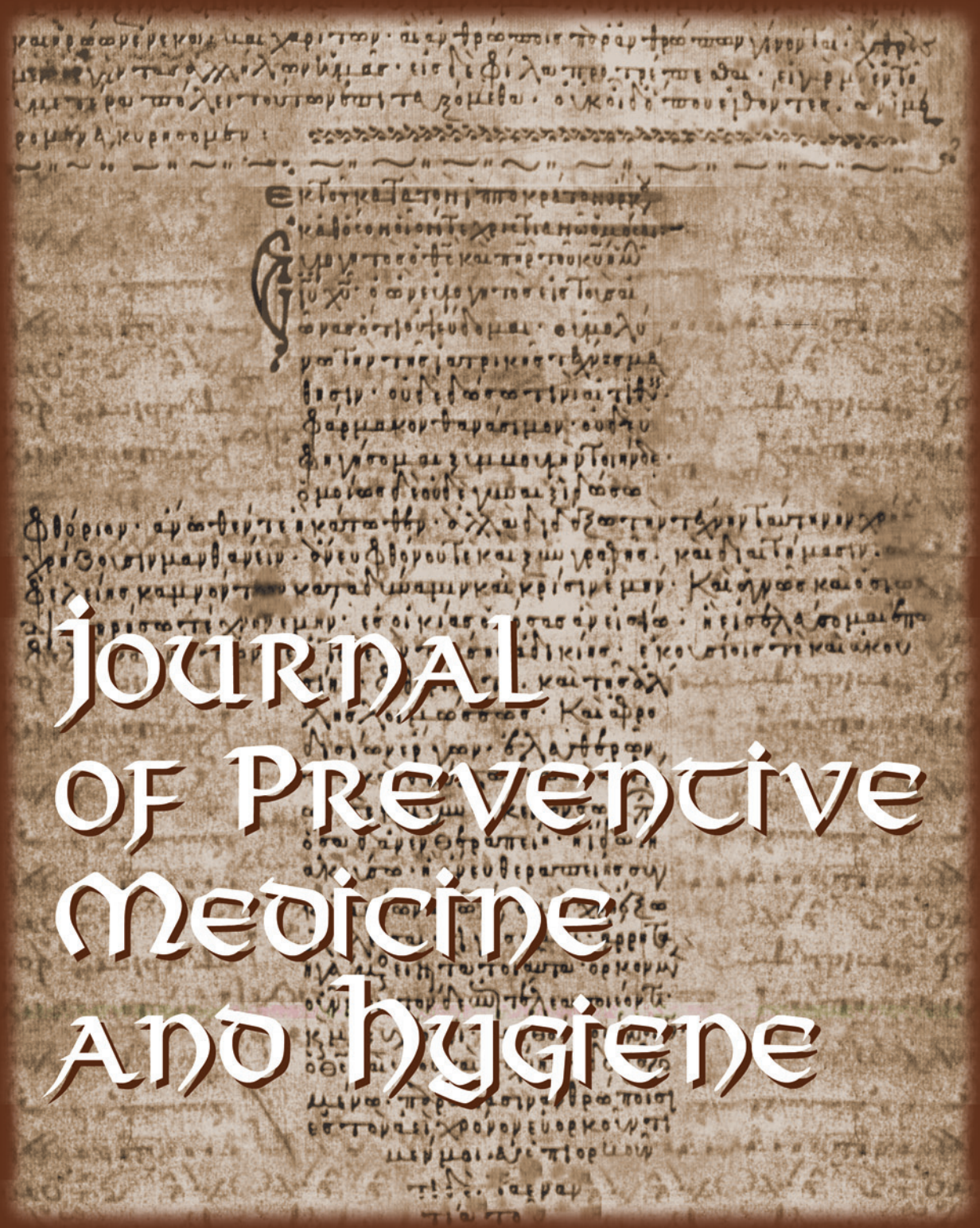
ISSN 1121-2233

vol. 55 n. 4

December 2014

Cited in Index Medicus / Medline
NLM ID 921440 (Pub-Med)



Journal of Preventive Medicine and Hygiene

The original document of Hippocrates' oath

Periodico trimestrale. - Aut. Trib. di Genova n. 507 del 6/10/60

The Journal has been accreditated, on occasion of the 17th December 2004 Meeting of the Executive and Scientific SItI Councils, by the Italian Society of Hygiene, Preventive Medicine and Public Health

PACINI EDITORE MEDICINA

JOURNAL OF PREVENTIVE MEDICINE AND HYGIENE

http://www.jpmh.org

Volume 55 - Issue 4 December 2014

CONTENTS

Review

Compounds with anti-influenza activity: present and future of strategies for the optimal treatment and management of influenza

Part II: Future compounds against influenza virus

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Key words

Influenza • Antivirals • Experimental drugs

Summary

In the first part of this overview, we described the life cycle of the influenza virus and the pharmacological action of the currently available drugs. This second part provides an overview of the molecular mechanisms and targets of still-experimental drugs for the treatment and management of influenza.
Briefly, we can distinguish between compounds with anti-influenza activity that target influenza virus proteins or genes, and molecules that target host components that are essential for viral replication and propagation. These latter compounds have been developed quite recently. Among the first group, we will focus especially on hemagglutinin, M2 channel and neuraminidase inhibitors. The second group of compounds may pave the way for personalized treatment and influenza management. Combination therapies are also discussed.
In recent decades, few antiviral molecules against influenza virus infections have been available; this has conditioned their use during human and animal outbreaks. Indeed, during seasonal and pandemic outbreaks, antiviral drugs have usually been administered in mono-therapy and, sometimes, in an uncontrolled manner to farm animals. This has led to the emergence of viral strains displaying resistance, especially to compounds of the amantadane family. For this reason, it is particularly important to develop new antiviral drugs against influenza viruses. Indeed, although vaccination is the most powerful means of mitigating the effects of influenza epidemics, antiviral drugs can be very useful, particularly in delaying the spread of new pandemic viruses, thereby enabling manufacturers to prepare large quantities of pandemic vaccine. In addition, antiviral drugs are particularly valuable in complicated cases of influenza, especially in hospitalized patients.
To write this overview, we mined various databases, including Embase, PubChem, DrugBank and Chemical Abstracts Service, and patent repositories.

In the first part of this overview [1], we described the life cycle of the influenza virus and the pharmacological action of the currently available drugs. In this second part, we will overview the molecular mechanisms and the targets of still-experimental drugs for the treatment and management of influenza. Figure 1 shows the attack points of several potential antiviral drugs.

Antiviral drug research is a particularly active field and new approaches have been developed. Briefly, we can distinguish between compounds with anti-influenza activity that directly target influenza virus proteins or genes, and molecules that target host components that are essential to viral replication and propagation. Among the former group, we will focus especially on hemagglutinin (HA), Matrix protein 2 (M2) and neuraminidase (NA) inhibitors (HAIs, NAIs). The latter molecules have been implemented quite recently and may pave the way for personalized treatment and management of influenza. Moreover, it is expected that the inhibition of host factors (such as single molecules) and/or complex mechanisms (such as intracellular signaling cascades and pathways) may act against different influenza virus strains and may be less prone to the emergence of drug resistance than the inhibition of viral components [2, 3]. Therapies that combine two or more compounds belonging to the same group or different groups are also discussed.

To write this overview, we mined various chemical databases, including Embase [4], PubChem [5, 6], DrugBank [7] and Chemical Abstracts Service (CAS) [8], as well as patent repositories and clinical trials registries [9]. We also scanned extant reviews and consulted the gray literature (books, proceedings, conference abstracts, posters and congress communications) in order to increase coverage of the anti-influenza drugs included in the present article. With regard to the search strategy, we used a mining approach similar to that described in Eyer and Hruska [10]. No time or language filters were applied.

To the best of our knowledge, this article constitutes the most comprehensive and up-to-date overview of anti-influenza compounds in the literature. It can be used

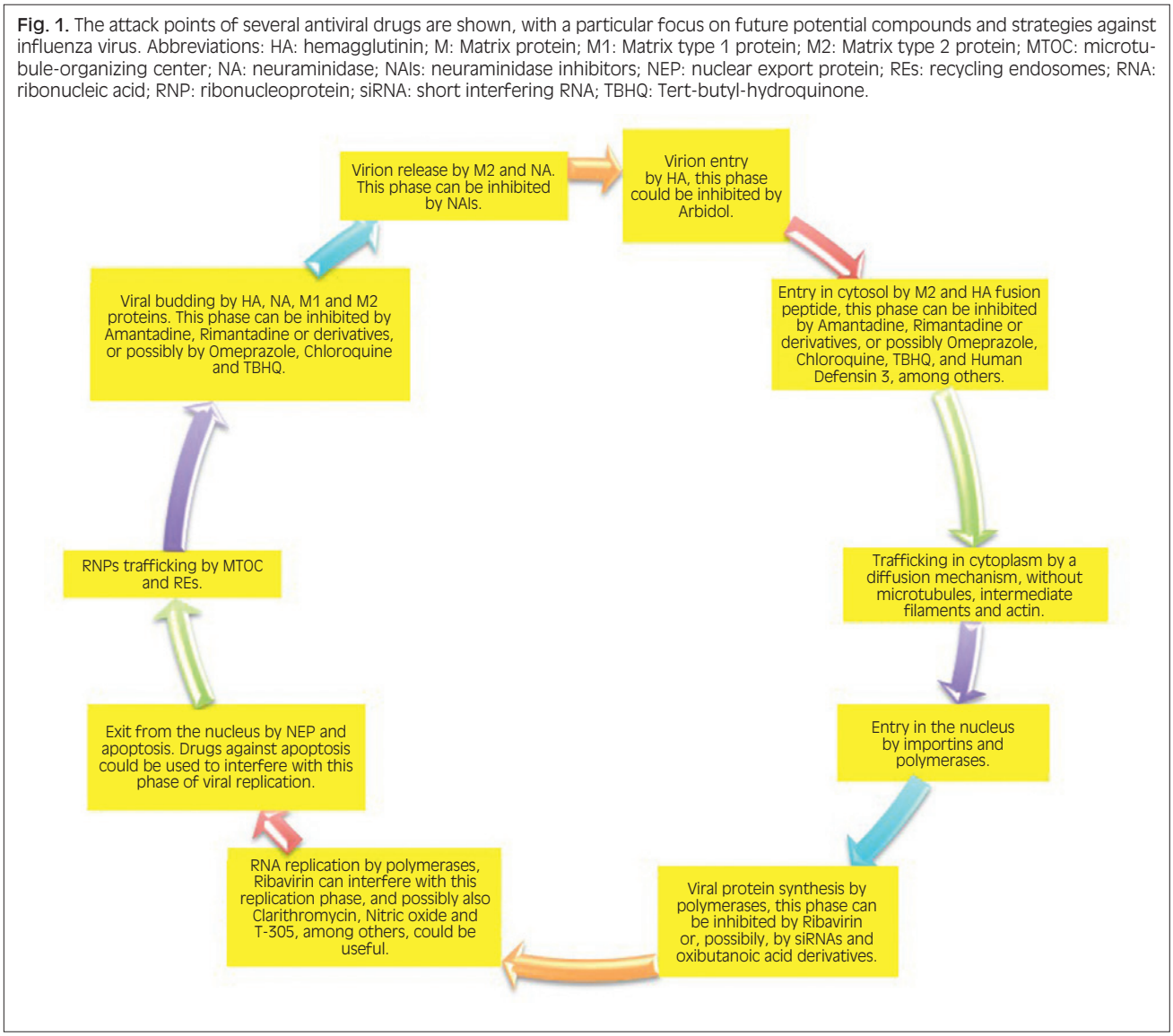

Fig. 1. The attack points of several antiviral drugs are shown, with a particular focus on future potential compounds and strategies against influenza virus. Abbreviations: HA: hemagglutinin; M: Matrix protein; M1: Matrix type 1 protein; M2: Matrix type 2 protein; MTOC: microtubule-organizing center; NA: neuraminidase; NAIs: neuraminidase inhibitors; NEP: nuclear export protein; REs: recycling endosomes; RNA: ribonucleic acid; RNP: ribonucleoprotein; siRNA: short interfering RNA; TBHQ: Tert-butyl-hydroquinone.

also as a working bibliography and a mapping review for scholars doing research in the field.
Along with this paper, a database is currently being designed and developed and will be accessible at the CIRI-IT institutional website [11].

Entry and Attachment Inhibitors

Effective antiviral compounds that interfere with the attachment and entry of the influenza virus into the host cell include triterpenoids [12] such as glycyrrhizic acid (GA) [13], glycyrrhizin (GR) [14], glycyrrhetinic acid [15] and further derivatives extracted from licorice and present in some Chinese medicaments. GR is the most active of these molecules and can repress the replication of H3N2 and H5N1, as well as of several viruses [16]. It can be delivered as an approved parenteral GR preparation (Stronger Neo-Minophafen C, SNMC), and glutamyl-tryptophan can be added in order to increase its activity [17, 18]. GR is able to inhibit entry of the virus into the host cell, and reduces the level of pro-inflammatory molecules such as chemokine (C-X-C motif) ligand type 10 (CXCL10), interleukin 6 (IL6), CC chemokine ligand type 2 (CCL2), and CC chemokine ligand type 5 (CCL5) [19, 20]. It also exerts an anti-apoptotic action. In addition, GR hinders monocyte recruitment and has anti-oxidant activities, inhibiting the formation of influenza virus-induced reactive oxygen species (ROS) [21]. It extensively modulates gene expression, activating interferon-gamma (IFN-gamma) and reducing the expression of Nuclear factor kappa B (NFκB), c-Jun N-terminal kinase (JNK), and p38. Furthermore, GR reduces high-mobility-group box type 1 (HMGB1) [22]. Promising glycyrrhizin derivatives include spacer-linked 1-thioglucuronide analogues [23]. GA inhibits influenza virus growth and replication in embryonated eggs [24]. Moreover, it can be used as an adjuvant in the preparation of anti-influenza vaccines [25].
Other triterpenoids [26], such as the saponins and uralsaponins M-Y from the roots of *Glycyrrhiza ura-*

lensis [27], exhibit anti-influenza and anti-HIV activities. Moreover, saponins can be used as vaccine adjuvants [28-31] and modulate the expression of cytokines and chemokines [32, 33]. Further triterpenoid derivatives share broad antiviral actions [34-38].

Dextran sulphate (DS) is a negatively charged sulphated polysaccharide. Besides inhibiting virus entry and attachment, it represses HA-dependent fusion activity [39-41] and NA-dependent activity [42]. However, mutations conferring resistance to DS are described in the literature [43]. Oxidized dextran can be administered as a prevention [44-46].

Other sulphated molecules include the sulphated syalil lipid NMS03, which is effective against IAV, Human Metapneumovirus (HMPV) and picoRNAvirus. It is assumed that it interferes with fusion, but the precise nature of its mechanism is still unknown [47].

Another potential fusion inhibitor is BTA9881, which has shown promising activity against RSV [48, 49].

Lysosomotropic agents, such as concanamycin A [50-53], the macrolide antibiotic bafilomycin A1 [54, 55], saliphenylhalamide [56], N,N'-Dicyclohexylcarbodiimide [52], and chloroquine [57-64], inhibit vacuolar ATPase (V-ATPase) and reduce endosome acidification and lysosome number. They act on the CME pathway, but are unable to block clathrin caveolae-independent endocytosis. It should be stressed that the anti-influenzal activity of these compounds strongly depends on the pH of the cellular environment and that some scholars have reported conflicting findings about their *in vivo* effectiveness [65].

Extract from milk thistle seeds, known as silymarin, a complex mixture of flavonolignans, and its main component silibinin are active against influenza [66]. Also silybin and its derivative can block virus entry and regulate autophagy, repressing the formation of oxidative stress species and triggering activation of the extracellular signal-regulated kinase (ERK)/p38 mitogen-activated protein kinase (MAPK) and IκB kinase (IKK) cascades [67]. Other silybin derivatives include silybin fatty acid conjugates, which have strong anti-oxidant properties [68].

Compounds from *Melaleuca alternifolia* (tea tree) oil (TTO) concentrate (MAC) [69, 70] have a broad antimicrobial activity. *In silico* simulations have shown that these compounds can interfere with virus entry and fusion of the influenza virus [71, 72].

Other potential compounds include *Amaryllidaceae* alkaloids from *Lycoris radiate*, such as lycorine, hippeastrine, hemanthamine and 11-hydroxy vittatine, which can also inhibit the nuclear-to-cytoplasmic export of the ribonucleoprotein (RNP) complex [73].

Curcumin is able to inhibit virus entry and HA [74]. It also has antioxidant, anti-inflammatory, anticancer, antiviral, antibacterial and antidiabetic properties, among others [75]. Curcumin acts against a large array of targets [76]. Curcumin is also active against other viruses [75, 77]. Rajput and collaborators showed that animals on a diet enriched in curcumin displayed an improved immune response [78]. Surprisingly, curcumin derivatives do not exhibit anti-influenza activity [79].

LADANIA067, extracted from the leaves of the wild blackcurrant (*Ribes nigrum folium*) [80, 81], has shown antiviral activities both *in vivo* and *in vitro*, without having any effect on influenza virus metabolism or growth/proliferation.

Fattiviracin A1 is a recently discovered antiviral [82]. Besides inhibiting both IAV and IBV, it is active against HIV, HSV and VZV [83].

Lignans exert a good anti-influenza activity [84, 85] Germacrone is a molecule purified from *Rhizoma curcuma*. It can be effectively combined with oseltamivir [86].

Akt inhibitors are also effective entry inhibitors. These include peptide "Akt in", which may be TCL1- or TCL1b-based, MK2206 [87, 88] and Ma-xing-shi-gan-tang (MXSGT), a traditional Chinese herbal decoction [89]. Everolimus, an inhibitor of the PI3K-Akt-mTOR pathway, is also a valuable tool against influenza [90].

Among anti-attachment drugs, Fludase (DAS181) has potential anti-influenza virus properties [91-103]. This medication, which has proved capable of inhibiting human and avian influenza viruses in pre-clinical studies, acts by mimicking NA and destroying the molecules of sialic acid receptors on the host cell surface. It is also effective against NA-resistant influenza strains [92, 93, 103].

HA Inhibitors

An effective class of HAIs is that of the amide derivatives [104-107].

Gossypol is a natural phenolic aldehyde extracted from the cotton plant and blocks the dehydrogenase family enzymes [108, 109]. Its antiviral properties emerged during a 1970 study, in which an experimental model of influenza pneumonia was used [108]. In particular, chiral (+)-gossypol is more active than (–)-gossypol [110, 111].

Another antiviral against HA is Entry Block-peptide (EB-peptide), a peptide derived from fibroblast growth factor 4 (FGF4) [112]. EB-peptide can inhibit virus entry and attachment, being effective even when administered post-infection. Besides repressing influenza viruses, EB-peptide is also active against other viruses [113]. It can also be used as an adjuvant in the formalin-inactivated influenza whole-virus vaccine, triggering phagocytosis of influenza virions. Other peptides similar to EB-peptide are the FluPep (FP) peptides, such as FP1 (Tkip) and FP2-FP9 [114]. Tkip was designed as a mimetic of the suppressor of the cytokine signaling (SOCS) protein, which is involved in mediating the immune response to influenza. Furthermore, peptide NDFRSKT has strong antiviral properties, but with unknown therapeutic characteristics [115, 116].

