organization as a screening tool for the identification of at-risk drinkers [15-17].
The questionnaire consists of 10 items that inquire into alcohol consumption, ways of alcohol intake and alcohol-related problems in the previous 12 months. One of the items was about binge-drinking.
For each question there were multiple entries and a score between 0 and 4 was associated to each entries. The sum of all entries' score quantifies the possible risk a subject has in his relationship with alcohol. This final score is stratified into three groups:

- 0-8: negative, no problems to report;
- 8-14: the consumer can be considered "at risk subject", he/she has or has had alcohol-related problems (accidents or occasional heavy drinking), but he/she probably hasn't a physical addiction to alcohol, yet;
- equal to or greater than 15: he/she has alcohol-related problems and/or is an alcohol-dependent subject.

A written informed consent was obtained from women who participated to the survey. In addition to the questionnaire, the enrolled subjects were asked to fill out a

Tab. I. Distribution of sample by age group.

Age group	N	%
< 18	9	8.4
18 - 60	48	44.9
> 60	50	46.7
Total	107	100.0

form which investigated age, marital status, educational attainment and job.
Questionnaires were submitted from March to November 2012.
The sample was composed of 150 women older than 13 years of age, living in the city of Barletta, selected by stratified sampling based on age group. The sample was divided into three strata: the first, which included women younger than 18 years (A Group), the second including women between 18 and 60 years (B Group), the third including women aged over 60 years (C Group). Each stratum consisted of 50 units.
Data from filled questionnaires were entered into a File Maker Pro 10 database and analyzed with the statistical software STATA MP11.

Results

107 women were enrolled from a 150 units sample under investigation. The response rate amounted to 71.3% (Tab. I).
Five of younger than 18 women have not filled out the form for personal details; another woman did not indicate age. Therefore, the average age of the sample was calculated for 101 subjects and was equal to 54.4 years; the description of personal characteristics, instead, was carried out on 102 subjects; analysis of the questionnaires answers was performed for 107 subjects.
The lowest response rate (18%) was reported among women younger than 18 years. This low participation was owed to the fact that a parental consent was required to the girls in order to join the study and a small number of parents let their daughters to participate and answer the questionnaire.
The educational attainment of the enrolled women was assessed considering the highest qualification achieved. Graduated women were the smaller proportion of the sample (7.8%), while 12.7% have no educational qualifications (Fig. 1).
Analyzing the distribution of educational attainment by age group, the total of women with low qualification or no title was older than 60 years, while women with a higher educational attainment were in the group aged between 18 and 60 years old.
Considering the job of enrolled women, the majority of women (54%) was reported to be housewife. Self-employed (2%) and unemployed women (3%) are less frequent (Fig. 2).
As regard as their marital status, 49% of women reported to be widowed, 11% to be married, 37% is unmarried, while 3% is separated or divorced.
The first question of the AUDIT questionnaire investigated the frequency of alcoholic beverages intake. 37% of women reported that they have never drunk alcohol, about 1% reported to have drunk alcohol more than four times a week. The remaining 62% consumes alcoholic beverages with a frequency that goes from 2-3 times a week to less than once a month. Table II shows the frequency of alcoholic beverages consumption in the different age groups.

Fig. 1. Distribution of sample by educational qualification.