Other molecules which bind to HA are collectins (CLs) [117]. Human CLs and bovine conglutinin, CL-

43 and CL-46 confer protection against influenza infection [118-122].
A related group of molecules is the ficolins (such as H-ficolin and L-ficolin), present at high concentrations in serum and in bronchoalveolar secretions [123]. They bind not only to HA but also to NA in vitro models [124]. These proteins can be engineered in such a way as to become more active against influenza virus; for example, Chang and collaborators designed recombinant chimeric lectins consisting of mannose-binding lectin (MBL) and L-ficolin [125]. However, because of their role in the inflammatory response, their potential use in humans requires more complete analysis. Recently, agglutinins such as NICTABA, UDA [126] and protectins like protectin D1 [127-130] have been found to have anti-influenza propriety [131].
An interesting compound, which binds to specific high-mannose oligosaccharides of HA is Cyanovirin-N (CVN) [132]. In 2003, O'Keefe *et al.* demonstrated its potent *in vitro* antiviral activity against a wide range of IAVs and IBVs, including NA-resistant strains, though resistance induced by mutations that affect the glycosilation site of HA seems to arise quite naturally [133].
Clarithromycin (CAM), able to inhibit influenza virus replication *in vitro* and in cell cultures, appears to have 3 mechanisms of action against type A seasonal Influenza virus. It was recently showed that CAM reduces the expression of human influenza virus receptors on the mucosal surface of the airways, reduces the production of nuclear factor-kB (NF-kB), and increases pH inside the endosomes [134, 135].
Norakin (Triperiden) is an anticholinergic drug that interacts with HA [136, 137]. This interaction may be indirect, being mediated by an increase in the internal pH in the pre-lysosomal compartment [138-140]. However, strains resistant to Norakin have been described [141-144]. Also Norakin derivatives seem to be effective antiviral compounds [145].
Another interesting compound is nitazoxanide [146-151], useful for the treatment of protozoal and bacterial infections and is active against hepatitis and influenza viruses or rotaviruses. Further thiazolides act at the post-translational level by selectively blocking the maturation of viral HA at a stage preceding that of resistance to endoglycosidase H digestion, thus interfering with HA intracellular trafficking and insertion into the host plasma membrane, which is a key step in the correct assembly and exit of the virus from the host cell.
Bacillus intermedius ribonuclease (BINASE) shows a good anti-influenza activity. BINASE and HA interact with sialic acid on the cell surface and penetrate into the host cell. Subsequently, viral RNA is released and cleaved by BINASE [152, 153].
High mannose-binding lectins (HMBL) are powerful influenza and HIV inhibitors [154].
Rutin, quercetin, and related compounds, extracted from elderberry fruit (*Sambucus nigra L.*) [155-161] are other HA inhibitors. Xylopine and rosmaricin have an amine group that interacts with HA [162, 163].
Theaflavins (TFs) from black tea have a strong anti-influenza activity, inhibiting HA and reducing the level of IL6, thus exerting an anti-inflammatory and anti-apoptotic action [164-166].

M2 Inhibitors

M2 inhibitors can be basically divided into 2 groups. The first includes compounds derived from the leads of amantadine and rimantadine and its hydroxylated derivatives [167-172]. The second includes non-adamantane derivatives, which are promising drugs against influenza viruses [173]. Some of these compounds have been specifically designed for some important mutants of the M2 ion channel of IAV [174-177].
Regarding molecules putatively capable of blocking the ion pump, Gasparini and coworkers recently conducted a field investigation into the effect of omeprazole family compounds (OFC) [178] on Influenza-like Illness (ILIs). The results showed that subjects treated with omeprazole family compounds displayed a lower risk of catching ILI (OR_{adj} = 0.29, 95% CI: 0.15-0.52) than non-treated subjects. Molecular docking and molecular dynamics (MD) simulations, which are a common method of searching for new potential drugs, seem to confirm these findings [179]. The M2 Protein – Protein Data Bank (PDB) code *3C9J* [180] – was simulated as being embedded in a dipalmitoylphosphatidylcholine (DPPC) membrane in complex, with its ligands amantadine and rimantadine being used as positive controls and omeprazole as a putative ligand. The thermodynamic integration method was used in order to estimate binding free energies of the ligands. Free-energy calculations imply omeprazole as a potent anti-viral drug. Also another study has suggested the antiviral properties of omeprazole against Ebolavirus [181].
Polyamines such as spermine [182, 183], spermidine and putrescine have recently been identified as intrinsic rectifiers of potassium channels. Indeed, the M2 protein has a binding site for polyamines, which is different from the amantadine binding site [184]. Polyamines have quite recently been exploited in designing anti-influenza vaccines [185, 186].
Spiropiperidine M2 inhibitor and its derivatives appear promising in acting against amantadine-resistant viruses; in particular, spiropiperidine-9 seems to be the most active [187].
Among natural products, pinanamine derivatives [188] and 24-E-ferulate [188] have a good influenza activity.

Endosomal and lysosomal inhibitors

Substituted salicylanilides appear promising antiviral agents [190-193]. In particular, Niclosamide [192], which is approved for human use against helminthic infections, besides being active against influenza viruses, has also shown anti-neoplastic and broad antiviral ef-

fects, being active against SARS-related coronavirus and Human Rhinovirus (HRV).
Lysosomotropic agents [50-64] have also been already discussed. Further compounds include molecules obtained from TTO [69-72], which have already been mentioned.

Protease inhibitors

The cleavage of HA can be blocked not only by anti-M2 protein compounds, but also by inhibition of the necessary proteases [194]. Given the great importance of the proteases in the viral replication cycle, many authors [195, 196] have directed their research towards anti-protease medications that could block, or at least mitigate, the consequences of HA cleavage. HA can also be blocked by natural products such as Hepatocyte growth factor activator inhibitor 2 (HAI-2) [197]. Several anti-protease drugs have been studied in *in vitro* models, animals and humans, such as Camostat mesilate [198], epsilon-aminocapronic acid [199], leupeptin [200] and Aprotinin [201], which has been approved for topical use in a small-particle aerosol formulation in Russia. A theoretical advantage of antiviral activity against enzymatic activities of the host is that these molecules would not lead to the selection of resistant viral variants.
Other molecules can interfere with the mechanism of fusion of the endosomal and viral membranes [202]. Indeed, numerous small molecules that block virus infectivity by inhibiting the conformational changes required for HA-mediated membrane fusion have been identified. Russell *et al.* [194] have demonstrated that TBHQ (*Tert-butyl-hydroquinone)* stabilizes the neutral pH structure and, in this way, presumably, inhibits the conformational rearrangements required for membrane fusion. Furthermore, Leikina *et al.* [203] have demonstrated that human β-defensin 3, a lectin, can inhibit HA-mediated influenza viral fusion.
Regarding the compounds targeted against the transcription and replication of vRNA, one of the first drugs developed is Ribavirin (RIB). RIB, also known by the trade name "Virazole", is a nucleoside analog [204]. Its mechanism of action is not completely known. However, Inosine 5'-monoposphate dehydrogenase (IMPDH) appears to be the principal target of the molecule. This inhibition diminishes the intracellular concentration of GTP (Guanosine-5'-triphosphate), and this is thought to stop viral protein synthesis and limit vRNA replication. Crotty *et al*. also demonstrated that RIB is a lethal vRNA mutagen [205]. However, the need for high doses of the drug in order to have obtain good clinical results has limited the use of RIB as an anti-influenza drug, and a recent revision of the literature by Chan-Tack *et al.* suggests that there are no conclusive results on the beneficial use of Virazole for the treatment of influenza [206]. RIB can also be delivered as a liposome encapsulated with muramyl tripeptide (MTP-PE) [207].
α(1)-antitrypsin (AAT) [208] is a serine protease inhibitor of elastase and proteinase-3 (PR-3). This protein is produced by the liver and its expression increases particularly during the acute-phase response. It also has immunomodulatory, anti-inflammatory and tissue-protective properties, reducing influenza-related complications and morbidities. As an immunomodulator, AAT mediates the maturation and differentiation of dendritic cells (DCs) and T regulatory cells (T_{reg}s), activating the IL1 receptor antagonist (IL1RA) and inducing IL10 release. Moreover, it exerts an anti-apoptotic effect, inhibiting caspases-1 and -3. The role of AAT in inhibiting influenza viruses is consistent with the clinical observations that subjects with AAT deficiency are exposed to the risk of severe influenza-related complications and should therefore be vaccinated [209, 210].
Stachyflin, acetylstachyflin and its phosphate esther or oxo derivatives [211, 212] exert their inhibitory activity on a variety of HA subtypes of IAV (H1, H2, H5 and H6, among others) but have no activity on H3 subtype IAV or on IBV [213-217]. The metabolites of stachyflin and its derivatives include compounds such as *cis*-fused decalin [214]. Stachyflin compounds can be delivered intranasally or orally, using PEG 400 as vehicle [211]. However, some amino acid substitutions confer resistance to stachyflin [212].
BMY-27709, a salicylamide derivative, and its analogues are other useful compounds [218, 219].
Thiobenzamide derivatives have a good activity profile. In particular, the axial disposition of the thioamide moiety has proved to be crucial to inhibitory activity [220].
Ulinastatin [221] is a protease inhibitor, which also protects lysosome integrity. Its use has been suggested for the treatment of avian influenza [221] and severe influenza-related complications, such as encephalopathy [222] and acute respiratory distress syndrome (ARDS) [223, 224]. Indeed, a recently published meta-analysis has shown that this drug is effective in managing acute lung injury (ALI) and ARDS [225].
The ubiquitin-specific peptidase type 18 (USP18) protease inhibitor ISG15 is another promising molecule [226]. ISG15 is part of the interferon-regulated cellular cascade. USP18 was found to be one of seven genes which predict a response to influenza virus [227]. This finding was reproduced by Liu and collaborators [228].

Polymerase inhibitors

Other antiviral strategies have been directed against the viral RNA polymerase [229, 230]. The trimeric polymerase complex has multiple enzymatic activities and can thus be targeted at different sites of action. For instance, nucleoside/nucleotide compounds have been developed against other viruses, namely HIV, HBV, etc.
A historical compound is moroxydine [231-233]. It is also active against HSV and VZV.
The most thoroughly studied of these molecules is Favipiravir (T-705). *In vitro* studies have demonstrated the high antiviral potency of the drug and mouse studies have demonstrated its protective efficacy against a wide

range of influenza viruses A and B. This molecule also seems to be effective against other viruses [234-238].
More recently, other compounds directed towards anti-nucleasic activities have been studied, such as the series of hydroxypyridinone, which appears to have antiviral activity in cells [230].
On studying 33 different kinds of phytochemicals, other scholars have identified a family of drugs called marchantines, which appear to interact with the PA subunit of the endonuclease [239].
An attractive strategy for developing anti-polymerase compounds appears to be that of interfering with the subunit binding interfaces of PB1 and PA, which are very well conserved in different Influenza virus strains [240]. Thus, these compounds would reduce the transcriptional activity of the viral RNA polymerase. One such promising compound is AL18, which is also active against human cytomegalovirus [241].
Furthermore, the recent definition of the PB1/PB2 binding interface by means of crystallography [242] has prompted researchers to study synthetic peptides, such as peptide 1-37 and peptide 731-757, which seem to inhibit the interaction between PB1 and PB2 [243-247].
Azaindole VX-787, an inhibitor of PB2 [248-251], is able to interfere with the cap-snatching activity of the polymerase complex of the influenza virus. The small GTPase Rac1 inhibitor NSC23766 exhibits a similar activity profile [252].

Nuclear pathway inhibitors

Leptomycin B (LMB) inhibits nuclear export signal (NES)-mediated vRNP export, as well as NES-receptor CRM1/exportin-1 (XPO-1); however, it is somewhat toxic [253].
Verdinexor (KPT-335) [254] is a new-generation XPO-1 antagonist that is well tolerated in animal models and seems to be effective against both IAV and IBV. It is a selective inhibitor of nuclear export (SINE).

NP inhibitors

Given the fundamental importance of the NP in modulating the replication cycle of the virus, many authors have investigated strategies for preventing its production. Moreover, molecules that prevent the functional polymerization of the NP monomers have also been studied, such as, for example, Nucleozin (NCZ) [255]. It also blocks viral RNA and protein synthesis and targets vRNP nuclear export and its cytoplasmatic trafficking. As a final result, fewer and smaller influenza viral particles are released. NCZ derivatives include a quite effective compound, namely 3061 (FA-2), which has been shown to inhibit the replication of the influenza A/WSN/33 (H1N1) virus, though NP-mutant strains have displayed resistance to this drug [256].
Jiang and collaborators screened a peptide library and discovered that the NP-binding proline-rich peptide was particularly effective against influenza viruses [257].
Another interesting molecule is the interferon-inducible Mx1 protein [258, 259].
Cycloheximide (CHX), which is also active against enterovirus-71 (EV-71), coxsackievirus B, and actinomycin D, are quite effective chemicals [260-262].
Intriguingly, clinically licensed anti-cyclooxygenase-2 (COX-2) Naproxen also appears to inhibit the functional polymerization of NP monomers. Its derivatives, such as naproxen A and C0, also appear quite promising [263].
Another drug directed against the NP is Ingavirin, which has been licensed in Russia. Indeed, Ingavirin interacts with the transport of newly synthesized NPs from the cytoplasm to the nucleus [264-272]. It is also active against parainfluenza virus, adenoviruses and human metapneumovirus [273].

NA inhibitors

NAIs include peramivir and lanimamivir derivatives [274-289].
Baicalin induces autophagy and acts against both NA [290] and NS1 [291-293].
Isoscutellarein is another compound that inhibits influenza virus sialidase. Its derivative is also active against influenza [294, 295].

NS1 inhibitors

Another potential strategy against influenza is to block the NS1 protein, a non-structural protein that is very important during the viral replication cycle. Indeed, the NS1 protein down-regulates the cellular production of IFN α/β. Furthermore, it has been demonstrated that NS1 also modulates other crucial aspects of influenza virus replication, namely viral RNA replication, viral protein synthesis, and general host-cell physiology [1, 296]. Finally, NS1 probably has an anti-apoptotic function in the early phases of replication. The meaning of apoptosis during influenza A virus replication is ambiguous, although it is usually considered to be a cellular antiviral defense that limits virus replication. Therefore, influenza viruses have acquired different ways of procrastinating this seeming host strategy [1]. Nonetheless, cellular pro-apoptotic factors favor the effective replication of influenza viruses, and some viral proteins, such as NA and PB1-F2, carry out pro-apoptotic tasks [1, 297]. Furthermore, some compounds that act against the NS1 protein have been studied. In this perspective, peptide-mediated inhibition of NS1 – CPSF30 has been proposed as a strategy for mitigating viral replication [298, 299]. Unfortunately, this virus-specific approach leads to viral mutation and the occurrence of drug resistance.
More recently, Jablonski *et al.* studied a class of molecules derived from the NSC125044 compound, which

displayed NS1 protein inhibition in viral replication assays [300].
Regulated in development and DNA damage responses-1 (REDD1) is a molecule that has recently emerged from comprehensive biochemical screening. Moreover, REDD1 inhibits the mTOR pathway [301].

Other RNA synthesis inhibitors

Cordycepins extracted from *Cordyceps*, a genus of ascomycete fungi, are used for diverse medicinal purposes because of their different pharmacological actions with hypothetical anti-viral activity [302].

Caspase inhibitors

Apoptosis plays a major role in the influenza virus life cycle [303-307]. Indeed, in order to replicate, the virus activates the mechanism of apoptosis through the activation of caspase 3. Cellular inhibitors of apoptosis proteins (cIAPs) are essential regulators of cell death and immunity. Nucleotide-binding oligomerization domain-like receptor type 1 (NLRX1) [308] binds to viral protein PB1-F2, preventing IAV-induced macrophage apoptosis and promoting both macrophage survival and type I IFN signaling. Interestingly, compounds that inhibit this enzymatic activity could be useful as anti-influenza antivirals. Indeed, Wurzer *et al.* have shown that apoptotic activation by caspase 3 is required for efficient virus production [306]. Furman and collaborators have demonstrated that the apoptotic index is a predictive biomarker of influenza vaccine responsiveness [309]. However, the question of whether apoptosis is beneficial to the viral reproductive cycle or to host cells is still under debate. Moreover, Hinshaw *et al.* [307] demonstrated that, on inhibiting apoptosis during viral infection, influenza virus RNP complexes were retained in the nucleus. Therefore, the use of caspase 3 inhibitors could have good potential as anti-influenza drugs [310].

Autophagy

Autophagy (or autophagocytosis) is a catabolic mechanism that involves cellular breakdown of dysfunctional cell components through the involvement of lysosomes. Procyanidin has an anti-IAV activity [311].

Glucosidase, mannosidase and glycosilation inhibitors

L-fructose and L-xylulose can inhibit influenza virus replication [312]. Glucosidase I and glucosidase II inhibitors include iminosugars, which alter glycan processing of influenza HA and NA [313].

Pathway inhibitors

Raf/MEK/ERK pathway inhibitors include compounds, which act as an inhibitor of MEK1 and MEK2 [3]. NFKB inhibitors include Bortezomib [3], among others. These proteasome inhibitors are also effective against paramyxoviruses, HRV, poliovirus, coxsackievirus, HSV and HIV.

Phospholipase inhibitors

Lipid metabolism plays a fundamental role during influenza virus replication: membranes and their components, such as sphingolipids, are crucial to all steps of the viral life cycle, from attachment and membrane fusion, to intracellular transport, replication, protein sorting and budding. Infection by influenza virus stimulates phospholipase D (PLD) activity [314].

Release inhibitors

HDAC6 is an anti-IAV host factor that negatively regulates the trafficking of viral components to the host cell plasma membrane via its substrate, acetylated microtubules [315].
As an anti-influenza chemical, cyclosporin A does not act through its classical targets, namely cyclophilin A (CypA), cyclophilin B (CypB) and P-glycoprotein (Pgp) [316], but by inhibiting influenza virus release. Ching-fang-pai-tu-san (CFPTS) has a similar action [317].

Anti-oxidants, anti-inflammatory compounds and immunomodulators

Oxidation plays a major role in influenza virus life cycle and replication [318]. With regard to anti-influenza drugs that act subsequently to the various stages of viral replication, after the formation of vRNPs, it is worth considering that Resveratrol may be useful as an anti-influenza drug. Indeed, this compound could interfere with the translocation of RNPs from the nucleus to the cytoplasm [319-321]. Dehydroascorbic acid also has antiviral properties [322, 323].
Calcitriol prior to/or post-H1N1 exposure does not affect viral clearance but significantly reduces autophagy and restores the increased apoptosis seen on H1N1 infection to its constitutive level. However, it significantly reduces the levels of H1N1-induced TNF-α (tumor necrosis factor-alpha), RANTES, IL8, IFN-β (interferon-beta) and IFN-stimulated gene-15 (ISG15). 1,25[OH]2 D3 treatment prior to/or post-H1N1 infection significantly downregulates both IL-8 and IL-6 RNA levels [324, 325].
Publications on antiviral drugs are often devoted to the use of statins as anti-flu drugs [326-328]. In particular, Fedson has suggested treating patients affected by H5N1 with statins [326, 327]. Studies *in vitro*, in animals and

in the field seem to support this strategy. Statins are held to act through various mechanisms: through immunomodulatory and anti-inflammatory activity, by interfering with the proteins of the cytoskeleton and the interaction between these and the lipid rafts, and by reducing the availability of intracellular cholesterol. The balanced content of cholesterol in the cell is critical to the replication of IAV. Indeed, a reduction in cholesterol could impair the infectivity of progeny influenza viruses, probably by reducing the cholesterol content of the viral envelope [328]. However, some studies have found statins to be ineffective against influenza viruses [329, 330].

Extracts from *Epimedium koreanum Nakai* have immunomodulatory properties [331], also against HSV, VSV and Newcastle Disease Virus (NDV). Carrageenan [332] extracted from edible red seaweeds can be administered as a nasal spray [333]. In particular, iota-carrageenan appears to be the most effective against influenza.

Cycloferon [334-336], amixin, Larifan, Kagocel and Ragosin stimulate B cells and macrophages to produce IFN-alpha [337]. They are widely used in Russia.

Apocynin, a NADPH oxidase type 2 (NOX2) inhibitor, stimulates cell superoxide production. However, in certain conditions, it can also act as a ROS production stimulator in non-phagocyte cells [338]. By contrast, NADPH oxidase type 1 (NOX1) has anti-inflammatory activity and inhibits ROS production [339, 340].