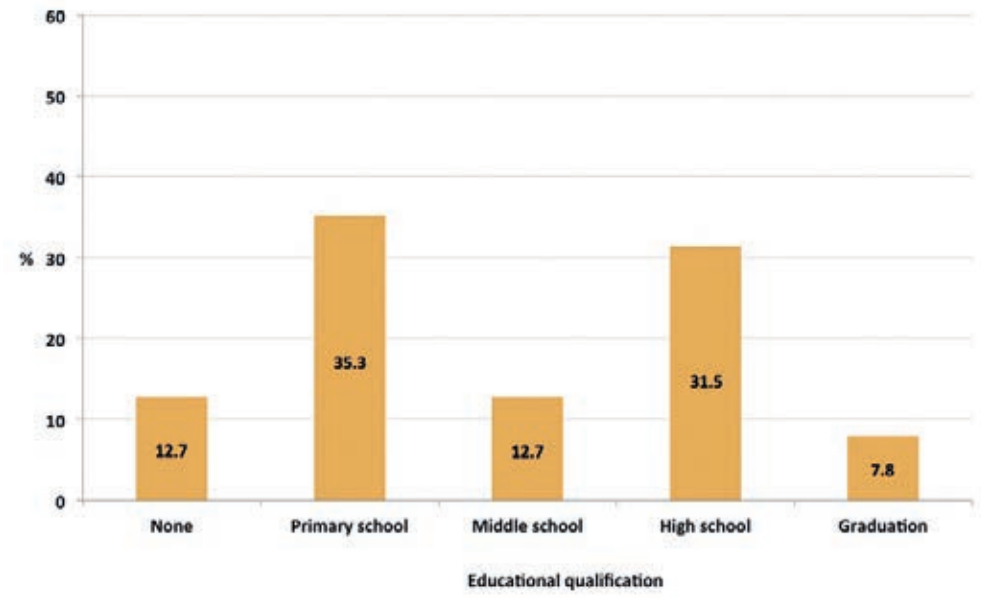
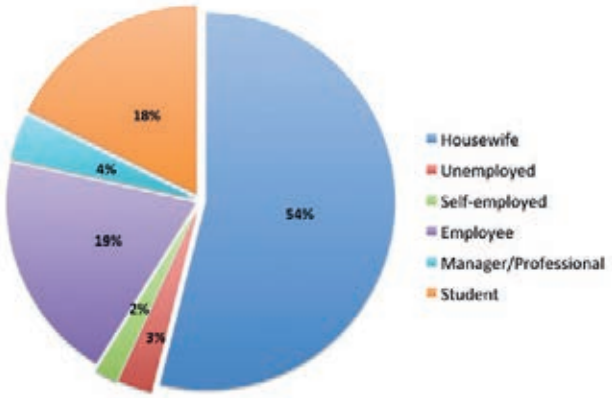


Fig. 2. Distribution of sample by job.



61.7% of the women stated they consumed 1 or 2 alcoholic units when they drank, less than 1% consumed 3 or more units.
The binge-drinking was reported by 5% of women, with a frequency of less than once a month.
The proportion of women who reported alcohol-related risk behaviors is less than 3% (Fig. 3) and such behaviors occur less than once a month.
All the women who reported alcohol-related hazardous behaviors were single and between 18 and 60 years old. In particular, the two women who were not able to stop drinking once started are an employee and unemployed woman; one of the three women who were not able to do daily activities because of drinking is a professional and two are unemployed. Two more women reported to feel guilty or regret after drinking and they were both unemployed. One of the three women who said they did not remember the events of the previous evening, due to the intake of alcohol, is an employee and two are unemployed.
All of the enrolled women stated they never received any advice to quit drinking by family, friends and health professionals.
The final score, calculated for all the women from their questionnaire answers, was not higher than 8. The average score was 1.3 (SD = 1.5; range: 0 to 2.8).
Women under 18 had an average score of 0.4, those aged 18 to 60 had an average score of 1, the average score of over 60 women was 1.8. The comparison of the average scores of the three groups showed a statistically significant difference ($F = 5.8$, $p = 0.004$). There were no significant differences between the average, grouped by educational attainment of the enrolled women ($F = 2.4$, $p = 0.052$).
There was no correlation between the score and the age of the enrolled women.

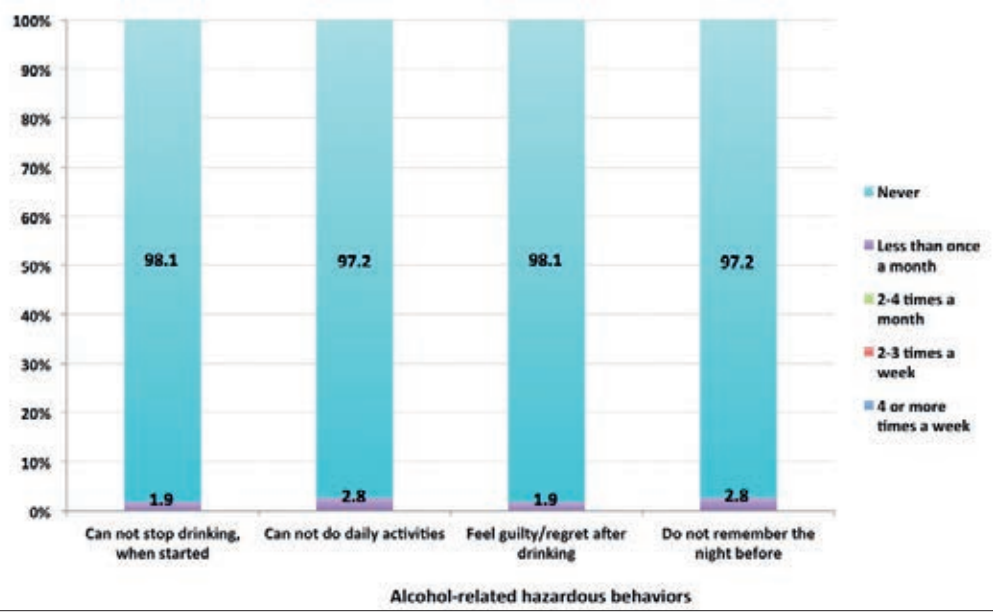
Discussion and conclusions

Data from literature clearly showed a change in the habits of alcohol intake by the global [2], European [4, 5] and also Italian [6, 7] population, this last one being less important [5]. These changes also affect and involve female. In fact, while in the past the woman used to drink at home, often alone and because of peculiar family and/or social situations, nowadays it is much common for women to have the same drinking habits men typically have [8]. Even the types of alcohol beverages the women consumes have changed: the abuse of alcopops, beer and spirits, that is not typical of the Mediterranean tradition, is increasing [7].
The results of our study showed that the alcohol consumption among women from Barletta is quite moderate, along with reported literature data [1, 5]. More than a third of the enrolled women said they never take alcoholic drinks, others said they only occasionally consume more than 1-2 alcohol units. The hazardous alcohol consumption was found in a small proportion of women (1%), with lower values compared to national [5] and regional ones [14], even if the binge-drinking involves a higher proportion (5%) of women than the national average [5].
Only ten of the women with alcohol risky consumption (3%) reported alcohol-related hazardous behaviors. They are all unmarried women between the ages of 18 and 60 years. Statistical significance was found for the age (women older than 60 years consume much alcohol) and the lack of stable relationships. This suggests that seniority and solitude could induce the alcohol consumption.
Despite the small sized sample and the lower response in the under 18 years old age group, which doesn't allow to have information about alcohol consumption in the youngest women, the study demonstrated that alcohol consumption is still moderate among women from the city of Barletta and not associated to harmful behavior and onset of alcohol-related problem. Nevertheless it suggests that much attention should be paid to women at risk because of their age and single-status, which seemed to be risk factors for alcohol related harmful be-

Tab. II. Distribution of sample by frequency of alcoholic beverages consumption and age group.

Frequency of alcoholic beverages consumption	< 18		18 - 60		> 60		Total	
	n	%	n	%	n	%	n	%
Never	6	66.7	8	16.7	26	52.0	40	37.4
Less than once a month	2	22.2	17	35.4	8	16.0	27	25.2
2-4 times a month	1	11.1	13	27.1	5	10.0	19	17.8
2-3 times a week	0	0.0	10	20.8	10	20.0	20	18.7
4 or more times a week	0	0.0	0	0.0	1	2.0	1	0.9
Total	9	100.0	48	100.0	50	100.0	107	100.0

Fig. 3. Proportion of women who reported alcohol-related risky behaviors, by frequency of hazardous behavior.



havior and problems, and they should represent the main subject of future social interventions to prevent alcohol related problems in the city of Barletta.

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ORIGINAL ARTICLE

Association of objective health factors with self-reported health

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Key words

Self-reported health status • Cardiovascular risk • Risk factors • Primary care

Summary

Objectives. *There is a strong relationship between subjective health and mortality, level of functional ability and medical consumption. The aim of this study was to describe the correlation of objective health-related factors with self-reported health (SRH) of a sample of the Belgian population.*
Methods. *Participants were recruited during an exhibition at the Brussels Exhibition Centre. They completed a visual analogue scale assessing their SRH. Medical history and health related parameters of the participants were recorded.*
Results. *In total 974 visitors participated. From the multivariate analysis we found an association between low SRH and diabetes (OR 0.23-0.80), increased body mass index (OR 0.52-0.74), coronary heart disease (OR 0.28-0.97), smoking (OR 0.38-0.89), speaking Dutch (OR 0.40-0.92), not knowing length (OR 0.36-0.99), family history of breast cancer (OR 0.41-0.94), family history of coronary heart disease (OR 0.45-095) and aging (OR 0.84-0.99). Following a cholesterol-lowering diet was associated with a high SRH (OR 1.10-2.44).*
Conclusions. *Most of the factors associated with low SRH are known and confirm what has previously been reported in literature. However, the associations between low SRH and not knowing your length, speaking Dutch or having a family history of breast or colon cancer, as well as the association between high SRH and being on a cholesterol-lowering diet are interesting new findings.*