Rolipram, a selective phosphodiesterase-4 (PDE-4) inhibitor with antidepressant properties, and sertraline, a selective serotonin reuptake inhibitor (SSRI), exhibit strong antiviral activities if combined with oseltamivir [341]. The rationale for using PDE-4 is that it belongs to a family of enzymes that metabolize cyclic adenosin monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), which are commonly found during inflammatory and immune responses. By reducing bronchospasm and bronchoconstriction, it reduces mortality and morbidity in a mouse model. SSRI downregulates the expression of interferon-alpha, TNF-alpha, IL-6, IL-10 and T helper 1 (Th1) cells, and modulates immune responses from the Th1 toward the Th2 phenotype.

Sphingosine mimetics are able to finely modulate the release of cytokines and chemokines. In one study [342], neutralizing antibody and cytotoxic T cell responses were seen to be reduced, though still protective. As a result, the infiltration of PML and macrophages into the lung was markedly reduced, and thus also pulmonary tissue injury. DC maturation was suppressed, which limited the proliferation of specific antiviral T cells in the lung and draining lymph nodes. Furthermore, they were effective in controlling CD8(+) T cell accumulation in the lungs even when given 4 days after the onset of influenza virus infection.

Leucomycin A3 (LMA3), a macrolide antibiotic, inhibits neutrophil myeloperoxidase (MPO), which contributes to the pathogenesis and progression of severe influenza-induced pneumonia, and mediates the production of hypochlorous acid, a potent tissue injury factor [343].

BG-777, derived from leukotriene B4, exerts both antiviral and stimulatory activities on the host defence system. It is also active against HIV, RSV and Coronaviruses. It recruits leukocytes and fosters the release of chemokines such as MIP1-beta and defensins [344].

QS-21 is a molecule with immunomodulatory properties, and is currently being investigated as an adjuvant for vaccines against influenza [345]. Thymalfasin (Zadaxin), which is derived from thymosin alpha-1, is another powerful adjuvant [346-348]. Canakinumab (Ilaris), an IL1-beta blocking antibody, is also a promising compound in immunotherapy [349].

Some observations should be made on influenza therapy with non-steroidal anti-inflammatory drugs. Seasonal flu is normally treated with over-the-counter (OTC) drugs, which are designed to relieve symptoms. The most common are paracetamol, acetylsalicylic acid (which, however, is contraindicated in individuals under 18 years of age) and ibuprofen or other NSAIDs. Coughing is usually mitigated by means of drugs that use dextromethorphan or acetylcistein as their active ingredient [350-357].

The inflammation driven by innate immunity is usually sufficient to cure the disease. However, especially when the virus is particularly virulent or during pandemics, immunity may be dysregulated (cytokine-storm), which may give rise to very severe forms of influenza. The treatment of both seasonal and pandemic influenza therefore utilises appropriate and timely anti-inflammatory therapy. Some of the above-mentioned drugs, such as statins and naproxen, have anti-inflammatory properties; however, they are probably also able to exert a real antiviral activity.

In the light of the human cases of infection by the H5N1 strain and the lethal cases caused by the H1N1pdm virus, the need for modulators of innate immunity is of particular importance. Indeed, patients with severe or fatal human infections due to the H1N1pdm virus, for instance, have high pro-inflammatory responses early in the illness.

For the above-mentioned reasons, the literature often reports *in vitro* and animals studies which demonstrate the therapeutic utility of anti-inflammatory and immunemodulatory compounds, such as fibrates, against influenza.

Gene therapies

Gene therapy consists of modulating (up-regulating or down-regulating) genes and/or their products involved in the response to influenza [358].

microRNAs (miRNAs) are small non-coding RNA molecules (containing about 22 nucleotides) which function in RNA silencing or RNA interference (RNAi) and in the post-transcriptional regulation of gene expression. Host miRNAs are able to down-regulate the expression of viral genes. Therefore, miRNA modulation could be a promising approach in influenza treatment, despite

the difficulties of delivering miRNAs to cells efficiently [359-363].
Small interfering RNAs (siRNAs) are also mediators of RNAi. They are short (19-26 nucleotides) and induce sequence-specific degradation of homologous mRNA [364-366].
Long non-coding RNAs (lncRNAs) modulate various biological processes [367]. One lncRNA, in particular, plays a major role; it acts as a negative regulator of antiviral response (NRAV) and is down-regulated during influenza infection. NRAV negatively regulates the transcription of multiple critical interferon-stimulated genes (ISGs), by remodeling chromatin [368].

Compounds with unknown mechanisms

In the case of some compounds, the precise nature of their pharmacological activity against influenza is still unknown and requires further research.
Nanoparticles are a promising nanobiotechnological tool that can act as carriers of non-conjugated nanoparticles. Silver nanoparticles [369, 370] modulate SP-A and SP-D [371], and can be used to deliver RNAi [372]. Poly(gamma-glutamic) acid [373], fullerenes [374], chitosan or N-trimethyl chitosan (TMC) [375] and polymeric nanoparticles have also been investigated as vaccine adjuvants [376, 377]. However, single-walled carbon nanotubes (SWCNTs) seem to increase influenza virus pathogenicity and infectivity [378].

Combination therapies

Combination therapies (CTs) can be divided into associations of two or more drugs directly targeting viral components, and associations of a direct-acting viral compound and a molecule targeting host components. CTs may improve clinical outcomes, reduce the risk of respiratory complications, mortality and morbidity, reduce the risks of using single drugs (such as resistance, dose-related toxicity or other side-effects) and may potentiate and enhance antiviral activity [379, 380]. CTs can, in turn, be further divided into early combination chemotherapy (ECC) and sequential multidrug chemotherapy (SMC). Furthermore, many studies have evaluated the efficacy of combining anti-inflammatory drugs with antiviral drugs in comparison with single-drug treatment. However, not all combination therapies, for instance the combination of oseltamivir with zanamivir or simvastatin with oseltamivir, are superior to monotherapy [102, 379, 380].
CTs can also exploit various chimeric monoclonal antibodies [381].

Conclusions

In the last few decades, few antiviral molecules against influenza virus infections have been available. This has conditioned their use during human and animal outbreaks. Indeed, during seasonal and pandemic outbreaks, antiviral drugs have usually been administered in mono-therapy and, sometimes, in an uncontrolled manner to farm animals. This has led to the emergence of viral strains displaying resistance, especially to compounds of the amantadane family. For this reason, it is particularly important to develop new antiviral drugs against influenza viruses. Indeed, although vaccination is the most powerful means of mitigating the effects of influenza epidemics, antiviral drugs can be very useful, particularly in delaying the spread of new pandemic viruses, thereby enabling manufacturers to prepare large quantities of pandemic vaccine. In addition, antiviral drugs are particularly valuable in complicated cases of influenza, especially in hospitalized patients. This latter are individuals at risk, such as the elderly or patients with chronic respiratory diseases. For these subjects, it would be particularly important to have more antivirals to be administered in appropriate manner.
In the light of the extensive experience gained through the use of anti-influenza drugs, and in the light of the considerable advances in the search for new effective molecules against influenza viruses, many important considerations can be made.
Firstly, the study of new compounds should be conducted in a more rational way. Indeed, the models and methods used by various scholars display marked differences. These studies often involve *in vitro* cell cultures and usually use Madin–Darby canine kidney (MDCK) cells and African green monkey kidney Vero cells. However, human tracheal epithelial cell cultures are sometimes used. While some authors have assessed the inhibition of viral growth by applying the haemagglutination test to the supernatant of the cell monolayer, others have used the inhibition of the virus-induced Cytopathic Effect (CPE). Furthermore, more sophisticated tests have been used – for instance, qPCR with the aim of amplifying sequences of viral genes, such as the M2 gene, NP gene, etc., or RT-PCR with the aim of quantifying IAV RNA after *in vitro* antiviral treatment of cell cultures exposed to different influenza virus strains. In addition, the murine model is the most widely used to study influenza compounds, as influenza causes fatal pneumonia in the mouse. Obviously, the human is the best, but results in humans are available only if clinical trials have been performed or if the drug has been licensed. However, as it is very costly to develop a new compound for commercialization, preliminary evaluations *in vitro* and in animal models are very important. In some cases, it is also useful to carry out epidemiological studies on drugs used for other purposes, in order to investigate their possible therapeutic efficacy against influenza.
To optimise the development of influenza antivirals, it is very important to define standardized methods for the evaluation of the molecules that have been hypothesized to have a potential antiviral effect. In *in vitro* studies, for instance, it is important to define the cell line that should be used (MDCK, or VERO, or THE cell line), the standard virus that should be tested (PR8 and/or High

pathogenic virus, such as H5N1) and the antiviral assay that should be performed (Haemagglutination, CPE inhibition, RT-PCR). Likewise, in *in vivo* tests, the choice of which animal to utilize should be established, while in human studies it is important to determine the number and age of the subjects to be studied. Only if standardized methods are defined, will it be possible to correctly evaluate the antiviral potential of the compound under examination. In this perspective, it is also important to compare the antiviral activity of the hypothetical antiviral with that of reference drugs (amantadine, oseltamivir, etc) in order to ascertain the influenza antiviral index of the new molecule. In *in vitro* studies, it is also advisable to evaluate the capacity of the antiviral under study to induce viral resistance.

In the field of medicinal chemistry, the discovery and development of a completely New Molecular Entity (NME) or compound is particularly expensive in terms of time and costs. Research could therefore be carried out along two different lines: designing/optimising new derivatives from an existing lead (such as the second-generation NAI laninamivir and peramivir); and repurposing/repositioning existing drugs for new potential clinical applications [382, 383]. The latter approach, also termed drug retasking or reprofiling, has already yielded promising results. While drug retargeting was initially serendipitous, it was later more systematically developed and exploited, not least by combining advanced biochemical, biophysical and bioinformatics/cheminformatics techniques. Viroinformatics [384] and computational systems biology [385] can suggest rational inhibitors of viral transcription, replication, protein synthesis, nuclear export and assembly/release. Other strategies may emerge from gene data mining. In this regard, Bao and collaborators used a prioritizing gene approach in order to find the most important genes involved in host resistance to influenza virus [386]. They found that the response was controlled by two TNF-mediated pathways: apoptosis and TNF receptor-2 signaling pathways. In addition, systems pharmacometrics and systems pharmacology [387] could identify valuable CTs by studying drug synergy.

Secondly, the available anti-influenza drugs should be used in an appropriate manner, in order to impede or to mitigate the phenomenon of viral resistance. In this regard, the first question is: what anti-inflammatory drug should be chosen? The answer should take into account the age of the patient, the toxicity and tolerability of the drug and its efficacy in alleviating the patient's symptoms. Obviously, therapy should be initiated as soon as possible, and an NSAID (aspirin only for subjects over 18 years, ibuprofen, naproxen or paracetamol [acetaminophen]) should be chosen. These compounds not only relieve the symptoms, but also equilibrate the patient's innate immunity and sometimes have a direct or indirect antiviral effect. For instance, it is interesting that reducing pro-inflammatory cytokines diminishes the activity of proteases involved in HA cleavage. In addition, the administration of acetylcysteine is useful not only because of its mucolytic action, but also on account of its antioxidant activity.

The choice of the antiviral should take into account the broad resistance of influenza viruses to amantadane drugs and also the fact that mono-therapy can easily lead to the emergence of novel viral resistance. In this perspective, topic drugs, such as zanamivir, have proved to generate less resistant viral strains than drugs administered orally. In addition, other antivirals, such as anti-protease drugs, could be useful in influenza therapy. These compounds could have advantages in that, being inhibitors of cellular proteins, they should be less prone to selecting resistant viral strains. However, it should be borne in mind that disturbing the cellular environment in order to disrupt viral functions could have adverse side effects. Furthermore, it has been proposed that therapeutic protocols involving a combination of two or more antivirals should be drawn up in order to reduce the development of drug-resistant viral strains and, at the same time, administer lower drug doses. Another hypothesis could be to administer two or more different antivirals alternately.

Finally, the use of antivirals in the veterinary field (for example, chicken flocks) should be carefully controlled, and in this case the combined or alternated administration of at least two antiviral drugs should be the rule. It is important to realise that this implies a *one world, one health, one medicine, one science* approach [382, 383], in which human and veterinary medicine cooperate in the interest of global health in an increasingly interconnected world.

References

[1] Gasparini R, Amicizia D, Lai PL, et al. *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*. J Prev Med Hyg 2014;55:69-85.

[2] Müller KH, Kakkola L, Nagaraj AS, et al. *Emerging cellular targets for influenza antiviral agents*. Trends Pharmacol Sci 2012;33:89-99.

[3] Planz O. *Development of cellular signaling pathway inhibitors as new antivirals against influenza*. Antiviral Res 2013;98:457-68.

[4] Embase. Accessible at http://www.elsevier.com/online-tools/embase (last accessed: 16/07/2014).

[5] PubChem. Accessible at http://www.ncbi.nlm.nih.gov/pcsubstance/ (last accessed: 16/07/2014).

[6] PubChem. Accessible at http://www.ncbi.nlm.nih.gov/pccompound/ (last accessed: 16/09/2014).

[7] DrugBank. Accessible at http://www.drugbank.ca (last accessed: 16/07/2014).

[8] Chemical Abstracts Service. Accessible at https://scifinder.cas.org/ (last accessed: 16/07/2014).

[9] Clinical trials registries. Accessible at https://clinicaltrials.gov/ct2/home (last accessed: 16/07/2014).

[10] Eyer L, Hruska K. *Antiviral agents targeting the influenza virus: a review and publication analysis*. Veterinarni Medicina 2013;58:113-85.

[11] CIRI-IT. Accessible at http://www.cirinet.it/jm/ (last accessed: 16/09/2014).

[12] Pu JY, He L, Wu SY, et al. *Anti-virus research of triterpenoids in licorice*. Bing Du Xue Bao 2013;29:673-9.

[13] Jia W, Wang C, Wang Y, et al. *Qualitative and quantitative analysis of the major constituents in chinese medical preparation Lianhua-Qingwen capsule by UPLC-DAD-QTOF-MS*. Scientific World Journal 2015;2015:731-65.

[14] Utsunomiya T, Kobayashi M, Pollard RB, et al. *Glycyrrhizin, an active component of licorice roots, reduces morbidity and mortality of mice infected with lethal doses of influenza virus.* Antimicrob Agents Chemother 1997;41:551-6.

[15] Fiore C, Eisenhut M, Krausse R, et al. *Antiviral effects of Glycyrrhiza species*. Phytother Res 2008;22:141-8.

[16] Harada S. *The broad anti-viral agent glycyrrhizin directly modulates the fluidity of plasma membrane and HIV-1 envelope*. Biochem J 2005;392:191-9.

[17] Smirnov VS, Garshinina AV, Guseva VM, et al. *The anti-viral activity of the complex glycyrrhizic acid-alpha-glutamyl-tryptophan against experimental lethal influenza infection in white mice caused by oseltamivir-resistant strain of the virus*. Vopr Virusol 2013;58:19-26.

[18] Smirnov VS, Zarubaev VV, Anfimov PM, et al. *Effect of a combination of glutamyl-tryptophan and glycyrrhizic acid on the course of acute infection caused by influenza (H3H2) virus in mice*. Vopr Virusol 2012;57:23-7.

[19] Wolkerstorfer A, Kurz H, Bachhofner N, et al. *Glycyrrhizin inhibits influenza A virus uptake into the cell*. Antiviral Res 2009;83:171-8.

[20] Michaelis M, Geiler J, Naczk P, et al. *Glycyrrhizin inhibits highly pathogenic H5N1 influenza A virus-induced pro-inflammatory cytokine and chemokine expression in human macrophages*. Med Microbiol Immunol 2010;199:291-7

[21] Michaelis M, Geiler J, Naczk P, et al. *Glycyrrhizin exerts antioxidative effects in H5N1 influenza A virus-infected cells and inhibits virus replication and pro-inflammatory gene expression*. PLoS One 2011;6:e19705.

[22] Moisy D, Avilov SV, Jacob Y, et al. *HMGB1 protein binds to influenza virus nucleoprotein and promotes viral replication*. J Virol 2012;86:9122-33.

[23] Stanetty C, Wolkerstorfer A, Amer H, et al. *Synthesis and antiviral activities of spacer-linked 1-thioglucuronide analogues of glycyrrhizin*. Beilstein J Org Chem 2012;8:705-11.

[24] Pompei R, Paghi L, Ingianni A, et al. *Glycyrrhizic acid inhibits influenza virus growth in embryonated eggs*. Microbiologica 1983;6:247-50.

[25] Scherließ R, Ajmera A, Dennis M, et al. *Induction of protective immunity against H1N1 influenza A(H1N1)pdm09 with spray-dried and electron-beam sterilised vaccines in non-human primates*. Vaccine 2014;32:2231-40.

[26] Song G, Yang S, Zhang W, et al. *Discovery of the first series of small molecule H5N1 entry inhibitors*. J Med Chem 2009;52:7368-71.

[27] Song W, Si L, Ji S, et al. *Uralsaponins M-Y, antiviral triterpenoid saponins from the roots of Glycyrrhiza uralensis*. J Nat Prod 2014;77:1632-43.

[28] Song X, Chen J, Sakwiwatkul K, et al. *Enhancement of immune responses to influenza vaccine (H3N2) by ginsenoside Re*. Int Immunopharmacol 2010;10:351-6.

[29] Barr IG, Mitchell GF. *ISCOMs (immunostimulating complexes): the first decade*. Immunol Cell Biol 1996;74:8-25.

[30] Liu H, Patil HP, de Vries-Idema J, et al. *Enhancement of the immunogenicity and protective efficacy of a mucosal influenza subunit vaccine by the saponin adjuvant GPI-0100*. PLoS One 2012;7:e52135.

[31] Liu H, de Vries-Idema J, Ter Veer W, et al. *Influenza virosomes supplemented with GPI-0100 adjuvant: a potent vaccine formulation for antigen dose sparing*. Med Microbiol Immunol 2014;203:47-55.

[32] Zhai L, Li Y, Wang W, et al. *Enhancement of humoral immune responses to inactivated Newcastle disease and avian influenza vaccines by oral administration of ginseng stem-and-leaf saponins in chickens*. Poult Sci 2011;90:1955-9.

[33] Sun H, He S, Shi M. *Adjuvant-active fraction from Albizia julibrissin saponins improves immune responses by inducing cytokine and chemokine at the site of injection*. Int Immunopharmacol 2014;22:346-55.

[34] Kazakova OB, Giniyatullina GV, Yamansarov EY, et al. *Betulin and ursolic acid synthetic derivatives as inhibitors of Papilloma virus*. Bioorg Med Chem Lett 2010;20:4088-90.

[35] Kazakova OB, Medvedeva NI, Baĭkova IP, et al. *Synthesis of triterpenoid acylates - an effective reproduction inhibitors of influenza A (H1N1) and papilloma viruses*. Bioorg Khim 2010;36:841-8.

[36] Flekhter OB, Medvedeva NI, Kukovinets OS, et al. *Synthesis and antiviral activity of lupane triterpenoids with modified cycle E*. Bioorg Khim 2007;33:629-34.

[37] Baltina LA, Flekhter OB, Nigmatullina LR, et al. *Lupane triterpenes and derivatives with antiviral activity*. Bioorg Med Chem Lett 2003;13:3549-52.

[38] Grishko VV, Galaiko NV, Tolmacheva IA, et al. *Functionalization, cyclization and antiviral activity of A-secotriterpenoids*. Eur J Med Chem 2014;83:601-8.

[39] Krumbiegel M, Dimitrov DS, Puri A, et al. *Dextran sulfate inhibits fusion of influenza virus and cells expressing influenza hemagglutinin with red blood cells.* Biochim Biophys Acta 1992;1110:158-64.