Introduction

Good health seems to be an important determinant for the well-being and quality of life of people. The context of health is not limited to the physical well-being of individuals or communities but should take into account the emotional, socio-economic, mental, spiritual and cultural well-being of the individuals in the community. The most widely used definition of health is that provided by the World Health Organization (WHO): "Health is a complete state of physical, mental and social well-being, and not merely the absence of disease or infirmity" [1].
We aimed to measure the subjective health status in one question, which means that our measurements included not only physical health, but also social and psychological health components. We may assume that such a subjective health status will be affected by the presence of symptoms or specific complaints, but also by the medical diagnoses and risk factors known by the participant [2].
In the past, several studies have been set up to identify the determinants of subjective health. However, it has never been possible to develop an accurate conceptual description of subjective health status [3].
According to the second goal of the WHO Millennium Development Goals, everyone should have the possibility to develop one's own health potential. The subjective assessment of one's own health is considered to be a good indicator in this context, both at the individual level and at the level of society.
Many studies were able to demonstrate a strong cohesion between subjective health and mortality [4]. Subjective health also seems to be a good predictor of morbidity, the level of functional ability and medical consumption [5-10]. The subjective perception of health status is therefore a useful tool to detect high-risk persons and to estimate care requirements.
This study aimed to describe the correlation of objective health-related factors with the perceived health of a sample of the Belgian population.

Methods

Participants

Participants were recruited during a food exhibition at the Brussels Exhibition Centre from October 6th to October 21th 2012. All adult visitors were invited to participate. Exclusion criteria were: pregnancy, taking vitamin K antagonists, showing signs of addiction to alcohol, medication or drugs, or being intolerant to blood and / or finger pricks.

Questionnaire

To evaluate the self-reported health (SRH) the part of the EQ-5D questionnaire measuring SRH using a visual analogue scale (VAS) was used [11]. We did not use

the other scales from the EQ-5D questionnaire. Participants were asked to score their perceived health status on a scale between 0 and 100 with 0 corresponding with the worst health participants could possibly imagine and 100 meaning the best imaginable health.
Scoring on the VAS was performed after recording the participants' age, gender and zip-code but before other health-related questions were asked.
Participants were asked if they knew their length, weight, abdominal circumference, blood pressure, cholesterol level and blood sugar level. Subsequently these parameters were also measured. Furthermore, participants were asked about their medical history (coronary heart disease, hypercholesterolemia, diabetes, hypertension, other diseases) and their family history (breast cancer, coronary heart disease, diabetes, colon cancer). They were also asked about their latest tetanus vaccination.

Measurements

Some parameters of the enrolled subjects were measured. We respectively used a digital personal scale Seca Sensa 804 to measure weight, a Seca 206 wall-mounted measuring tape for height, a Seca 201 ergonomic circumference measuring tape for abdominal circumference, a calibrated DS-54 WelchAllyn sphygmomanometer blood pressure device for blood pressure, a OneTouch device using capillary blood for blood sugar and an Accutrend Plus monitor using capillary blood for total cholesterol. Capillary blood was obtained by pricking the index finger. A short medical history was obtained, focusing on food and beverage intake during the two hours before the measurement.

Approval of the ethical comity

The study protocol was approved by the ethical committee of the University Hospital Brussels. Visitors of the exhibition were allowed to participate after they had read the patient-information leaflet and had signed an informed consent form. After completing the questionnaire, participants were offered personalised health advice.

Statistical processing

Data were anonymously recorded in a mySQL database using an online custom-made PHP-based interface, hosted by the Faculty of Medicine and Pharmacy of the Vrije Universiteit Brussel. Incomplete data sets were eliminated from the database. From the entered data, body mass index (BMI) was generated by the system. Cardiovascular risk was estimated by the system using the Belgian SCORE risk tables [12]. Participants were assigned to either a low-SRH-group or a high SRH-group, using the SRH median (7.14) as an arbitrary cutoff. This permitted us to compare two groups of a similar size.
Statistical analyses were performed using SPSS 22, using cross-tables and the Chi-Square test for discrete variables. A t-test was used to compare the means of two groups and the one-way ANOVA was used to compare the means of three groups.

A logistic regression was performed to determine variables linked with high or low SRH. The following variables were entered: gender, age-groups, language, region, hypertension, hypercholesterolemia, diabetes, coronary heart disease, no disease, family history of coronary heart disease, family history of colon cancer, family history of diabetes, family history of breast cancer, no family history, tetanus vaccination up-to-date, weight, length, abdominal circumference, does know blood pressure, does know blood sugar level, does know cholesterol level, no treatment or diet for cholesterol, diet for cholesterol, statin for cholesterol, plant stanol for cholesterol, smoker, alcohol abuse, physical activity, BMI (4 groups), cardiovascular risk groups (SCORE low, intermediate and high).

Results

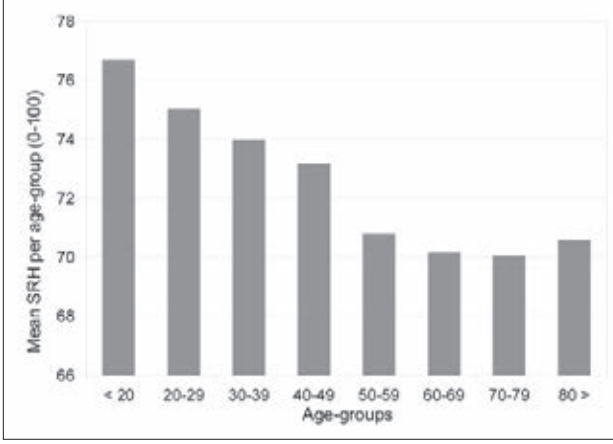
Study population

In total 974 visitors participated: 31% men and 69% women. Ages ranged from 18 to 90 years, with an average of 53.3 years. Most of the participants (77%) were from the Flemish region, 18% from the Brussels Region and 5% from the Walloon Region.
The median SRH was 71.4%: 499 participants had a SRH of 71% or lower and 475 had a SRH of more than 71%.
The mean SRH was the highest among the very young (< 20 year) and decreased gradually until the age group of 50 to 59 years, to remain stable in the older age groups (Fig. 1).

Mean self-reported health

The mean SRH did not differ between men (72%) and women (71%) (Tab. I.). Neither was there a significant difference between regions, although Dutch-speaking participants had a lower SRH than French-speaking participants.
SRH was lower among participants with known hypertension, diabetes and coronary heart disease compared to those without these conditions.

Fig. 1. Mean self-reported health (SRH) per age-group.



Tab. I. Mean self-reported health (SRH) per group.