[40] Herrmann A, Korte T, Arnold K, et al. *The influence of dextran sulfate on influenza A virus fusion with erythrocyte membranes*. Antiviral Res 1992;19:295-311.

[41] Lüscher-Mattli M, Glück R, Kempf C, et al. *A comparative study of the effect of dextran sulfate on the fusion and the in vitro replication of influenza A and B, Semliki Forest, vesicular stomatitis, rabies, Sendai, and mumps virus*. Arch Virol 1993;130:317-26.

[42] Yamada H, Moriishi E, Haredy AM, et al. *Influenza virus neuraminidase contributes to the dextran sulfate-dependent suppressive replication of some influenza A virus strains*. Antiviral Res 2012;96:344-52.

[43] Yamada H, Nagao C, Haredy AM, et al. *Dextran sulfate-resistant A/Puerto Rico/8/34 influenza virus is associated with the emergence of specific mutations in the neuraminidase glycoprotein*. Antiviral Res 2014;111:69-77.

[44] Shkurupy VA, Potapova OV, Sharkova TV, et al. *Experimental study of the efficiency of oxidized dextran for prevention of influenza A/H5N1*. Bull Exp Biol Med 2014;158:112-4.

[45] Shkurupy VA, Potapova OV, Sharkova TV, et al. *Effects of Preventive Administration of Oxidized Dextran on Liver Injury and Reparative Regeneration in Mice Infected with Influenza A/H5N1 Virus*. Bull Exp Biol Med 2015;158:483-8.

[46] Potapova OV, Shkurupiy VA, Sharkova TV, et al. *Preventive efficacy of oxidized dextran and pathomorphological processes in mouse lungs in avian influenza A/H5N1*. Bull Exp Biol Med 2011;150:707-10.

[47] De Clercq E. *Highlights in the development of new antiviral agents*. Mini Rev Med Chem 2002;2:163-75.

[48] Bond S, Draffan AG, Fenner JE, et al. *1,2,3,9b-Tetrahydro-5H-imidazo,1-a]isoindol-5-ones as a new class of respiratory syncytial virus (RSV) fusion inhibitors. Part 2: identification of BTA9881 as a preclinical candidate*. Bioorg Med Chem Lett 2015;25:976-81.

[49] Weisman LE. *Respiratory syncytial virus (RSV) prevention and treatment: past, present, and future*. Cardiovasc Hematol Agents Med Chem 2009;7:223-33.

[50] Guinea R, Carrasco L. *Concanamycin A blocks influenza virus entry into cells under acidic conditions*. FEBS Lett 1994;349:327-30.

[51] Guinea R, Carrasco L. *Concanamycin A: a powerful inhibitor of enveloped animal-virus entry into cells*. Biochem Biophys Res Commun 1994;201:1270-8.

[52] Guinea R, Carrasco L. *Requirement for vacuolar proton-ATPase activity during entry of influenza virus into cells*. J Virol 1995;69:2306-12.

[53] Müller KH, Kainov DE, El Bakkouri K, et al. *The proton translocation domain of cellular vacuolar ATPase provides a target for the treatment of influenza A virus infections*. Br J Pharmacol 2011;164:344-57

[54] Yeganeh B, Ghavami S, Kroeker AL, et al. *Suppression of influenza A virus replication in human lung epithelial cells by noncytotoxic concentrations bafilomycin A1*. Am J Physiol Lung Cell Mol Physiol 2015;308:L270-86.

[55] Ochiai H, Sakai S, Hirabayashi T, et al. *Inhibitory effect of bafilomycin A1, a specific inhibitor of vacuolar-type proton pump, on the growth of influenza A and B viruses in MDCK cells*. Antiviral Res 1995;27:425-30.

[56] Bimbo LM, Denisova OV, Mäkilä E, et al. *Inhibition of influenza A virus infection in vitro by saliphenylhalamide-loaded porous silicon nanoparticles*. ACS Nano 2013;7:6884-93.

[57] Paton NI, Lee L, Xu Y, et al. *Chloroquine for influenza prevention: a randomised, double-blind, placebo controlled trial*. Lancet Infect Dis 2011;11:677-83.

[58] Savarino A. *Use of chloroquine in viral diseases*. Lancet Infect Dis 2011;11:653-4.

[59] Ooi EE, Chew JS, Loh JP, et al. *In vitro inhibition of human influenza A virus replication by chloroquine*. Virol J 2006;3:39.

[60] Di Trani L, Savarino A, Campitelli L, et al. *Different pH requirements are associated with divergent inhibitory effects of chloroquine on human and avian influenza A viruses*. Virol J 2007;4:39.

[61] Vigerust DJ, McCullers JA. *Chloroquine is effective against influenza A virus in vitro but not in vivo*. Influenza Other Respir Viruses 2007;1:189-92.

[62] Yan Y, Zou Z, Sun Y, et al. *Anti-malaria drug chloroquine is highly effective in treating avian influenza A H5N1 virus infection in an animal model*. Cell Res 2013;23:300-2.

[63] Garulli B, Di Mario G, Sciaraffia E, et al. *Enhancement of T cell-mediated immune responses to whole inactivated influenza virus by chloroquine treatment in vivo*. Vaccine 2013;31:1717-24.

[64] Wu L, Dai J, Zhao X, et al. *Chloroquine enhances replication of influenza A virus A/WSN/33 (H1N1) in dose-, time-, and MOI-dependent manners in human lung epithelial cells A549*. J Med Virol 2015, in press.

[65] De Clercq E. *A Cutting-edge view on the current state of antiviral drug development*. Med Res Rev 2013;33:1249–1277.

[66] Blaising J, Lévy PL, Gondeau C, et al. *Silibinin inhibits hepatitis C virus entry into hepatocytes by hindering clathrin-dependent trafficking*. Cell Microbiol 2013;15:1866-82.

[67] Dai JP, Wu LQ, Li R, et al. *Identification of 23-(s)-2-amino-3-phenylpropanoyl-silybin as an antiviral agent for influenza A virus infection in vitro and in vivo*. Antimicrob Agents Chemother 2013;57:4433-43.

[68] Gazák R, Purchartová K, Marhol P, et al. *Antioxidant and antiviral activities of silybin fatty acid conjugates*. Eur J Med Chem 2010;45:1059-67.

[69] Garozzo A, Timpanaro R, Bisignano B, et al. *In vitro antiviral activity of Melaleuca alternifolia essential oil*. Lett Appl Microbiol 2009;49:806-8.

[70] Garozzo A, Timpanaro R, Stivala A, et al. *Activity of Melaleuca alternifolia (tea tree) oil on Influenza virus A/PR/8: study on the mechanism of action*. Antiviral Res 2011;89:83-8.

[71] Li X, Duan S, Chu C, et al. *Melaleuca alternifolia concentrate inhibits in vitro entry of influenza virus into host cells*. Molecules 2013;18:9550-66.

[72] Mantil E, Daly G, Avis TJ. *Effect of tea tree (Melaleuca alternifolia) oil as a natural antimicrobial agent in lipophilic formulations*. Can J Microbiol 2015;61:82-8.

[73] He J, Qi WB, Wang L, et al. *Amaryllidaceae alkaloids inhibit nuclear-to-cytoplasmic export of ribonucleoprotein (RNP) complex of highly pathogenic avian influenza virus H5N1*. Influenza Other Respir Viruses 2013;7:922-31.

[74] Aggarwal BB, Deb L, Prasad S. *Curcumin differs from tetrahydrocurcumin for molecular targets, signaling pathways and cellular responses*. Molecules 2014;20:185-205.

[75] Chen TY, Chen DY, Wen HW, et al. *Inhibition of enveloped viruses infectivity by curcumin*. PLoS One 2013;8:e62482.

[76] Shuto T. *Regulation of expression, function, and inflammatory responses of innate immune receptor Toll-like receptor-2 (TLR2) during inflammatory responses against infection*. Yakugaku Zasshi 2013;133:1401-9.

[77] Kim K, Kim KH, Kim HY, et al. *Curcumin inhibits hepatitis C virus replication via suppressing the Akt-SREBP-1 pathway*. FEBS Lett. 2010;584:707-12.

[78] Rajput N, Naeem M, Ali S, et al. *The effect of dietary supplementation with the natural carotenoids curcumin and lutein on broiler pigmentation and immunity*. Poult Sci 2013;92:1177-85.

[79] Ou JL, Mizushina Y, Wang SY, et al. *Structure-activity relationship analysis of curcumin analogues on anti-influenza virus activity*. FEBS J 2013;280:5829-40.

[80] Haasbach E, Hartmayer C, Hettler A, et al. *Antiviral activity of Ladania067, an extract from wild black currant leaves against influenza A virus in vitro and in vivo*. Front Microbiol 2014;5:171.

[81] Ehrhardt C, Dudek SE, Holzberg M, et al. *A plant extract of Ribes nigrum folium possesses anti-influenza virus activity in vitro and in vivo by preventing virus entry to host cells*. PLoS One 2013;8:e63657.

[82] Yokomizo K, Miyamoto Y, Nagao K, et al. *Fattiviracin A1, a novel antiviral agent produced by Streptomyces microflavus strain No. 2445. II. Biological properties*. J Antibiot (Tokyo) 1998;51:1035-9.

[83] Habib ES, Yokomizo K, Nagao K, et al. *Antiviral activity of fattiviracin FV-8 against human immunodeficiency virus type 1 (HIV-1)*. Biosci Biotechnol Biochem 2001;65:683-5.

[84] Tanaka T, Ikeda T, Kaku M, et al. *A new lignan glycoside and phenylethanoid glycosides from Strobilanthes cusia BREMEK*. Chem Pharm Bull (Tokyo) 2004;52:1242-5.

[85] Uyeda M. *Metabolites produced by actinomycetes-antiviral antibiotics and enzyme inhibitors*. Yakugaku Zasshi 2004;124:469-79.

[86] Liao Q, Qian Z, Liu R, et al. *Germacrone inhibits early stages of influenza virus infection*. Antiviral Res 2013;100:578-88.

[87] Denisova OV, Söderholm S, Virtanen S, et al. *Akt inhibitor MK2206 prevents influenza pH1N1 virus infection in vitro*. Antimicrob Agents Chemother 2014;58:3689-96.

[88] Hirata N, Suizu F, Matsuda-Lennikov M, et al. *Inhibition of Akt kinase activity suppresses entryand replication of influenza virus*. Biochem Biophys Res Commun 2014;450:891-8.

[89] Hsieh CF, Lo CW, Liu CH, et al. *Mechanism by which ma-xing-shi-gan-tang inhibits the entry of influenza virus*. J Ethnopharmacol 2012;143:57-67.

[90] Murray JL, McDonald NJ, Sheng J, et al. *Inhibition of influenza A virus replication by antagonism of a PI3K-AKT-mTOR pathway member identified by gene-trap insertional mutagenesis*. Antivir Chem Chemother 2012;22:205-15.

[91] Chan RW, Chan MC, Wong AC, et al. *DAS181 inhibits H5N1 influenza virus infection of human lung tissues*. Antimicrob Agents Chemother 2009;53:3935-41.

[92] Triana-Baltzer GB, Gubareva LV, Nicholls JM, et al. *Novel pandemic influenza A(H1N1) viruses are potently inhibited by DAS181, a sialidase fusion protein*. PLoS One 2009;4:e7788.

[93] Triana-Baltzer GB, Gubareva LV, Klimov AI, et al. *Inhibition of neuraminidase inhibitor-resistant influenza virus by DAS181, a novel sialidase fusion protein*. PLoS One 2009;4:e7838.

[94] Triana-Baltzer GB, Babizki M, Chan MC, et al. *DAS181, a sialidase fusion protein, protects human airway epithelium against influenza virus infection: an in vitro pharmacodynamic analysis*. J Antimicrob Chemother 2010;65:275-84.

[95] Moss RB, Hansen C, Sanders RL, et al. *A phase II study of DAS181, a novel host directed antiviral for the treatment of influenza infection*. J Infect Dis 2012;206:1844-51.

[96] Ison MG. *Expanding the armamentarium against respiratory viral infections: DAS181*. J Infect Dis 2012;206:1806-8.

[97] Zhang H. *DAS181 and H5N1 virus infection*. J Infect Dis 2009;199:1250, author reply 1250-1.

[98] Moscona A, Porotto M, Palmer S, et al. *A recombinant sialidase fusion protein effectively inhibits human parainfluenza viral infection in vitro and in vivo*. J Infect Dis 2010;202:234-41.

[99] Jones BG, Hayden RT, Hurwitz JL. *Inhibition of primary clinical isolates of human parainfluenza virus by DAS181 in cell culture and in a cotton rat model*. Antiviral Res 2013;100:562-6.

[100] Malakhov MP, Aschenbrenner LM, Smee DF, et al. *Sialidase fusion protein as a novel broad-spectrum inhibitor of influenza virus infection*. Antimicrob Agents Chemother 2006;50:1470-9.

[101] Hayden F. *Developing new antiviral agents for influenza treatment: what does the future hold?* Clin Infect Dis 2009;48 Suppl 1:S3-13.

[102] Belser JA, Lu X, Szretter KJ, et al. *DAS181, a novel sialidase fusion protein, protects mice from lethal avian influenza H5N1 virus infection.* J Infect Dis 2007;196:1493-9.

[103] Marjuki H, Mishin VP, Chesnokov AP, et al. *An investigational antiviral drug, DAS181, effectively inhibits replication of zoonotic influenza A virus subtype H7N9 and protects mice from lethality*. J Infect Dis 2014;210:435-40.

[104] Zhu L, Li Y, Li S, et al. *Inhibition of influenza A virus (H1N1) fusion by benzenesulfonamide derivatives targeting viral hemagglutinin*. PLoS One 2011;6:e29120.

[105] Hsieh HP, Hsu JT. *Strategies of development of antiviral agents directed against influenza virus replication*. Curr Pharm Des 2007;13:3531-42.

[106] Shigeta S. *Current status of research and development for anti-influenza virus drugs--chemotherapy for influenza*. Nihon Rinsho 1997;55:2758-64.

[107] Luo G, Torri A, Harte WE, et al. *Molecular mechanism underlying the action of a novel fusion inhibitor of influenza A virus*. J Virol 1997;71:4062-70.

[108] Vichkanova SA, Oïfa AI, Goriunova LV. *Antiviral properties of gossypol in experimental influenza pneumonia*. Antibiotiki 1970;15:1071-3.

[109] Krylov VF. *Treatment of patients with influenza*. Ter Arkh 1975;47:49-55.

[110] Yang J, Zhang F, Li J, et al. *Synthesis and antiviral activities of novel gossypol derivatives*. Bioorg Med Chem Lett 2012;22:1415-20.

[111] Yang J, Chen G, Li LL, et al. *Synthesis and anti-H5N1 activity of chiral gossypol derivatives and its analogs implicated by a viral entry blocking mechanism*. Bioorg Med Chem Lett 2013;23:2619-23.

[112] Jones JC, Turpin EA, Bultmann H, et al. *Inhibition of influenza virus infection by a novel antiviral peptide that targets viral attachment to cells.* J Virol 2006;80:11960-7.

[113] Altmann SE, Brandt CR, Jahrling PB, et al. *Antiviral activity of the EB peptide against zoonotic poxviruses*. Virol J 2012;9:6.

[114] Nicol MQ, Ligertwood Y, Bacon MN, et al. *A novel family of peptides with potent activity against influenza A viruses*. J Gen Virol 2012;93:980-6.

[115] Rajik M, Jahanshiri F, Omar AR, et al. *Identification and characterisation of a novel anti-viral peptide against avian influenza virus H9N2.* Virol J 2009;6:74.

[116] Matsubara T. *Potential of peptides as inhibitors and mimotopes: selection of carbohydrate-mimetic peptides from phage display libraries*. J Nucleic Acids. 2012;2012:740982.

[117] Selman L, Hansen S. *Structure and function of collectin liver 1 (CL-L1) and collectin 11 (CL-11, CL-K1).* Immunobiology 2012;217:851-63.

[118] Ling MT, Tu W, Han Y, et al. *Mannose-binding lectin contributes to deleterious inflammatory response in pandemic H1N1 and avian H9N2 infection*. J Infect Dis 2012;205:44-53.

[119] Dec M, Wernicki A. *Conglutinin, CL-43 and CL-46-three bovine collectins*. Pol J Vet Sci 2006;9:265-75.

[120] Kawai T, Kase T, Suzuki Y, et al. *Anti-influenza A virus activities of mannan-binding lectins and bovine conglutinin*. J Vet Med Sci 2007;69:221-4.

[121] Malhotra R, Haurum JS, Thiel S, et al. *Binding of human collectins (SP-A and MBP) to influenza virus*. Biochem J 1994;304:455-61.

[122] Hartshorn KL, Sastry K, Brown D, et al. *Conglutinin acts as an opsonin for influenza A viruses*. J Immunol 1993;151:6265-73.

[123] Verma A, White M, Vathipadiekal V, et al. *Human H-ficolin inhibits replication of seasonal and pandemic influenza A viruses*. J Immunol 2012;189:2478-87.

[124] Pan Q, Chen H, Wang F, et al. *L-ficolin binds to the glycoproteins hemagglutinin and neuraminidase and inhibits influenza A virus infection both in vitro and in vivo*. J Innate Immun 2012;4:312-24.

[125] Chang WC, Hartshorn KL, White MR, et al. *Recombinant chimeric lectins consisting of mannose-binding lectin and L-ficolin are potent inhibitors of influenza A virus compared with mannose-binding lectin*. Biochem Pharmacol 2011;81:388-95.

[126] Gordts SC, Renders M, Férir G, et al. *NICTABA and UDA, two GlcNAc-binding lectins with unique antiviral activity profiles*. J Antimicrob Chemother 2015, in press.

[127] Pustylnikov S, Sagar D, Jain P, et al. *Targeting the C-type lectins-mediated host-pathogen interactions with dextran*. J Pharm Pharm Sci 2014;17:371-92.

[128] Morita M, Kuba K, Ichikawa A, et al. *The lipid mediator protectin D1 inhibits influenza virus replication and improves severe influenza*. Cell 2013;153:112-25.

[129] Zhang C, Xu Y, Jia L, et al. *A new therapeutic strategy for lung tissue injury induced by influenza with CR2 targeting complement inhibitor*. Virol J 2010;7:30.

[130] Imai Y. *Role of omega-3 PUFA-derived mediators, the protectins, in influenza virus infection*. Biochim Biophys Acta 2015;1851:496-502.

[131] Takahashi M, Mori S, Shigeta S, et al. *Role of MBL-associated serine protease (MASP) on activation of the lectin complement pathway*. Adv Exp Med Biol. 2007;598:93-104.

[132] Smee DF, Bailey KW, Wong MH, et al. *Treatment of influenza A (H1N1) virus infections in mice and ferrets with cyanovirin-N*. Antiviral Res 2008;80:266-71.

[133] O'Keefe BR, Smee DF, Turpin JA, et al. *Potent anti-influenza activity of cyanovirin-N and interactions with viral hemagglutinin.* Antimicrob Agents Chemother 2003;47:2518-25.

[134] Miyamoto D, Hasegawa S, Sriwilaijaroen N, et al. *Clarithromycin inhibits progeny virus production from human influenza virus-infected host cells.* Biol Pharm Bull 2008;31:217-22.

[135] Yamaya M, Shinya K, Hatachi Y, et al. *Clarithromycin inhibits type a seasonal influenza virus infection in human airway epithelial cells.* J Pharmacol Exp Ther 2010;333:81-90.

[136] Ghendon Y, Markushin S, Heider H, et al. *Haemagglutinin of influenza A virus is a target for the antiviral effect of Norak*in. J Gen Virol 1986;67:1115-22.

[137] Ott S, Wunderli-Allenspach H. *Effect of the virostatic Norakin (triperiden) on influenza virus activities*. Antiviral Res 1994;24:37-42.

[138] Presber HW, Schroeder C, Hegenscheid B, et al. *Antiviral activity of Norakin (triperiden) and related anticholinergic antiparkinsonism drugs*. Acta Virol 1984;28:501-7.

[139] Heider H, Markushin S, Schroeder C, Ghendon Y. *The influence of Norakin on the reproduction of influenza A and B viruses*. Arch Virol 1985;86:283-90.

[140] Schroeder C, Heider H, Hegenscheid B, et al. *The anticholinergic anti-Parkinson drug Norakin selectively inhibits influenza virus replication*. Antiviral Res 1985;(Suppl 1):95-9.

[141] Prösch S, Heider H, Schroeder C, et al. *Mutations in the hemagglutinin gene associated with influenza virus resistance to norakin*. Arch Virol 1988;102:125-9.

[142] Prösch S, Heider H, Schroeder C, et al. *Mapping mutations in influenza A virus resistant to norakin*. FEBS Lett 1990;267:19-21.

[143] Klimov AI, Markushin SG, Prösch S, et al. *Relation between drug resistance and antigenicity among norakin-resistant mutants of influenza A (fowl plague) virus*. Arch Virol 1992;124:147-55.

[144] Markushin SG, Ginzburg VP, Khaĭder AM, et al. *Factors that cause a change in the antigenic structure of of the influenza virus hemagglutinin*. Vopr Virusol 1992;37:196-9.

[145] Oka M, Ishiwata Y, Iwata N, et al. *Synthesis and anti-influenza virus activity of tricyclic compounds with a unique amine moiety*. Chem Pharm Bull (Tokyo) 2001;49:379-83.

[146] Rossignol JF. *Nitazoxanide. A first-in-class broad-spectrum antiviral agent.* Antiviral Res 2014, in press.

[147] Ashiru O, Howe JD, Butters TD. *Nitazoxanide, an antiviral thiazolide, depletes ATP-sensitive intracellular Ca(2+) stores*. Virology 2014;462-463:135-48.

[148] Belardo G, Cenciarelli O, La Frazia S, et al. *Synergistic effect of nitazoxanide with neuraminidase inhibitors against influenza A viruses in vitro*. Antimicrob Agents Chemother 2015;59:1061-9.

[149] Täubel J, Lorch U, Rossignol JF, et al. *Analyzing the relationship of QT interval and exposure to nitazoxanide, a prospective candidate for influenza antiviral therapy – A formal TQT study*. J Clin Pharmacol 2014;54:987-94.

[150] Haffizulla J, Hartman A, Hoppers M, et al. *Effect of nitazoxanide in adults and adolescents with acute uncomplicated influenza: a double-blind, randomised, placebo-controlled, phase 2b/3 trial*. Lancet Infect Dis 2014;14:609-18.

[151] Ashton LV, Callan RL, Rao S, et al. *In vitro susceptibility of canine influenza A (H3N8) virus to nitazoxanide and tizoxanide*. Vet Med Int 2010;2010.

[152] Ulyanova V, Vershinina V, Ilinskaya O. *Barnase and binase: twins with distinct fates*. FEBS J 2011;278:3633-43.

[153] Shah Mahmud R, Ilinskaya ON. *Antiviral Activity of Binase against the Pandemic Influenza A (H1N1) Virus*. Acta Naturae 2013;5:44-51.

[154] Sato Y, Hirayama M, Morimoto K, et al. *High mannose-binding lectin with preference for the cluster of alpha1-2-mannose from the green alga Boodlea coacta is a potent entry inhibitor of HIV-1 and influenza viruses*. J Biol Chem 2011;286:19446-58.

[155] Savov VM, Galabov AS, Tantcheva LP, et al. *Effects of rutin and quercetin on monooxygenase activities in experimental influenza virus infection*. Exp Toxicol Pathol 2006;58:59-64.

[156] Davis JM, Murphy EA, McClellan JL, et al. *Quercetin reduces susceptibility to influenza infection following stressful exercise*. Am J Physiol Regul Integr Comp Physiol 2008;295:R505-9.

[157] Choi HJ, Song JH, Park KS, et al. *Inhibitory effects of quercetin 3-rhamnoside on influenza A virus replication*. Eur J Pharm Sci 2009;37:329-33.

[158] Kumar P, Khanna M, Srivastava V, et al. *Effect of quercetin supplementation on lung antioxidants after experimental influenza virus infection*. Exp Lung Res 2005;31:449-59.

[159] Raju TA, Lakshmi AN, Anand T, et al. *Protective effects of quercetin during influenza virus-induced oxidative stress*. Asia Pac J Clin Nutr 2000;9:314-7.

[160] Friel H, Lederman H. *A nutritional supplement formula for influenza A (H5N1) infection in humans*. Med Hypotheses 2006;67:578-87.

[161] Eşanu V, Prahoveanu E, Crişan I, et al. *The effect of an aqueous propolis extract, of rutin and of a rutin-quercetin mixture on experimental influenza virus infection in mice*. Virologie 1981;32:213-5.

[162] Chang SS, Huang HJ, Chen CY. *Two birds with one stone? Possible dual-targeting H1N1 inhibitors from traditional Chinese medicine*. PLoS Comput Biol 2011;7:e1002315.

[163] Chang TT, Sun MF, Chen HY, et al. *Screening from the world's largest TCM database against H1N1 virus*. J Biomol Struct Dyn 2011;28:773-86.

[164] Nakayama M, Suzuki K, Toda M, et al. *Inhibition of the infectivity of influenza virus by tea polyphenols*. Antiviral Res 1993;21:289-99.

[165] Yang ZF, Bai LP, Huang WB, et al. *Comparison of in vitro antiviral activity of tea polyphenols against influenza A and B viruses and structure-activity relationship analysis*. Fitoterapia 2014;93:47-53.

[166] Zu M, Yang F, Zhou W, et al. *In vitro anti-influenza virus and anti-inflammatory activities of theaflavin derivatives*. Antiviral Res 2012;94:217-24.

[167] Hayden FG, Aoki FY. *Amantadine, rimatadine, and related agents.* In: Barriere SL, editor. *Antimicrobial Therapy and Vaccines.* Baltimore: Williams & Williams 1999, pp 1344-1365.

[168] Wang C, Takeuchi K, Pinto LH, et al. *Ion channel activity of influenza A virus M2 protein: characterization of the amantadine block.* J Virol 1993;675585-94.

[169] Ruigrok RW, Hirst EM, Hay AJ. *The specific inhibition of influenza A virus maturation by amantadine: an electron microscopic examination.* J Gen Virol 1991;72:191-4.

[170] Sheu TG, Fry AM, Garten RJ, et al. *Dual resistance to adamantanes and oseltamivir among seasonal influenza A(H1N1) viruses: 2008-2010.* J Infect Dis 2011;203:13-7.

[171] Smirnova TD, Danilenko DM, Eropkin MIu, et al. *Influence of rimantadine, ribavirine and triazavirine on influenza A virus replication in human monolayer and lymphoblastoid cell lines*. Antibiot Khimioter 2011;56:11-6.

[172] Karpenko I, Deev S, Kiselev O, et al. *Antiviral properties, metabolism, and pharmacokinetics of a novel azolo-1,2,4-triazine-derived inhibitor of influenza A and B virus replication*. Antimicrob Agents Chemother 2010;54:2017-22.

[173] Tanner JA, Zheng BJ, Zhou J, et al. *The adamantane-derived bananins are potent inhibitors of the helicase activities and replication of SARS coronavirus.* Chem Biol 2005;12:303-11.

[174] Moorthy NS, Poongavanam V, Pratheepa V. *Viral M2 ion channel protein: a promising target for anti-influenza drug discovery*. Mini Rev Med Chem 2014;14:819-30.

[175] Rey-Carrizo M, Torres E, Ma C, et al. *3-Azatetracyclo.2.1.1(5,8).0(1,5)]undecane derivatives: from wild-type inhibitors of the M2 ion channel of influenza A virus to derivatives with potent activity against the V27A mutant*. J Med Chem 2013;56:9265-74.

[176] Wang J, Wu Y, Ma C, et al. *Structure and inhibition of the drug-resistant S31N mutant of the M2 ion channel of influenza A virus*. Proc Natl Acad Sci U S A 2013;110:1315-20.

[177] Wang J, Ma C, Wang J, et al. *Discovery of novel dual inhibitors of the wild-type and the most prevalent drug-resistant mutant, S31N, of the M2 proton channel from influenza A virus*. J Med Chem 2013;56:2804-12.

[178] Gasparini R, Lai PL, Casabona F, et al. *Do the omeprazole family compounds exert a protective effect against influenza-like illness?* BMC Infect Dis 2014;14:297.

[179] Bozdaganyan M, Orekhov P, Bragazzi NL, et al. *Docking and molecular dynamics (MD) simulations in potential drugs discovery: an application to influenza virus M2 protein*. Am J Biochem Biotechnol 2014;10:180-88.

[180] *3C9J. The Crystal structure of Transmembrane domain of M2 protein and Amantadine complex.* Accesible at http://www.rcsb.org/pdb/explore.do?structureId=3c9j.

[181] Long J, Wright E, Molesti E, et al. *Antiviral therapies against Ebola and other emerging viral diseases using existing medicines that block virus entry*. F1000Research 2015;4:30.

[182] Bachrach U, Don S. *Inactivation of influenza and Newcastle disease viruses by oxidized spermine*. Isr J Med Sci 1970;6:435-7.

[183] Bachrach U. *Antiviral activity of oxidized polyamines*. Amino Acids 2007;33:267-72.

[184] Lin TI, Heider H, Schroeder C. *Different modes of inhibition by adamantane amine derivatives and natural polyamines of the functionally reconstituted influenza virus M2 proton channel protein*. J Gen Virol 1997;78:767-74.

[185] Even-Or O, Samira S, Rochlin E, et al. *Immunogenicity, protective efficacy and mechanism of novel CCS adjuvanted influenza vaccine*. Vaccine 2010;28:6527-41.

[186] Even-Or O, Joseph A, Itskovitz-Cooper N, et al. *A new intranasal influenza vaccine based on a novel polycationic lipid-ceramide carbamoyl-spermine (CCS). II. Studies in mice and ferrets and mechanism of adjuvanticity*. Vaccine 2011;29:2474-86.

[187] Fytas C, Kolocouris A, Fytas G, et al. *Influence of an additional amino group on the potency of aminoadamantanes against influenza virus A. II - Synthesis of spiropiperazines and in vitro activity against influenza A H3N2 virus*. Bioorg Chem 2010;38:247-51.

[188] Zhao X, Jie Y, Rosenberg MR, et al. *Design and synthesis of pinanamine derivatives as anti-influenza A M2 ion channel inhibitors*. Antiviral Res 2012;96:91-9.

[189] Serkedjieva J, Manolova N, Bankova V. *Anti-influenza virus effect of some propolis constituents and their analogues (esters of substituted cinnamic acids)*. J Nat Prod 1992;55:294-302.

[190] Kesel AJ. *Synthesis of novel test compounds for antiviral chemotherapy of severe acute respiratory syndrome (SARS).* Curr Med Chem 2005;12:2095-162.

[191] Iwai A, Shiozaki T, Miyazaki T. *Relevance of signaling molecules for apoptosis induction on influenza A virus replication*. Biochem Biophys Res Commun 2013;441:531-7.

[192] Jurgeit A, McDowell R, Moese S, et al. *Niclosamide is a proton carrier and targets acidic endosomes with broad antiviral effects.* PLoS Pathog 2012;8:e1002976.

[193] Krátký M, Vinšová J. *Antiviral activity of substituted salicylanilides – a review*. Mini Rev Med Chem 2011;11:956-67.

[194] Russell RJ, Kerry PS, Stevens DJ, et al. *Structure of influenza hemagglutinin in complex with an inhibitor of membrane fusion*. Proc Natl Acad Sci U S A 2008;105:17736-41.

[195] Hosoya M, Matsuyama S, Baba M, et al. *Effects of protease inhibitors on replication of various myxoviruses.* Antimicrob Agents Chemother 1992;36:1432-6.

[196] Bahgat MM, Blazejewska P, Schughart K. *Inhibition of lung serine proteases in mice: a potentially new approach to control influenza infection.* Virol J 2011;8:27.

[197] Hamilton BS, Chung C, Cyphers SY, et al. *Inhibition of influenza virus infection and hemagglutinin cleavage by the protease inhibitor HAI-2.* Biochem Biophys Res Commun 2014;450:1070-5.

[198] Lee MG, Kim KH, Park KY, et al. *Evaluation of anti-influenza effects of camostat in mice infected with non-adapted human influenza viruses.* Arch Virol 1996;141:1979-89.

[199] Puzis LE, Lozitsky VP. *Action of epsilon-aminocaproic acid on the proteolysis system during experimental influenza in mice*. Acta Virol 1988;32:515-21.

[200] Tashiro M, Klenk HD, Rott R. *Inhibitory effect of a protease inhibitor, leupeptin, on the development of influenza pneumonia, mediated by concomitant bacteria.* J Gen Virol 1987;68:2039-41.

[201] Zhirnov OP, Klenk HD, Wright PF. *Aprotinin and similar protease inhibitors as drugs against influenza*. Antiviral Res 2011;92:27-36.

[202] Zhou Y, Wu C, Zhao L, et al. *Exploring the early stages of the pH-induced conformational change of influenza hemagglutinin*. Proteins 2014;82:2412-28.

[203] Leikina E, Delanoe-Ayari H, Melikov K, et al. *Carbohydrate-binding molecules inhibit viral fusion and entry by crosslinking membrane glycoproteins.* Nat Immunol 2005;6:995-1001.

[204] Streeter DG, Witkowski JT, Khare GP, et al. *Mechanism of action of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole), a new broad-spectrum antiviral agent.* Proc Natl Acad Sci U S A 1973;70:1174-8.

[205] Crotty S, Cameron C, Andino R. *Ribavirin's antiviral mechanism of action: lethal mutagenesis?* J Mol Med (Berl) 2002;80:86-95.

[206] Chan-Tack KM, Murray JS, Birnkrant DB. *Use of ribavirin to treat influenza*. N Engl J Med 2009;361:1713-4.

[207] Gangemi JD, Nachtigal M, Barnhart D, et al. *Therapeutic efficacy of liposome-encapsulated ribavirin and muramyl tripeptide in experimental infection with influenza or herpes simplex virus*. J Infect Dis 1987;155:510-7.

[208] Smee DF, Hurst BL, Day CW, et al. *Influenza Virus H1N1 inhibition by serine protease inhibitor (serpin) antithrombin III*. Int Trends Immun 2014;2:83-86.

[209] Stoller JK, Lacbawan FL, Aboussouan LS. *Alpha-1 Antitrypsin Deficiency*. In: Pagon RA, Adam MP, Ardinger HH, et al, editors. *GeneReviews* ® [Internet]. Seattle: University of Washington, 1993-2014. 2006 Oct 27 [updated 2014 May 01].

[210] Campos MA, Alazemi S, Zhang G, et al. *Influenza vaccination in subjects with alpha1-antitrypsin deficiency*. Chest 2008;133:49-55.

[211] Yagi S, Ono J, Yoshimoto J, et al. *Development of anti-influenza virus drugs I: improvement of oral absorption and in vivo anti-influenza activity of Stachyflin and its derivatives.* Pharm Res 1999;16:1041-6.

[212] Yoshimoto J, Yagi S, Ono J, et al. *Development of anti-influenza drugs: II. Improvement of oral and intranasal absorption and the anti-influenza activity of stachyflin derivatives*. J Pharm Pharmacol 2000;52:1247-55.

[213] Minagawa K, Kouzuki S, Yoshimoto J, et al. *Stachyflin and acetylstachyflin, novel anti-influenza A virus substances, produced by Stachybotrys sp. RF-7260. I. Isolation, structure elucidation and biological activities.* J Antibiot (Tokyo) 2002;55:155-64.

[214] Minagawa K, Kouzuki S, Kamigauchi T. *Stachyflin and acetylstachyflin, novel anti-influenza A virus substances, produced by Stachybotrys sp. RF-7260. II. Synthesis and preliminary structure-activity relationships of stachyflin derivatives.* J Antibiot (Tokyo) 2002;55:165-71.

[215] Motohashi Y, Igarashi M, Okamatsu M, et al *Antiviral activity of stachyflin on influenza A viruses of different hemagglutinin subtypes.* Virol J 2013;10:118.

[216] Watanabe K, Sakurai J, Abe H, et al. *Total synthesis of (+)-stachyflin: a potential anti-influenza A virus agen*t. Chem Commun (Camb) 2010;46:4055-7.

[217] Nakatani M, Nakamura M, Suzuki A, et al. *A new strategy toward the total synthesis of stachyflin, a potent anti-influenza A virus agent: concise route to the tetracyclic core structure*. Org Lett 2002;4:4483-6.

[218] Combrink KD, Gulgeze HB, Yu KL, et al. *Salicylamide inhibitors of influenza virus fusion.* Bioorg Med Chem Lett 2000;10:1649-52.

[219] Zhu L, Li Y, Li S, et al. *Inhibition of influenza A virus (H1N1)*

fusion by benzenesulfonamide derivatives targeting viral hemagglutinin. PLoS One 2011;6:e29120.

[220] Yu KL, Torri AF, Luo G, et al. *Structure-activity relationships for a series of thiobenzamide influenza fusion inhibitors derived from 1,3,3-trimethyl-5-hydroxy-cyclohexylmethylamine.* Bioorg Med Chem Lett 2002;12:3379-82.

[221] Yuan S. *Drugs to cure avian influenza infection-multiple ways to prevent cell death*. Cell Death Dis 2013;4:e835

[222] Ye M, Zheng JB, Yu KJ, et al. *Effects of high dose ulinastatin treatment in patients with severe pneumonia complicating influenza A H1N1 infection*. Zhongguo Wei Zhong Bing Ji Jiu Yi Xue 2011;23:48-9.

[223] Naganuma A, Mizuma H, Doi I, et al. *A case of acute respiratory distress syndrome induced by fulminant influenza A (H3 N2) pneumonia*. Nihon Kokyuki Gakkai Zasshi 2000;38:783-7.

[224] Munakata M, Kato R, Yokoyama H, et al. *Combined therapy with hypothermia and anticytokine agents in influenza A encephalopathy*. Brain Dev 2000;22:373-7.

[225] Leng YX, Yang SG, Song YH, et al. *Ulinastatin for acute lung injury and acute respiratory distress syndrome: A systematic review and meta-analysis*. World J Crit Care Med 2014;3:34-41.

[226] Ketscher L, Hannß R, Morales DJ, et al. *Selective inactivation of USP18 isopeptidase activity in vivo enhances ISG15 conjugation and viral resistance*. Proc Natl Acad Sci U S A 2015;112:1577-82.

[227] Uchida Y, Watanabe C, Takemae N, et al. *Identification of host genes linked with the survivability of chickens infected with recombinant viruses possessing H5N1 surface antigens from a highly pathogenic avian influenza virus*. J Virol 2012;86:2686-95.

[228] Liu AL, Li YF, Qi W, et al. *Comparative analysis of selected innate immune-related genes following infection of immortal DF-1 cells with highly pathogenic (H5N1) and low pathogenic (H9N2) avian influenza viruses*. Virus Genes 2015, in press.

[229] Loregian A, Mercorelli B, Nannetti G, et al. *Antiviral strategies against influenza virus: towards new therapeutic approaches.* Cell Mol Life Sci 2014, in press.

[230] Bauman JD, Patel D, Baker SF, et al. *Crystallographic fragment screening and structure-based optimization yields a new class of influenza endonuclease inhibitors.* ACS Chem Biol 2013;8:2501-8.

[231] Sheppard S. *Moroxydine: the story of a mislaid antiviral*. Acta Derm Venereol Suppl (Stockh) 1994;183:1-9.

[232] Mertens T, Eggers HJ. *Moroxydine*. Dtsch Med Wochenschr 1980;105:184.

[233] Dreyfus P. *Treatment of influenzal infections by a moroxydine derivative*. Sem Ther 1966;42:51-2.