	N	Mean SRH	Std. Dev.	p-value
Men	299	72.36	15.113	0.160
Women	675	71.00	13.314	
Brussels Region	174	71.36	17.274	0.176*
Flemish region	749	71.19	13.030	
Walloon region	51	74.94	13.143	
Dutch	857	70.75	13.746	< 0.001
French	117	76.34	14.069	
No hypertension	749	72.44	13.148	< 0.001
Hypertension	225	68.04	15.707	
No hypercholesterolemia	656	71.98	13.891	0.072
Hypercholesterolemia	318	70.27	13.860	
No diabetes	912	71.85	13.592	< 0.001
Diabetes	62	65.11	16.686	
No coronary heart disease	919	72.02	13.236	< 0.001
Coronary heart disease	55	61.35	19.798	
No disease	528	73.15	12.941	< 0.001
Some disease	446	69.38	14.704	
No family history of coronary heart disease	824	72.11	13.466	< 0.001
Family history of coronary heart disease	150	67.65	15.578	
No Family history of colon cancer	874	71.55	14.015	0.376
Family history of colon cancer	100	70.33	12.840	
No family history of diabetes	736	72.13	13.417	0.005
Family history of diabetes	238	69.23	15.107	
No family history of breast cancer	861	71.67	14.000	0.109
Family history of breast cancer	113	69.55	12.990	
No Family history of no disease	527	72.83	13.393	0.001
Family history of some disease	447	69.76	14.308	
Tetanus vaccination not up-to-date or unknown	422	71.32	13.308	0.849
Tetanus vaccination up-to-date or unknown	552	71.49	14.343	
Does not know weight	21	69.57	12.424	0.499
Does know weight	953	71.46	13.931	
Does not know length	78	67.68	16.289	0.035
Does know length	896	71.75	13.631	
Does not know abdominal circumference	892	71.62	13.666	0.197
Does know abdominal circumference	82	69.23	16.135	
Does not know blood pressure	140	73.31	12.662	0.062
Does know blood pressure	834	71.10	14.076	
Does not know blood sugar level	321	72.29	14.058	0.174
Does know blood sugar level	653	70.99	13.808	
Does not know cholesterol level	330	72.36	14.311	0.136
Does know cholesterol level	644	70.94	13.666	
No diet or treatment for cholesterol	622	71.72	13.926	0.369
Diet or treatment for cholesterol	352	70.89	13.849	
No diet for cholesterol	846	71.28	13.996	0.390
Diet for cholesterol	128	72.37	13.241	
No statin treatment for cholesterol	794	72.12	13.553	0.001
Statin treatment for cholesterol	180	68.32	14.974	
No plant stanol treatment for cholesterol	822	71.14	14.111	0.117
Plant stanol treatment for cholesterol	152	72.93	12.617	
Non-smokers	865	71.75	13.897	0.034
Smokers	109	68.77	13.676	
Non-alcohol abusers	944	71.55	13.784	0.200
Alcohol abusers	30	67.47	16.870	
No physical activity	678	70.78	14.353	0.029
Physical activity	296	72.90	12.692	
Underweight (BMI < 18.5)	39	74.74	10.371	< 0.001*
Normal weight (18.5 < BMI < 25)	433	73.47	14.231	
Overweight (25 < BMI < 30)	360	70.87	13.167	
Obesity (BMI > 30	142	65.66	13.846	
Low cardiovascular risk SCORE	690	72.67	13.095	< 0.001*
Intermediate cardiovascular risk SCORE	149	71.88	12.054	
High cardiovascular risk SCORE	135	64.51	17.430	

* p = one way Anova

Having a disease was related to a lower SRH than not having a disease. Having a family history of diabetes or coronary heart disease was also related to a lower SRH. However, having a family history of breast cancer or colon cancer was not related to a lower SRH. But having a family history of a hereditary disease was related to a lower SRH than not having family history of such a disease.
Several other health parameters were assessed but only few were related to a lower SRH: not knowing one's own length, taking a statin, smoking and having little physical activity.
There was a linear relationship between BMI and SRH: the higher the BMI the lower the SRH. A similar relationship was found between cardiovascular risk and SRH: the higher the risk, the lower the SRH.

Mean values for parameters

In the group with low SRH, median age, mean previous and actual systolic blood pressures, previous and actual weights, previous and actual blood sugar levels, actual BMI and actual abdominal circumferences were higher than in the high SRH group. In the group with high SRH, mean previous and actual lengths were higher (Tab. II).

Logistic regression

Where possible, parameters were dichotomized and included in a logistic regression (Tab. III.). Participants were divided into age groups of 10 years, four BMI groups and three cardiovascular risk groups. A multivariate analysis confirmed the relationships between low SRH and diabetes (OR 0.23-0.80), increased BMI (OR 0.52-0.74), coronary heart disease (OR 0.28-0.97), smoking (OR 0.38-0.89), speaking Dutch (OR 0.40-0.92), not knowing length (OR 0.36-0.99), family history of breast cancer (OR 0.41-0.94), family history of coronary heart disease (OR 0.45-095) and aging (OR 0.84-0.99). Following a cholesterol-lowering diet was associated with a high SRH (OR 1.10-2.44).

Tab. II. Mean values for parameters for low and high self-reported health (SRH).