[234] Furuta Y, Takahashi K, Fukuda Y, et al. *In vitro and in vivo activities of anti-influenza virus compound T-705.* Antimicrob Agents Chemother. 2002;46:977-81.

[235] Caroline AL, Powell DS, Bethel LM, et al. *Broad spectrum antiviral activity of favipiravir (T-705): protection from highly lethal inhalational Rift Valley Fever*. PLoS Negl Trop Dis 2014;8:e2790.

[236] Oestereich L, Rieger T, Neumann M, et al. *Evaluation of antiviral efficacy of ribavirin, arbidol, and T-705 (favipiravir) in a mouse model for Crimean-Congo hemorrhagic fever.* PLoS Negl Trop Dis 2014;8:e2804.

[237] Smither SJ, Eastaugh LS, Steward JA, et al. *Post-exposure efficacy of oral T-705 (Favipiravir) against inhalational Ebola virus infection in a mouse model.* Antiviral Res 2014;104:153-5.

[238] Safronetz D, Falzarano D, Scott DP, et al. *Antiviral efficacy of favipiravir against two prominent etiological agents of hantavirus pulmonary syndrome*. Antimicrob Agents Chemother 2013;57:4673-80.

[239] Iwai Y, Murakami K, Gomi Y, et al. *Anti-influenza activity of marchantins, macrocyclic bisbibenzyls contained in liverworts.* PLoS One 2011;6:e19825.

[240] Shaw ML, Klumpp K. *Successes and challenges in the antiviral field.* Curr Opin Virol 2013;3:483-6.

[241] Loregian A, Coen DM. *Selective anti-cytomegalovirus compounds discovered by screening for inhibitors of subunit interactions of the viral polymerase.* Chem Biol 2006;13:191-200.

[242] Fukuoka M, Minakuchi M, Kawaguchi A, et al *Structure-based discovery of anti-influenza virus A compounds among medicines.* Biochim Biophys Acta 2012;1820:90-5

[243] Sugiyama K, Obayashi E, Kawaguchi A, et al. *Structural insight into the essential PB1-PB2 subunit contact of the influenza virus RNA polymerase.* EMBO J 2009;28:1803-11.

[244] Chase G, Wunderlich K, Reuther P, et al. *Identification of influenza virus inhibitors which disrupt of viral polymerase protein-protein interactions.* Methods 2011;55:188-91.

[245] Li C, Ba Q, Wu A, et al. *A peptide derived from the C-terminus of PB1 inhibits influenza virus replication by interfering with viral polymerase assembly.* FEBS J 2013;280:1139-49.

[246] Nasser EH, Judd AK, Sanchez A, et al. *Antiviral activity of influenza virus M1 zinc finger peptides*. J Virol 1996;70:8639-44.

[247] Li L, Chang S, Xiang J, et al. *Screen anti-influenza lead compounds that target the PA(C) subunit of H5N1 viral RNA polymerase.* PLoS One 2012;7:e35234.

[248] Clark MP, Ledeboer MW, Davies I, et al. *Discovery of a novel, first-in-class, orally bioavailable azaindole inhibitor (VX-787) of influenza PB2.* J Med Chem 2014;57:6668-78.

[249] Pagano M, Castagnolo D, Bernardini M, et al. *The fight against the influenza A virus H1N1: synthesis, molecular modeling, and biological evaluation of benzofurazan derivatives as viral RNA polymerase inhibitors*. Chem Med Chem 2014;9:129-50.

[250] Lepri S, Nannetti G, Muratore G, et al. *Optimization of small-molecule inhibitors of influenza virus polymerase: from thiophene-3-carboxamide to polyamido scaffolds.* J Med Chem 2014;57:4337-50.

[251] Gao J, Luo X, Li Y, et al. *Synthesis and biological evaluation of 2-oxo-pyrazine-3-carboxamide-yl nucleoside analogues and their epimers as inhibitors of influenza A viruses*. Chem Biol Drug Des 2014, in press.

[252] Dierkes R, Warnking K, Liedmann S, et al. *The Rac1 inhibitor NSC23766 exerts anti-influenza virus properties by affecting the viral polymerase complex activity*. PLoS One 2014;9:e88520.

[253] Elton D, Simpson-Holley M, Archer K, et al. *Interaction of the influenza virus nucleoprotein with the cellular CRM1-mediated nuclear export pathway*. J Virol 2001;75:408-19.

[254] Perwitasari O, Johnson S, Yan X, et al. *Verdinexor, a novel selective inhibitor of nuclear export, reduces influenza a virus replication in vitro and in vivo.* J Virol 2014;88:10228-43.

[255] Amorim MJ, Kao RY, Digard P. *Nucleozin targets cytoplasmic trafficking of viral ribonucleoprotein-Rab11 complexes in influenza A virus infection.* J Virol 2013;87:4694-703.

[256] Su CY, Cheng TJ, Lin MI, et al. *High-throughput identification of compounds targeting influenza RNA-dependent RNA polymerase activity*. Proc Natl Acad Sci USA 2010;107:19151-6.

[257] Jiang H, Xu Y, Li L, et al. *Inhibition of influenza virus replication by constrained peptides targeting nucleoprotein*. Antivir Chem Chemother 2011;22:119-30.

[258] Verhelst J, Parthoens E, Schepens B, et al. *Interferon-inducible protein Mx1 inhibits influenza virus by interfering with functional viral ribonucleoprotein complex assembly*. J Virol 2012;86:13445-55.

[259] Cianci C, Gerritz SW, Deminie C, et al. *Influenza nucleoprotein: promising target for antiviral chemotherapy*. Antivir Chem Chemother 2012;23:77-91.

[260] Pons M. *Effect of actinomycin D on the replication of influenza virus and influenza virus RNA*. Virology 1967;33:150-4.

[261] Vogel U, Scholtissek C. *Inhibition of the intracellular transport of influenza viral RNA by actinomycin D.* Arch Virol 1995;140:1715-23.

[262] Pons MW. *The inhibition of influenza virus RNA synthesis by actinomycin D and cycloheximide*. Virology 1973;51:120-8.

[263] Lejal N, Tarus B, Bouguyon E, et al. *Structure-based discovery of the novel antiviral properties of naproxen against the nucleoprotein of influenza A virus.* Antimicrob Agents Chemother 2013;57:2231-42.

[264] Zarubaev VV, Beliaevskaia SV, Sirotkin AK, et al. *In vitro and in vivo effects of ingavirin on the ultrastructure and infectivity of influenza virus.* Vopr Virusol 2011;56:21-5.

[265] Zarubaev VV, Garshinina AV, Kalinina NA, et al. *Activity of Ingavirin (6 -(1H-Imidazol-4-yl)ethylamino]-5-oxohexanoic acid) against human respiratory viruses in vivo experiments.* Pharmaceuticals 2011;4:1518-1534.

[266] Semenova NP, Prokudina EN, Livov DK, et al. *Effect of the antiviral drug Ingaviruin on intracellular transformations and import into the nucleus of influenza A virus nucleocapsid protein.* Vopr Virusol 2010;55:17-20.

[267] Loginova SIa, Borisevich SV, Shkliaeva OM, et al. *Prophylactic and therapeutic efficacies of Ingavirin, a novel Russian chemotherapeutic, with respect to influenza pathogen A (H5N1).* Antibiot Khimioter 2010;55:10-2.

[268] Shishkina LN, Nebol'sin VE, Skarnovich MO, et al. *In vivo efficacy of Ingavirin against pandemic A (H1N1/09)v influenza virus*. Antibiot Khimioter 2010;55:32-5.

[269] Kolobukhina LV, Merkulova LN, Shchelkanov MI, et al. *Efficacy of ingavirin in adults with influenza*. Ter Arkh 2009;81:51-4.

[270] Galegov GA, Andronova VL, Nebol'sin VE. *Antiviral effect of Ingavirin against seasonal influenza virus A/H1N1 in MDCK cell culture*. Antibiot Khimioter 2009;54:19-22.

[271] Loginova SIa, Borisevich SV, Maksimov VA, et al. *Investigation of prophylactic activity of Ingavirin, a new Russian drug, against grippe A virus (H3N2).* Antibiot Khimioter 2008;53:19-21.

[272] Shul'diakov AA, Liapina EP, Kuznetsov VI. *Current principles in the chemoprophylaxis of acute respiratory viral infections.* Ter Arkh 2013;85:27-33.

[273] Isaeva EI, Nebol'sin VE, Kozulina IS, et al. *In vitro investigation of the antiviral activity of Ingavirin against human metapneumovirus*. Vopr Virusol 2012;57:34-8.

[274] Gubareva LV, Kaiser L, Hayden FG. *Influenza virus neuraminidase inhibitors.* Lancet 2000;355:827-35.

[275] Feng E, Ye D, Li J, et al. *Recent advances in neuraminidase inhibitor development as anti-influenza drugs.* ChemMedChem 2012;7:1527-36

[276] Verma RP, Hansch C. *A QSAR study on influenza neuraminidase inhibitors.* Bioorg Med Chem 2006;14:982-96.

[277] Barroso L, Treanor J, Gubareva L, et al. *Efficacy and tolerability of the oral neuraminidase inhibitor peramivir in experimental human influenza: randomized, controlled trials for prophylaxis and treatment.* Antivir Ther 2005;10:901-10.

[278] Birnkrant D, Cox E. *The Emergency Use Authorization of peramivir for treatment of 2009 H1N1 influenza*. N Engl J Med 2009;361:2204-7.

[279] Koyama K, Ogura Y, Nakai D, et al. *Identification of bioactivating enzymes involved in the hydrolysis of laninamivir octanoate, a long-acting neuraminidase inhibitor, in human pulmonary tissue.* Drug Metab Dispos 2014;42:1031-8.

[280] Weight AK, Haldar J, Alvarez de Cienfuegos L, et al. *Attaching zanamivir to a polymer markedly enhances its activity against drug-resistant strains of influenza a virus.* J Pharm Sci 2011;100:831-5.

[281] Hayden FG, Cote KM, Douglas RG Jr. *Plaque inhibition assay for drug susceptibility testing of influenza viruses.* Antimicrob Agents Chemother 1980;17:865-70.

[282] Jedrzejas MJ, Singh S, Brouillette WJ, et al. *Structures of aromatic inhibitors of influenza virus neuraminidase.* Biochemistry 1995;34:3144-51.

[283] Li Y, Silamkoti A, Kolavi G, et al. *Pyrrolidinobenzoic acid inhibitors of influenza virus neuraminidase: the hydrophobic side chain influences type A subtype selectivity.* Bioorg Med Chem 2012;20:4582-9.

[284] Kim CU, Lew W, Williams MA, et al. *Structure-activity relationship studies of novel carbocyclic influenza neuraminidase inhibitors.* J Med Chem 1998;41:2451-60.

[285] Kati WM, Saldivar AS, Mohamadi F, et al. *GS4071 is a slow-binding inhibitor of influenza neuraminidase from both A and B strains.* Biochem Biophys Res Commun 1998;244:408-13.

[286] Kim CU, Lew W, Williams MA, et al. *Influenza neuraminidase inhibitors possessing a novel hydrophobic interaction in the enzyme active site: design, synthesis, and structural analysis of carbocyclic sialic acid analogues with potent anti-influenza activity.* J Am Chem Soc 1997;119:681-90.

[287] Jang YJ, Achary R, Lee HW, et al. *Synthesis and anti-influenza virus activity of 4-oxo- or thioxo-4,5-dihydrofuro,4-c]pyridin-3(1H)-ones.* Antiviral Res 2014;107:66-75.

[288] Lou J, Yang X, Rao Z, et al. *Design and synthesis of 6-oxo-1,4,5,6-tetrahydropyrimidine-5-carboxylate derivatives as neuraminidase inhibitors.* Eur J Med Chem 2014;83:466-73.

[289] Li J, Zhang D, Zhu X, et al. *Studies on synthesis and structure-activity relationship (SAR) of derivatives of a new natural product from marine fungi as inhibitors of influenza virus neuraminidase.* Mar Drugs 2011;9:1887-901.

[290] Ding Y, Dou J, Teng Z, et al. *Antiviral activity of baicalin against influenza A (H1N1/H3N2) virus in cell culture and in mice and its inhibition of neuraminidase.* Arch Virol 2014;159:3269-78.

[291] Nayak MK, Agrawal AS, Bose S, et al. *Antiviral activity of baicalin against influenza virus H1N1-pdm09 is due to modulation of NS1-mediated cellular innate immune responses*. J Antimicrob Chemother 2014;69:1298-310.

[292] Wan Q, Wang H, Han X, et al. *Baicalin inhibits TLR7/MYD88 signaling pathway activation to suppress lung inflammation in mice infected with influenza A virus.* Biomed Rep 2014;2:437-441.

[293] Xu G, Dou J, Zhang L, et al. *Inhibitory effects of baicalein on the influenza virus in vivo is determined by baicalin in the serum*. Biol Pharm Bull 2010;33:238-43.

[294] Nagai T, Suzuki Y, Tomimori T, et al. *Antiviral activity of plant flavonoid, 5,7,4'-trihydroxy-8-methoxyflavone, from the roots of Scutellaria baicalensis against influenza A (H3N2) and B viruses*. Biol Pharm Bull 1995;18:295-9.

[295] Nagai T, Miyaichi Y, Tomimori T, et al. *In vivo anti-influenza virus activity of plant flavonoids possessing inhibitory activity for influenza virus sialidase*. Antiviral Res 1992;19:207-17.

[296] Hale BG, Randall RE, Ortìn J, et al. *The multifunctional NS1 protein of influenza A viruses.* J Gen Virol 2008;89:2359-76.

[297] Zhirnov OP, Konakova TE, Wolff T, et al. *NS1 protein of influenza A virus down-regulates apoptosis.* J Virol 2002;76:1617-25.

[298] Kong JQ, Shen JH, Huang Y, et al. *Development of a yeast two-hybrid screen for selection of A/H1N1 influenza NS1 non-structural protein and human CPSF30 protein interaction inhibitors*. Yao Xue Xue Bao 2010;45:388-94.

[299] Twu KY, Noah DL, Rao P, et al. *The CPSF30 binding site on the NS1A protein of influenza A virus is a potential antiviral target.* J Virol 2006;80:3957-65.

[300] Jablonski JJ, Basu D, Engel DA, et al. *Design, synthesis, and evaluation of novel small molecule inhibitors of the influenza virus protein NS1.* Bioorg Med Chem 2012;20:487-97.

[301] Mata MA, Satterly N, Versteeg GA, et al. *Chemical inhibition of RNA viruses reveals REDD1 as a host defense factor*. Nat Chem Biol 2011;7:712-9.

[302] Mahy BW, Cox NJ, Armstrong SJ, et al. *Multiplication of influenza virus in the presence of cordycepin, an inhibitor of cellular RNA synthesis*. Nat New Biol 1973;243:172-4.

[303] Kurokawa M, Koyama AH, Yasuoka S, et al. *Influenza virus overcomes apoptosis by rapid multiplication.* Int J Mol Med 1999;3:527-30.

[304] Zhirnov OP, Klenk HD. *Control of apoptosis in influenza virus-infected cells by up-regulation of Akt and p53 signaling.* Apoptosis 2007;12:1419-32.

[305] Palese P, Shaw ML. *Orthomyxoviridae: the viruses and their replication.* In: Knipe DM, Holey PM, editors. *Fields Virology.* 5th Edition. Vol. 2. Philadelphia: Lippincott Williams & Wilkins 2007, pp. 1647-1689.

[306] Wurzer WJ, Planz O, Ehrhardt C, et al. *Caspase 3 activation is essential for efficient influenza virus propagation.* EMBO J 2003;22:2717-28.

[307] Hinshaw VS, Olsen CW, Dybdahl-Sissoko N, et al. *Apoptosis: a mechanism of cell killing by influenza A and B viruses*. J Virol 1994;68:3667-73.

[308] Jaworska J, Coulombe F, Downey J, et al. *NLRX1 prevents mitochondrial induced apoptosis and enhances macrophage antiviral immunity by interacting with influenza virus PB1-F2 protein*. Proc Natl Acad Sci USA 2014;111:E2110-9.

[309] Furman D, Jojic V, Kidd B, et al. *Apoptosis and other immune biomarkers predict influenza vaccine responsiveness*. Mol Syst Biol 2014;10:750.

[310] Feldman T, Kabaleeswaran V, Jang SB, et al. *A class of allosteric caspase inhibitors identified by high-throughput screening.* Mol Cell 2012;47:585-95.

[311] Dai J, Wang G, Li W, et al. *High-throughput screening for anti-influenza A virus drugs and study of the mechanism of procyanidin on influenza A virus-induced autophagy*. J Biomol Screen 2012;17:605-17.

[312] Muniruzzaman S, Pan YT, Zeng Y, et al. *Inhibition of glycoprotein processing by L-fructose and L-xylulose*. Glycobiology 1996;6:795-803.

[313] Hussain S, Miller JL, Harvey DJ, et al. *Strain-specific antiviral activity of iminosugars against human influenza A viruses.* J Antimicrob Chemother 2014, in press.

[314] Oguin TH 3rd, Sharma S, Stuart AD, et al. *Phospholipase D facilitates efficient entry of influenza virus, allowing escape from innate immune inhibition*. J Biol Chem 2014;289:25405-17.

[315] Husain M, Cheung CY. *Histone deacetylase 6 inhibits influenza A virus release by downregulating the trafficking of viral components to the plasma membrane via its substrate, acetylated microtubules*. J Virol 2014;88:11229-39.

[316] Hamamoto I, Harazaki K, Inase N, et al. *Cyclosporin A inhibits the propagation of influenza virus by interfering with a late event in the virus life cycle*. Jpn J Infect Dis 2013;66:276-83.

[317] Hsieh CF, Yen HR, Liu CH, et al. *Ching-fang-pai-tu-san inhibits the release of influenza virus.* J Ethnopharmacol 2012;144:533-44.

[318] Buffinton GD, Christen S, Peterhans E, et al. *Oxidative stress in lungs of mice infected with influenza A virus*. Free Radic Res Commun 1992;16:99-110.

[319] Drago L, Nicola L, Ossola F, et al. *In vitro antiviral activity of resveratrol against respiratory viruses*. J Chemother 2008;20:393-4.

[320] Palamara AT, Nencioni L, Aquilano K, et al. *Inhibition of influenza A virus replication by resveratrol.* J Infect Dis 2005;191:1719-29.

[321] Li C, Fang JS, Lian WW, et al. *In vitro antiviral effects and 3D QSAR Study of resveratrol derivatives as potent inhibitors of influenza H1N1 neuraminidase*. Chem Biol Drug Des 2015;85:427-38.

[322] Furuya A, Uozaki M, Yamasaki H, et al. *Antiviral effects of ascorbic and dehydroascorbic acids in vitro*. Int J Mol Med 2008;22:541-5.

[323] Uozaki M, Ikeda K, Tsujimoto K, et al. *Antiviral effects of dehydroascorbic acid*. Exp Ther Med 2010;1:983-986.

[324] Khare D, Godbole NM, Pawar SD, et al. *Calcitriol [1, 25[OH]2 D3] pre- and post-treatment suppresses inflammatory response to influenza A (H1N1) infection in human lung A549 epithelial cells*. Eur J Nutr 2013;52:1405-15.

[325] Goldstein MR, Mascitelli L, Pezzetta F. *Pandemic influenza A (H1N1): mandatory vitamin D supplementation?* Med Hypotheses 2010;74:756.

[326] Fedson DS. *Pandemic influenza: a potential role for statins in treatment and prophylaxis.* Clin. Infect. Dis 2006;43:199-205.

[327] Fedson DS. *Treating influenza with statins and other immunomodulatory agents*. Antiviral Res 2013;99:417-35.

[328] Mehrbod P, Hair-Bejo M, Tengku Ibrahim TA, et al. *Simvastatin modulates cellular components in influenza A virus-infected cells*. Int J Mol Med 2014;34:61-73.

[329] Glück B, Schmidtke M, Walther M, et al. *Simvastatin treatment showed no prophylactic effect in influenza virus-infected mice*. J Med Virol 2013;85:1978-82.

[330] Magulick JP, Frei CR, Ali SK, et al. *The effect of statin therapy on the incidence of infections: a retrospective cohort analysis*. Am J Med Sci 2014;347:211-6.

[331] Cho WK, Weeratunga P, Lee BH, et al. *Epimedium koreanum Nakai displays broad spectrum of antiviral activity in vitro and in vivo by inducing cellular antiviral state*. Viruses 2015;7:352-77.

[332] Leibbrandt A, Meier C, König-Schuster M, et al. *Iota-carrageenan is a potent inhibitor of influenza A virus infection*. PLoS One 2010;5:e14320.

[333] Koenighofer M, Lion T, Bodenteich A, et al. *Carrageenan nasal spray in virus confirmed common cold: individual patient data analysis of two randomized controlled trials*. Multidiscip Respir Med 2014;9:57.

[334] Romantsov MG, Golofeevskiĭ SV. *Cycloferon efficacy in the treatment of acute respiratory tract viral infection and influenza during the morbidity outbreak in 2009-201*. Antibiot Khimioter 2010;55:30-5.

[335] Romantsov MG, Ershov FI, Kovalenko AL, et al. *The therapeutic efficacy of cycloferon and the pharmacological activity of interferon inducers*. Ter Arkh 2014;86:83-8.

[336] Sukhinin VP, Zarubaev VV, Platonov VG, et al. *Protective effect of cycloferon in experimental influenza*. Vopr Virusol 2000;45:26-30.

[337] Tazulakhova EB, Parshina OV, Guseva TS, et al. *Russian experience in screening, analysis, and clinical application of novel interferon inducers*. J Interferon Cytokine Res 2001;21:65-73.

[338] Ye S, Lowther S, Stambas J. *Inhibition of Reactive Oxygen Species Production Ameliorates Inflammation Induced by Influenza A Viruses via Upregulation of SOCS1 and SOCS3*. J Virol 2015;89:2672-83.

[339] Selemidis S, Seow HJ, Broughton BR, et al. *Nox1 oxidase suppresses influenza a virus-induced lung inflammation and oxidative stress*. PLoS One 2013;8:e60792.

[340] Vlahos R, Selemidis S. *NADPH oxidases as novel pharmacologic targets against influenza A virus infection*. Mol Pharmacol 2014;86:747-59.

[341] Sharma G, Sharma DC, Fen LH, et al. *Reduction of influenza virus-induced lung inflammation and mortality in animals treated with a phosophodisestrase-4 inhibitor and a selective serotonin reuptake inhibitor.* Emerging Microbes & Infections 2013;2:e54.

[342] Marsolais D, Hahm B, Walsh KB, et al. *A critical role for the sphingosine analog AAL-R in dampening the cytokine response during influenza virus infection.* Proc Natl Acad Sci USA 2009;106:1560-5.

[343] Sugamata R, Sugawara A, Nagao T, et al. *Leucomycin A3, a 16-membered macrolide antibiotic, inhibits influenza A vi-*

rus infection and disease progression. J Antibiot (Tokyo) 2014;67:213-22.

[344] BG-777. Accessible at http://www.drugbank.ca/drugs/DB05839.

[345] Mbawuike I, Zang Y, Couch RB. *Humoral and cell-mediated immune responses of humans to inactivated influenza vaccine with or without QS21 adjuvant*. Vaccine 2007;25:3263-9.

[346] Panatto D, Amicizia D, Lai PL, et al. *Utility of thymosin alpha-1 (Zadaxin) as a co-adjuvant in influenza vaccines: a review*. J Prev Med Hyg 2011;52:111-5.

[347] Carraro G, Naso A, Montomoli E, et al. *Thymosin-alpha 1 (Zadaxin) enhances the immunogenicity of an adjuvated pandemic H1N1v influenza vaccine (Focetria) in hemodialyzed patients: a pilot study*. Vaccine 2012;30:1170-80.

[348] Gravenstein S, Duthie EH, Miller BA, et al. *Augmentation of influenza antibody response in elderly men by thymosin alpha one. A double-blind placebo-controlled clinical study*. J Am Geriatr Soc 1989;37:1-8.

[349] Chioato A, Noseda E, Felix SD, et al. *Influenza and meningococcal vaccinations are effective in healthy subjects treated with the interleukin-1 beta-blocking antibody canakinumab: results of an open-label, parallel group, randomized, single-center study*. Clin Vaccine Immunol 2010;17:1952-7.

[350] Jefferson T, Jones MA, Doshi P, et al. *Neuraminidase inhibitors for preventing and treating influenza in healthy adults and children.* Cochrane Database Syst Rev 2014;4:CD008965.

[351] CDC. *Influenza antiviral medications: summary for clinicians (current for the 2013-14 influenza season).* Document available at: http://www.cdc.gov/flu/professionals/antivirals/summary-clinicians.htm. Accessed on 24th July 2014.

[352] WHO. *Global Alert and Response (GAR) antiviral drugs for pandemic (H1N1) 2009: definitions and use.* Document available at: http://www.who.int/csr/disease/swineflu/frequently_asked_questions/antivirals/definitions_use/en/. Accessed on 24th July 2014).

[353] Budd A, Alleva L, Alsharifi M, et al. *Increased survival after gemfibrozil treatment of severe mouse influenza.* Antimicrob Agents Chemother 2007;51:2965-8.

[354] Lin KL, Sweeney S, Kang BD, et al. *CCR2-antagonist prophylaxis reduces pulmonary immune pathology and markedly improves survival during influenza infection.* J Immunol 2011;186:508-15.

[355] Zheng BJ, Chan KW, Lin YP, et al. *Delayed antiviral plus immunomodulator treatment still reduces mortality in mice infected by high inoculum of influenza A/H5N1 virus.* Proc Natl Acad Sci USA 2008;105:8091-6.

[356] Ghezzi P, Ungheri D. *Synergistic combination of N-acetylcysteine and ribavirin to protect from lethal influenza viral infection in a mouse model.* Int J Immunopathol Pharmacol 2004;17:99-102.

[357] Ottolini M, Blanco J, Porter D, et al. *Combination anti-inflammatory and antiviral therapy of influenza in a cotton rat model.* Pediatr Pulmonol 2003;36:290-4.

[358] Khanna M, Saxena L, Rajput R, et al. *Gene silencing: a therapeutic approach to combat influenza virus infections*. Future Microbiol 2015;10:131-40.

[359] Wichadakul D, Mhuantong W, Jongkaewwattana A, et al. *A computational tool for the design of live attenuated virus vaccine based on microRNA-mediated gene silencing*. BMC Genomics 2012;13(Suppl 7):S15.

[360] Buggele WA, Johnson KE, Horvath CM. *Influenza A virus infection of human respiratory cells induces primary microRNA expression*. J Biol Chem 2012;287:31027-40.

[361] Langlois RA, Albrecht RA, Kimble B, et al. *MicroRNA-based strategy to mitigate the risk of gain-of-function influenza studies*. Nat Biotechnol 2013;31:844-7.

[362] Zhang H, Li Z, Li Y, et al. *A computational method for predicting regulation of human microRNAs on the influenza virus genome*. BMC Syst Biol 2013;7(Suppl 2):S3.

[363] Li Y, Chan EY, Li J, et al. *MicroRNA expression and virulence in pandemic influenza virus-infected mice*. J Virol 2010;84:3023-32.

[364] Betáková T, Svančarová P. *Role and application of RNA interference in replication of influenza viruses*. Acta Virol 2013;57:97-104.

[365] Seth S, Templin MV, Severson G, et al. *A potential therapeutic for pandemic influenza using RNA interference*. Methods Mol Biol 2010;623:397-422.

[366] Ge Q, Filip L, Bai A, et al. *Inhibition of influenza virus production in virus-infected mice by RNA interference.* Proc Natl Acad Sci USA 2004;101:8676-81.

[367] Winterling C, Koch M, Koeppel M, et al. *Evidence for a crucial role of a host non-coding RNA in influenza A virus replication*. RNA Biol 2014;11:66-75.

[368] Ouyang J, Zhu X, Chen Y, et al. *NRAV, a long noncoding RNA, modulates antiviral responses through suppression of interferon-stimulated gene transcription*. Cell Host Microbe 2014;16:616-26.

[369] Xiang DX, Chen Q, Pang L, et al. *Inhibitory effects of silver nanoparticles on H1N1 influenza A virus in vitro*. J Virol Methods 2011;178:137-42.

[370] Xiang D, Zheng Y, Duan W, et al. *Inhibition of A/Human/Hubei/3/2005 (H3N2) influenza virus infection by silver nanoparticles in vitro and in vivo*. Int J Nanomedicine 2013;8:4103-13.

[371] McKenzie Z, Kendall M, Mackay RM, et al. *Nanoparticles modulate surfactant protein A and D mediated protection against influenza A infection in vitro*. Philos Trans R Soc Lond B Biol Sci 2015;370:20140049.

[372] Torrecilla J, Rodríguez-Gascón A, Solinís MÁ, et al. *Lipid nanoparticles as carriers for RNAi against viral infections: current status and future perspectives*. Biomed Res Int 2014;2014:161794.

[373] Okamoto S, Yoshii H, Akagi T, et al. *Influenza hemagglutinin vaccine with poly(gamma-glutamic acid) nanoparticles enhances the protection against influenza virus infection through both humoral and cell-mediated immunity*. Vaccine 2007;25:8270-8.

[374] Shoji M, Takahashi E, Hatakeyama D, et al. *Anti-influenza activity of c60 fullerene derivatives*. PLoS One 2013;8:e66337.

[375] Amidi M, Romeijn SG, Verhoef JC, et al. *N-trimethyl chitosan (TMC) nanoparticles loaded with influenza subunit antigen for intranasal vaccination: biological properties and immunogenicity in a mouse model*. Vaccine 2007;25:144-53.

[376] Sawaengsak C, Mori Y, Yamanishi K, et al. *Chitosan nanoparticle encapsulated hemagglutinin-split influenza virus mucosal vaccine*. AAPS PharmSciTech 2014;15:317-25.

[377] Sawaengsak C, Mori Y, Yamanishi K, et al. *Intranasal chitosan-DNA vaccines that protect across influenza virus subtypes*. Int J Pharm 2014;473:113-25.

[378] Sanpui P, Zheng X, Loeb JC, et al. *Single-walled carbon nanotubes increase pandemic influenza A H1N1 virus infectivity of lung epithelial cells*. Part Fibre Toxicol 2014;11:66.

[379] Govorkova EA, McCullers JA. *Therapeutics against influenza*. Curr Top Microbiol Immunol 2013;370:273-300.

[380] Govorkova EA, Webster RG. *Combination chemotherapy for influenza*. Viruses 2010;2:1510-29.

[381] Prabakaran M, Prabhu N, He F, et al. *Combination therapy using chimeric monoclonal antibodies protects mice from lethal H5N1 infection and prevents formation of escape mutants*. PLoS One 2009;4:e5672.

[382] Bastos LF, Coelho MM. *Drug repositioning: playing dirty to kill pain*. CNS Drugs. 2014;28:45-61.

[383] Wilkinson GF, Pritchard K. *In vitro screening for drug repositioning*. J Biomol Screen 2015;20:167-79.

[384] Heldt FS, Frensing T, Pflugmacher A, et al. *Multiscale modeling of influenza A virus infection supports the development of direct-acting antivirals*. PLoS Comput Biol 2013;9:e1003372.

[385] Smith SB, Dampier W, Tozeren A, et al. *Identification of common biological pathways and drug targets across multiple respiratory viruses based on human host gene expression analysis*. PLoS One 2012;7:e33174.

[386] Law GL, Tisoncik-Go J, Korth MJ, et al. *Drug repurposing: a better approach for infectious disease drug discovery?* Curr Opin Immunol 2013;25:588-92.

[387] Sharma D, Priyadarshini P, Vrati S. *Unraveling the web of viroinformatics: computational tools and databases in virus research*. J Virol 2015;89:1489-501.

[388] Bao S, Zhou X, Zhang L, et al. *Prioritizing genes responsible for host resistance to influenza using network approaches*. BMC Genomics 2013;14:816.

[389] Li Z, Zhou H, Lu Y, et al. *A critical role for immune system response in mediating anti-influenza drug synergies assessed by mechanistic modeling*. CPT Pharmacometrics Syst Pharmacol 2014;3:e135.

[390] Frank D. *One world, one health, one medicine*. Can Vet J 2008;49:1063-5.

[391] Travis DA, Sriramarao P, Cardona C, et al. *One medicine one science: a framework for exploring challenges at the intersection of animals, humans, and the environment*. Ann N Y Acad Sci 2014;1334:26-44.

Abbreviations

AAT: alpha-1-antitrypsin; ALI: acute lung injury; ARDS: acute respiratory distress syndrome; Asp: aspartic acid; BINASE: Bacillus intermedius Ribonuclease; CAM: Clarihtromycin; cAMP: cyclic adenosin monophosphate; *CAS: Chemical Abstract Service; CBP: CREB binding protein; CCL: CC chemokine ligand; CCL2: CCL type2; CCL5: CCL type 5; CFPTS: Ching-fang-pai-tu-san; cGMP: cyclic guanosine monophosphate*; *CHX: cycloheximide; CI: confidence interval; cIAPs: cellular inhibitors of apoptosis; CL: collectin; CL-43: CL type 43; CL-46: CL type 46; CME: Clathrin-Mediated Endocytosis; COX: cyclooxigenase; COX-2: COX type 2; CPE: cytopathic effect; CRM: chromosomal maintenance; CRM1: CRM type 1; CTs: combination therapies; CVN: Cyanovirin-N; CXCL: chemokine (C-X-C motif) ligand; CXCL10: CXCL type 10; CypA: cyclophilin A; CypB: cyclophilin B; DC: dendritic cell; DNA: deoxyribonucleic acid; DPPC: dipalmitoylphosphatidylcholine; DS: dextran sulphate; EB-peptide: entry block peptide; ECC: early combination chemotherapy; ERK: extracellular signal-regulated kinase; EV: enterovirus; EV71: EV type 71; FGF: fibroblast growth factor; FGF4: FGF type 4; FP: FluPep; FP1: FP type 1, also known as Tkip; GA: glycyrrhizic acid; GR: glycyrrhizin; GTP: guanosine-5′-triphosphate; GTPase: GTP hydrolase; HA: hemagglutinin; HAI-2: Hepatocyte growth factor activator inhibitor 2; HAIs: HA inhibitors; HBV: hepatitis B virus; HCV: hepatitis C virus; HGF: hepatocyte growth factor; HIV: Human Immunodeficiency Virus; HMBL: High mannose-binding lectin; HMG: 3-hydroxy-3-methylglutaryl-coenzyme A; HMGB: high-mobility-group; HMGB1: HMGB type 1; HMPV: Human Metapneumovirus; HPV: Human Papillomavirus; HRV: Human Rhinovirus; HSV: Herpes Simplex Virus; HSV-1: HSV type 1; IAV: influenza A virus; IBV: influenza B virus; IFN: interferon; IFN-α: alpha IFN; IFN-β: beta IFN; IKK: IκB kinase; IL: interleukin; IL6: IL type 6; IL8: IL type 8; IL10: IL type 10; ILI: influenza-like illness; IL1RA: IL type 1 receptor antagonist; IMPDH: Inosine 5'-monoposphate dehydrogenase*; *IRF: interferon-regulatory factor; IRF3: IRF type 3; ISG: interferon-stimulated gene; ISG15: ISG type 15; JNK: c-Jun N-termninal kinase; LMA3: Leucomycin A3; LMB: Leptomycin B; lncRNA: long non-coding RNA; M protein: matrix protein; M1: Matrix type 1 protein; M2 protein: Matrix type 2 protein; MAC: Melaleuca alternifolia concentrate; MAPK: mitogen-activated protein kinase; MBL: mannose-binding lectin; MBP: mannose-binding protein; MD: molecular dynamics; MDCK: Madin Darby Canine Kidney cell line; MIP1-beta: macrophage inflammatory protein type 1 beta; miRNA: microRNA; MPO: myeloperoxidase; mRNA: messenger RNA; MTOC: microtubule organizing center; mTOR: mammalian target of rapamycin; MTP-PE: muramyl tripeptide; MXSGT: Ma-xing-shi-gan-tang; NA: neuraminidase; NAIs: NA inhibitors; NADPH: nicotinamide adenine dinucleotide phosphate reduced; NB-DNJ: N-butyl-deoxynojirimycin*; *NCZ: nucleozin; NDV: Newcastle Disease Virus; NEP: nuclear export protein; NES: nuclear-export signal; Neu5Ac-S-CH2-Lev: α-2-S-[m-(N-levulinyl)aminobenzyl]-5-N-acetylneuraminic acid; NFKB: nuclear factor kappa B; NOX1: NADPH oxidase type 1; NOX2: NADPH oxidase type 2; NLRX1: Nucleotide-binding oligomerization domain-like receptor type 1; NRAV: negative regulator of antiviral response; Nrf2: Nuclear factor (erythroid-derived 2)-like 2, also known as NFE2L2; NS: Non-Structural protein; NS1: NS type 1; NS1A: NS type 1A; NS1ABP: NS1A binding protein; NSAIDs: non-steroidal anti-inflammatory drugs; OFCs: omeprazole family compounds; OR_{adj}: adjusted odds ratio; OTC: over the counter; PA: polymerase acidic protein; PB: polymerase basic protein; PB1: PB type 1; PB1-F2: PB1 frame 2; PB2: PB type 2; PCR: polymerase chain reaction; PDB: Protein Data Bank; PDTC: pyrrolidine dithiocarbamate; Pet: petasiphenol; PGE2: prostaglandin E2; Pgp: P-glycoprotein; PI3K: phosphatidylinositol 3-kinase; PLD: phospholipase D; PR-3: proteinase 3; qPCR: quantitative PCR; RE: recycling endosome; REDD1: regulated in development and DNA damage responses-1; RIB: ribavirin; RNA: ribonucleic acid; RNAi: RNA interference; RNP: ribonucleoprotein; ROS: reactive oxygen species; RSV: Respiratory Syncytial Virus; RT-PCR; SA: sialic acid; SARS: Severe Acute Respiratory Syndrome; SINE: selective inhibitor of nuclear export; siRNA: short interfering RNA; SMC: sequential multidrug chemotherapy; SOCS: Suppressor of cytokine signaling; SOCS1: SOCS type 1; SP-A: surfactant protein A; SP-D: surfactant protein D; SREBP-1: sterol regulatory element-binding protein 1; SNMC: Stronger Neo-Minophafen C; SWCNTs: single-walled carbon nano-*

tubes; TBHQ: Tert-butyl-hydroquinone; TFs: theaflavins; Th1: T helper 1 cell; THC: tetrahydrocurcumin; TLR: Toll-like receptor; TLR2: TLR type 2; TLR7: TLR type 7; TMC: N-trimethyl chitosan; TNF: tumor necrosis factor; TNF-α: TNF type α; Treg: T regulatory cell; TTO: tea-tree oil; TZV: triazavirine; US: United States of America; USP: ubiquitin-specific peptidase; USP18: USP type 18; Val: valine; vATPase: VEGF: vascular endothelial growth factor; vRNA: viral RNA; vRNP: viral RNP; VZV: Varicella Zoster Virus; XPO-1: exportin-1.

■ Received on November 3, 2014. Accepted on December 5, 2014.