	SRH group	N	Mean	Std. Dev.	p-value
Age	Low	499	55.39	16.600	< 0.001
	High	475	51.07	18.156	
Last measured systolic blood pressure	Low	355	127.47	16.488	0.014
	High	304	124.69	11.734	
Last measured diastolic blood pressure	Low	355	78.26	9.245	0.54
	High	304	77.86	7.735	
Last measured weight	Low	483	73.62	15.651	< 0.001
	High	466	69.63	12.465	
Last measured length	Low	442	166.52	8.741	0.008
	High	448	168.06	8.594	
Last measured abdominal circumference	Low	30	95.63	12.164	0.165
	High	19	90.21	13.559	
Last measured blood sugar	Low	261	92.17	17.741	< 0.001
	High	258	85.91	10.267	
Last measured cholesterol	Low	265	196.26	34.851	0.091
	High	254	191.00	35.787	
Mean number of cigarets per day	Low	499	1.62	5.150	0.169
	High	475	1.19	4.614	
Mean number of alcoholic beverages per day	Low	499	4.29	7.673	0.277
	High	475	3.81	5.866	
Actual systolic blood pressure	Low	499	125.90	16.175	0.049
	High	475	124.00	13.773	
Actual diastolic blood pressure	Low	499	77.58	9.240	0.861
	High	475	77.68	7.933	
Actual blood sugar	Low	499	109.70	53.936	< 0.001
	High	475	98.71	22.465	
Actual body weight	Low	499	73.56	15.633	< 0.001
	High	475	69.62	12.412	
Actual length	Low	499	166.12	8.792	0.002
	High	475	167.85	8.789	
Actual total cholesterol	Low	499	181.02	33.648	0.460
	High	475	179.43	33.501	
Actual abdominal circumference	Low	499	92.82	13.878	< 0.001
	High	475	87.37	12.410	
Actual body mass index	Low	499	26.062	4.8485	< 0.001
	High	475	24.226	3.9512	

Tab. III. Logistic regression: factors related to high or low self-reported health (SRH).

	B	p-value	OR	95% C.I.for OR	
				Lower	Upper
Age-groups (per 10 years)	-0.092	0.037	0.912	0.836	0.995
Speaking Dutch (vs French)	-0.501	0.019	0.606	0.399	0.920
Diabetes (Y/N)	-0.839	0.008	0.432	0.232	0.803
Coronary heart disease (Y/N)	-0.658	0.041	0.518	0.275	0.974
Family history of coronary heart disease (Y/N)	-0.421	0.027	0.656	0.451	0.954
Family history of breast cancer (Y/N)	-0.479	0.025	0.619	0.407	0.942
Does not know length (Y/N)	-0.521	0.047	0.594	0.355	0.992
Does not know blood sugar level (Y/N)	-0.503	0.058	0.605	0.360	1.017
Does not know cholesterol level (Y/N)	0.483	0.077	1.621	0.949	2.770
Follows a cholesterol diet (Y/N)	0.491	0.016	1.634	1.097	2.435
Smoking (Y/N)	-0.547	0.013	0.579	0.377	0.889
Body mass index (4 groups underweigh > obesity)	-0.476	<0.001	0.621	0.520	0.742

Variable(s) entered on step 1: gender, age-groups, language, region, hypertension, hypercholesterolemia, diabetes, coronary heart disease, no disease, family history of coronary heart disease, family history of colon cancer, family history of diabetes, family history of breast cancer, no family history, tetanus vaccination up-to-date, weight, length, abdominal circumference, does know blood pressure, does know blood sugar level, does know cholesterol level, no treatment or diet for cholesterol, diet for cholesterol, statin for cholesterol, plant stanol for cholesterol, smoker, alcohol abuse, physical activity, body mass index (4 groups), cardiovascular risk groups (SCORE low, intermediate and high).

Discussion

Sample population

Women and participants from the Flemish region were overrepresented in our study. Extra bias was caused by the fact that all participants were visitors of a food exhibition, meaning that severely disabled or seriously ill people were less likely to participate. As we never aimed to include a representative sample of the Belgian population, this did not hamper the interpretation of our results. Our purpose was to describe the correlation of objective health-related factors with SRH in an arbitrary sample of the Belgian population.

Self-reported health

Health status was not assessed by an objective third party. Even though self-assessment is undoubtedly influenced by external factors, including the views of other people, it was ultimately the participant himself who answered the questions.

This type of subjective assessment could rather be an emotional reflection than a systematic, cognitive analysis. Moreover, the subjective measurement of health is without any doubt related to the participants' quality of life.

We were particularly interested in "general" health and not in "current" health. With this subtle difference we tried to reduce the influence of temporary health issues. However, it is not clear how well participants were able to distinguish their general health from their actual health.

The median SRH in our study was 71.4%. In a Finnish study, a similar population had a SRH of 70% [13]. A similar study in Singapore also reported a SRH of approximately 70% [14]. Another study among institutionalised elderly reported a SRH of 78 [15]. Some caution is needed while comparing SRH-results from different studies and different countries, because a subjective approach of health could be highly influenced by cultural diversity. However, such a subjective assessment is sometimes influenced by some cultural related tendencies to complain more or to reflex a rather pessimistic view. Also the functional status appears to be related to ethnic variation [3]. Thereupon, SRH seems to be more useful to monitor health over a period of time or before and after a treatment.

Another limitation of the SRH assessment using a VAS is the difficulty to differentiate participants considering themselves in good or bad health because every participant might use its own arbitrary cut-off between good and bad health. Assessment methods as proposed by the WHO or the one used in the National Health Interview Survey in de United States do not have this disadvantage [10, 16].

Comparison with other studies

In the Belgian Health Interview Survey (HIS) of 2008 – using the assessment method proposed by the WHO – 23% of the participants considered themselves not to be in good health [17]. As we used a VAS we cannot compare this figure with our results. However, some comparisons are possible. In the HIS more women (25%) than men (20%) reported a bad health. This was not confirmed by our findings. Neither could we confirm that inhabitants of the Flemish region complained less about bad health (21%) than the inhabitants of the Walloon region (26%) or Brussels region (26%).

In our study (very) young participants scored the highest mean SRH with a gradual decrease until the age of 50. The HIS showed a similar evolution. But contrary to our findings that SRH remained stable in the older age groups, the HIS reported a continuing decrease in SRH after the age of 60. This can be explained by the fact our study population was a selected "mobile" population and that disabled or seriously ill people were less likely to visit the exhibition.

Another reliable source on health is the Organisation for Economic Co-operation and Development (OECD) [18]. From the most recent data (2011), 74% of the Belgian adult population considers its health as good. In Portugal, for example, only 50% of the population considers its health as good. The highest SRH is found in the United States with 90%. The figures for Belgium are comparable with The Netherlands (76%) although some other West-European countries such as Germany (65%) and France (68%) report substantially lower figures.

Factors associated with self-reported health

This multivariate analysis reconfirmed the relationship between low SRH and known diabetes, as has already been reported in other studies [19, 20]. Several studies also found increased BMI to be related to low SRH [21-23]. Similar evidence exists for coronary heart disease and aging [24, 18]. There is even some evidence that SRH predicts the prognosis after percutaneous coronary stenting [25].

Regarding the association between smoking and SRH strong evidence exists. An Irish study showed that non-working status, no private health insurance, inability to afford enough food, no car, being non-married, low social participation, serious neighbourhood problems, low social support, smoking, no alcohol consumption, illicit drug use, low physical activity and obesity were associated with poor SRH [26].

Only little is known about the influence of language on the SRH. A study in Singapore showed that Chinese speaking participants reported a lower SRH than English speaking participants despite that only very few differences existed between both groups, except the language [14]. In our study Dutch-speaking participants reported a lower SRH than French-speaking participants. The importance of language as such is unclear, because important cultural and socio-economic differences exist between both groups.

Our study detected an association between low SRH and not knowing one's own length. Although only few participants didn't know their length, we found it to be a strong predictor of low SRH. To our knowledge no information exists on this association, but other studies do mention an association between knowing one's own medical condition and SRH [15]. It is remarkable that in our study this association was only found for length and not for blood pressure, weight or other evident parameters. Having a family history of breast cancer or coronary heart disease also seems to impact on SRH. Knowing that one has an increased risk for a hereditary disease seems to affect one's SRH. However, this was not confirmed for colon cancer.

In our study, being on a cholesterol-lowering diet was associated with a high SRH, confirming previous evidence that people who are on a diet report a higher SRH [27]. One might expect that the quality of life of people on a diet is lower than of those who are not. Leading a healthy lifestyle probably has a favourable impact on the SRH. However, people following a diet often have an unhealthy lifestyle and compensate with a diet.

Conclusions

Our findings on the associations between low SRH and diabetes, increased body mass index, coronary heart disease, smoking and aging are known and confirm what has previously been reported in literature. However, the associations between low SRH and speaking Dutch, not knowing your own length or having a family history of breast cancer or colon cancer as well as the association between high SRH and being on a cholesterol-lowering diet are new and interesting findings. This is important because these factors might have an impact on the assessment of SRH.

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Conflicts of interest

Logistical support for this study was provided by Unilever Belgium who funded two student workers.

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