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REVIEW

Evaluation of efficacy and effectiveness of live attenuated zoster vaccine

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Key words

Herpes Zoster • Prevention • Vaccine

Summary

Herpes zoster (HZ) is a viral disease characterized by a dermatologic and neurologic involvement caused by the reactivation of the latent varicella zoster virus (VZV) acquired during primary infection (varicella). HZ incidence increases with age and is related to waning specific cell-mediated immunity (CMI). The most frequent complication of HZ is post-herpetic neuralgia (PHN) characterized by chronic pain lasting at least 30 days, with impact on patients' quality of life. Available treatments are quite unsatisfactory in reducing pain and length of the disease. The evaluation of the epidemiology, the debilitating complications (PHN), the suboptimal available treatments and the costs related to the diagnosis and clinical/therapeutic management of HZ patients have been the rationale for the search of an adequate preventive measure against this disease. The target of this intervention is to reduce the frequency and severity of HZ and related complications by stimulating CMI. Prevention has recently become possible with the live attenuated vaccine Oka/Merck, with an antigen content at least 10-fold higher than the antigen content of pediatric varicella vaccines. Clinical studies show a good level of efficacy and effectiveness, particularly against the burden of illness and PHN in all age classes. Accordingly to the summary of the characteristics of the product the zoster vaccine is indicated for the prevention of HZ and PHN in individuals 50 years of age or older and is effective and safe in subjects with a positive history of HZ.

Introduction

Herpes zoster (HZ) is an acute infectious disease sustained by the reactivation of varicella zoster virus (VZV); this latter is an ubiquitous pathogen that, after primary infection (varicella), becomes latent in sensory ganglia [1].

VZV is an alpha-herpes virus characterized by a fast replication cycle, a rapid inter-cellular spreading and the ability to establish latency, mainly in dorsal root ganglia [2, 3]. The virus contains a double-stranded DNA genome, has an icosaedric capsid (with 162 capsomers), a tegument and an envelope [4]. Envelope glycoproteins allow the virus to adhere to human cells, mainly in the respiratory tract; then the virus, before becoming latent, infects peripheral blood mononuclear cells (PBMC) and epidermal cells, causing the typical rash [5, 6].

VZV reservoir is exclusively human; the virus is air-transmitted and is quite labile outside host cells [7]. It could be also transmitted by skin lesions of subjects affected by varicella or zoster. In about a quarter of infected individuals, mainly in adulthood, latent VZV reactivates causing HZ. About 10-30% of people infected by VZV will develop an episode of HZ during their lifetime; HZ incidence is particularly high in elderly and in immunocompromised subjects [8]. Reactivation is strictly related to a decrease in the cell-mediated immunity (CMI); this latter is inversely related to age. During reactivation, the virus replicates, causes neuronal damage and inflammation, and a vesicular rash with dermatomal distribution. The rash typically involves the dermatomal distribution of one single sensory nerve and, in immunocompetent subjects, lasts for 2-3 weeks with moderate to severe pain. A rate of HZ cases are associated with pain lasting some weeks to months, and even years. This medical case is called post-herpetic neuralgia (PHN), and is usually defined as a pain lasting more than 90 days after the healing of the skin rash. PHN has a high impact on patients' quality of life [9, 10].

Immunological aspects

VZV primary infection elicits innate immune response, characterized by IFN-α, IFN-γ and IL-6 release, as well as humoral and cell-mediated immunity [11]. CMI plays an important role in limiting viral replication and avoiding severe disease [12]; humoral immune response is probably less relevant, as suggested by un-complicated varicella cases in agammaglobulinemic patients [13, 14]. However, VZV primary infection elicits a long lasting antibody-mediated and cell-mediated immune response. There is an ample consensus on the crucial role played by CMI in preventing VZV reactivation. Immunosenescence or immunosuppression that imply a decrease of VZV-specific CMI are strictly related to the occurrence of HZ cases [15]. An international debate is ongoing on the role of exogenous and endogenous boosting of

VZV-specific CMI; it has been suggested that exposure to varicella, causing an increase of specific CMI, could decrease the risk of VZV reactivation [16, 17]. This hypothesis is supported by studies demonstrating a decreased risk of HZ in subjects with household or occupational exposition to varicella [18]. Other authors believe that endogenous booster plays a role in preventing HZ incidence, as an increase of HZ cases has not been demonstrated in subjects surely not exposed to exogenous boosting [19]. Anyway, a HZ case elicits an increase of specific CMI, and this is probably the reason why relapse of HZ is quite rare [20].

Clinical aspects

The clinical course of HZ consists of 4 phases: prodromic, acute, sub-acute and chronic [21]. The prodromic phase usually (70-80% of cases) starts 1-5 days before the onset of rash [22]; its symptoms are aspecific and include pruritus, burning sensations, fever, malaise and headache [23]. The acute phase is characterized by dermatomal skin rash with vesicles; the duration of the rash is related to the age of the subject (it increases with aging) and to the dermatomes involved. Vesicles evolve in crusts in few days and then lesions heal. VZV can be transmitted during the vesicular phase; contagiousness halts during the crusting phase [24]. Acute pain during rash is related to the neurotropism of the virus [25]. Pain in the acute phase is described as pulsating, shooting, burning or piercing; it can be continuous or intermittent, as well as it can be associated with pruritus, tingling and/or numbness. Many patients show allodynia, with pain due to a stimulus which does not normally provoke pain (e.g. contact of dresses on the skin) [26]; this latter may have an impact on quality of life and may be prognostic of incoming PHN [27]. Sub-acute phase usually comes before chronic disease (30-90 days after rash) [27]. Chronic phase is characterized by PHN, with a pain lasting up to months and even years [26]. Most patients classify this pain as moderate-severe, with a pain score ≥ 4 on a scale ranging between 0 and 10; they are usually treated with analgesics [28]. HZ can be severe, particularly in immunocompromised subjects; disseminated HZ, HZ ophtalmicus, encephalitis, facial palsy, Bell's palsy and Ramsay Hunt syndrome are the most common complications of HZ [29]. HZ ophtalmicus implies an involvement of the first branch of the trigeminal nerve; it occurs in the 1-10% of all HZ cases [30] and it may be related (at least in 1/3 of cases) to the Hutchison's sign (nasociliary skin lesion). This latter is prognostic of ocular inflammation and corneal sensory denervation [31]. A delayed contralateral hemiparesis following HZ ophtalmicus is quite rare, but it is related to a high risk of neurological sequelae and to a case fatality ratio equal to 20-25% [32, 33]. Recently, two researches, performed in UK, have demonstrated a higher risk of stroke, transient ischemic attack and myocardial infarction in subjects youngers than 40 years and affected by HZ; this risk is higher in subjects with HZ ophtalmicus [34-36].

Early diagnosis and timely therapy are essential in order to reduce frequency and severity of complications and to improve the outcome of infection. However, the therapeutic approach to HZ and its complications (PHN in particular) is quite difficult. Therapy should start as soon as possible (within max 72 hours from disease onset), in order to avoid a loss of efficacy [37]. Most of the therapeutic options are related to undesirable effects and allow to achieve only sub-optimal results. Therefore, PHN is difficult to prevent and to treat [38-41].

Epidemiology

Industrialized countries report a quite similar age-related incidence; 20-35% of subjects living in these countries has a HZ case during its lifetime [29]. Complications occur in 13-40% of cases [42]; 8-27% of subjects with HZ suffer of PHN [43]. HZ incidence increases with age, being four-fold higher in subjects ≥ 70 years of age than in < 60 year-old subjects [44].
In the USA 0.5-1 million HZ cases are estimated each year, accounting for an incidence equal to 2-3/1,000/year in the general population [45]. Incidence is low in subjects younger than 40 years of age (0.9-1.9/1,000/year); it increases to 2.5, 3.8, 6.1, 8.5 and 9.4 per 1,000 per year in subjects belonging to the age classes 40-49, 50-59, 60-69, 70-79 and ≥ 80 years, respectively [46, 47]. The estimates in Europe suggest that 1.7 ± 0.1 million of new cases occur every year; incidence rates increase with aging also in this geographical area (2/1,000 and 10/1,000 in < 40 and ≥ 80 year-old subjects, respectively) [48]. The female/male ratio is equal to 1.4, and incidence in females seems to increase with aging [49]; this pattern of incidence could be related to the greater attitude of females to look for medical advice [50].
In Italy, 157,000 new cases (annual incidence: 6.3/1,000 person-years) are estimated to occur each year; most cases (76.2%) are reported by ≥ 50 year-old subjects [51]. Twenty point six (20.6%) and 9.2% of HZ cases have PHN at 3 and 6 months, respectively [52]. In the period 1999-2005, 35,328 hospitalizations due to HZ have been reported (mean: 4,503/year); 62% of these hospitalizations involved subjects older than 65 years [53].
HZ and PHN have a negative impact on quality of life and on social life of affected people, reducing physical ability, implying malaise, fatigue, anorexia, weight loss, insomnia [54]. Besides, symptoms (skin lesions and pain) together with functional and social impairment related to HZ could have, particularly in case of chronic disease, an impact on patients' psychology [55, 56].

New preventive option: zoster vaccine

The burden in terms of morbidity and of short- and long-term complications, the sub-optimal therapeutic options and the high costs related to HZ has allowed the search of a new preventive approach by vaccination. Since many years it has been demonstrated that live attenuated VZV vaccines can boost VZV-specific CMI. In particular, live attenuated varicella vaccines, with a high anti-

gen content, elicit a significant increase of VZV-specific CMI in immunocompetent elderly subjects [57-61].
The zoster vaccine, developed by Merck and nowadays commercially available, has an antigen content higher than at least 19,400 PFU (Plaque-Forming Units), i.e. at least 10 times higher than the antigen content in pediatric varicella vaccine [62]. During the last years several studies on efficacy, effectiveness and safety of this vaccine have been performed.
Noteworthy, a phase III study is ongoing to evaluate the efficacy, safety and immunogenicity of GSK Biologicals' candidate Herpes Zoster vaccine in adults aged ≥ 50 years (NCT01165177 and NCT01165229).

Zoster vaccine: evaluation of efficacy

The efficacy of the new zoster vaccine has been evaluated in two phase III clinical trials involving more than 38,000 subjects ≥ 60 years of age (SPS: shingles prevention study) and 22,000 subjects 50-59 years of age (ZEST: Zoster efficacy and safety trial), respectively [63, 64].
The SPS has allowed to collect data useful to obtain vaccine licensure in USA and in Europe. The SPS has been a multicenter, double-blinded, placebo controlled, randomized clinical trial, performed in the USA, enrolling immunocompetent subjects ≥ 60 years of age with a positive anamnesis of varicella or residing for at least 30 years in a VZV-endemic area. The exclusion criteria were positive anamnesis of zoster, allergy to any vaccinal component, immunosuppression or any other condition that could interfere with the evaluation of results. Randomized subjects received one dose (0.5 ml) of the zoster vaccine (n = 19,270) or of placebo (n = 19,276). The mean age of both groups was equal to 69 years (46% and 6.5% of subjects were ≥ 70 and ≥ 80 year old, respectively). The follow up period lasted a mean of 3.1 years (range 1 day-4.9 years).
The primary end point of the study was the evaluation of safety and efficacy of the vaccine. In particular, vaccine efficacy was evaluated as the reduction of the burden of illness (BOI). This end point includes incidence, severity and duration of acute and chronic pain related to HZ during a follow-up period of at least 6 months. The secondary end point of the study was vaccine efficacy against the incidence of PHN (pain with a ≥ 3 score on a scale ranging from 0 to 10 and lasting at least 90 days after the onset of rash). Pain and discomfort have been evaluated and measured by a questionnaire filled in by patients after the onset of HZ (Brief Pain Zoster Inventory). A score ≥ 3 has been considered clinically significant, as it is related to a relevant decrease of normal daily activities [65, 66]. Another secondary end point was the efficacy against the incidence of HZ. More than 95% of enrolled subjects have completed the study; a total of 957 HZ cases occurred, 315 among immunized subjects and 642 in subjects receiving placebo. Concerning the primary end point, the efficacy against BOI was equal to 61.1% (95%CI: 51.1-69.1).
During the study, 107 cases of PHN have been registered, 27 in immunized subjects and 80 in the placebo group. The efficacy against PHN has been equal to 66.5% (95%CI: 47.5-79.2); the efficacy against PHN stratified by age has been 65.7% (95%CI: 20.4-86.7) and 66.8% (95%CI: 43.3-81.3) in the age groups 60-69 and ≥ 70 years, respectively. The level of efficacy against PHN increased accordingly to the definition of the duration of the chronic pain (58.9% and 72.9% for PHN defined as pain persisting 30 days and 182 days after rash onset, respectively).
The study has also demonstrated an efficacy against HZ equal to 51.3% (95%CI: 44.2- 57.6); the level of efficacy decreased in older subjects (63.9% and 18% in 60-69 and ≥ 80 year-old subjects).
The level of efficacy against HZ decreased in older subjects, while the efficacy against PHN and BOI was not related to the age group considered. HZ occurring in immunized subjects lasted for a shorter time than cases registered in the placebo group (21 *vs.* 24 days; p = 0.03) [63]. Another efficacy study, called ZEST (Zoster Efficacy and Safety Trial), was performed in North America and in Europe in the period October 2007-January 2010. It was a double-blinded, placebo controlled, randomized clinical trial that involved subjects 50-59 year-old subjects with a positive anamnesis of varicella or living for at least 30 years in a VZV-endemic area [64]. Exclusion criteria were quite similar to the ones adopted in the SPS trial; a total of 22,439 were enrolled to receive a dose of zoster vaccine (n = 11,211) or placebo (n = 11,228). The mean follow-up period was 1.3 years (range 0 days-2 years).
The end point of the trial was to assesses vaccine efficacy, safety and tolerability in immunized group compared to the placebo one. Efficacy against HZ was 69.8% (95%CI: 54.1-80.6); 30 and 99 HZ cases were registered in the immunized and in the placebo group, respectively ($p < 0.001$).
The efficacy of zoster vaccine in the ZEST study in the age group 50-59 years resulted similar to the one observed in the SPS trial in the age group 60-69 years (63.9%), and higher than in subjects ≥ 70 years of age (37.6%). The results obtained in the ZEST study were in line with those obtained during the SPS trial [63, 64]; the higher efficacy against HZ observed in the ZEST study is probably related to a better immune response of younger subjects [64].
The duration of efficacy has been evaluated as well in 2 persistence substudies: short-term persistence substudy (STPS) and long-term persistence substudy (LTPS).
The STPS started in October 2005; in this open-label study zoster vaccine was offered to subjects previously enrolled in the SPS placebo group. The follow-up in this substudy involved zoster vaccine recipients in the SPS as well. A total of 14,270 subjects were enrolled in the STPS substudy: 7,320 subjects were zoster vaccine recipients and 6,950 were placebo recipients in the SPS trial. These latter were offered one dose of zoster vaccine; the mean age was equal to 73.3 years and the follow-up lasted for a mean of 1.2 years (range 1 day-2.2 years). Efficacy in the STPS has been evaluated against the 3 end points already used in the SPS trial: BOI, PHN and HZ incidence. In the STPS the efficacy has been assessed on data basically collected 4-7 years after the

immunization performed in the SPS; 84 and 95 HZ cases occurred in the group of immunized subjects and in the placebo group, respectively.
The estimated efficacy in the STPS has been the following: 50.1% against BOI (95%CI: 14.1-71); 60.1% against PHN (95%CI: -9.8-86.7); 39.6% against HZ (95%CI: 18.2%-55.5).
Taking into account the combined results of SPS and STPS, vaccine zoster showed an efficacy equal to 58.6% (95%CI: 48.6-66.6), 64.9% (95%CI: 47.4-77.0) and 48.7% (95%CI: 42.0-54.7) against BOI, PHN and HZ, respectively. STPS vaccine efficacy for each end point was lower than in the SPS; anyway, a persistence of vaccine efficacy was demonstrated through year 5 after immunization [67].
The long-term persistence substudy (LTPS) evaluated 6,867 subjects that had been immunized during the SPS and the STPS [67, 68]; for this reason a control group was not available. The mean age at enrollment was equal to 74.5 years; the mean follow-up was 3.9 years (range 1 week-4.75 years). In the LTPS efficacy has been evaluated 7-10 years after immunization. The HZ incidence during the LTPS was 10.3/1,000 person-years and the efficacy was: 37% against BOI (95%CI: 27-46), 35% against PHN (95%CI: 9-56) and 21% against HZ (95%CI: 11-30).

Zoster vaccine: evaluation of effectiveness

Clinical trials (SPS, ZEST, STPS, LTPS) have demonstrated the efficacy and the safety of the new zoster vaccine. It is important to demonstrate that similar results are obtained in the "real life"; for this reason post-marketing effectiveness studies are relevant and have been performed.
In the period January 2007-December 2009, Tseng et al. have enrolled 2 groups of subjects included in the Kaiser Permanente Southern California health plan; the first one accounted for 75,761 subjects who received zoster vaccine, the second one accounted for 227,283 unimmunized subjects. The mean duration of the follow up was equal to 1.56 and 1.72 years for vaccinated and unvaccinated cohorts, respectively; during this period, 5,434 HZ cases occurred with an incidence equal to 13/1,000 person-years (95%CI: 12.6-13.3) and 6.4/1,000 person-years (95%CI: 5.9-6.8) in unimmunized and immunized subjects, respectively.
HZ incidence in unimmunized subjects resulted higher in older subjects (≥ 80 *vs.* 60-64 year old subjects, Hazard ratio (HR) 1.45, 95%CI: 1.3-1.63), lower in males (HR 0.75, 95%CI: 0.7-0.79), and in black people (HR 0.69, 95%CI: 0.62-0.76). HZ incidence was higher, even if not statistically significant, in unvaccinated subjects affected by lung (HR 1.34, 95%CI: 0.95-1.13), kidney (HR1.04, 95%CI: 0.95-1.13) and cardiac (HR 1.06, 95%CI: 0.97-1.16) diseases. Immunization was positively related to a decrease of the risk of HZ (HR 0.45, 95%CI: 0.42-0.48), HZ ophtalmicus (HR 0.37, 95%CI: 0.23-0.61), hospitalizations due to HZ (HR 0.35, 95%CI: 0.24-0.51). As a whole, immunization allowed to achieve a 55% reduction of the HZ incidence; this result is consistent with the one obtained during the SPS (51%). However, in this effectiveness study the positive impact of immunization did not change considering different age classes, supporting the recommendation to provide HZ vaccine even to oldest subjects [69].
Zhang et al. have evaluated the effectiveness of zoster vaccine in patients affected by immune-mediated diseases. The study, performed in the period January 2006-December 2009, involved 463,541 insured by Medicare and affected by rheumatoid arthritis (292,169), psoriatic arthritis (11,030), psoriasis (89,565), ankylosing spondylitis (4,026), inflammatory bowel disease (66,751). The inclusion criteria included: age ≥ 60 years, diagnosis of at least one of the previously mentioned diseases, inclusion in the Medicare since at least 6 months. Zoster vaccine was provided to 18,683 subjects (72.3% females, 86.3 white); the mean age of enrolled people was 74 years.
Eleven HZ cases occurred in vaccinated subjects, with an incidence rate of 7.8 cases/1,000 person-years (95%CI: 3.7-16.5). No varicella or HZ cases were registered in patients in treatment with biologics or with anti-TNF during the 42 days following immunization. After controlling for demographic data, type of immune-mediated disease, the accesses to health care, the use of biologic or nonbiologic disease-modifying antireumathic drugs (DMARDs) or oral glucocorticoids, the hazard ratio (HR) of HZ related to immunization resulted equal to 0.61 (95%CI: 0.52-0.71) and the vaccine effectiveness equal to 39%. This study has demonstrated that zoster vaccine is not related to an increased risk of varicella or HZ in patients under biologic treatment [70].
More recently, Langan et al. have studied a cohort of 766,330 subjects older than 65 years, enrolled in the period January 2006-December 2009, and involved in the Medicare programs A (covers inpatients care), B (covers physician services and facility costs) since at least 12 months and registered since at least 6 months in program D (drug benefit). As a whole, 29,758 subjects received zoster vaccine; 4,469 were immunosuppressed at the time of zoster immunization.
As a whole, 154 HZ cases occurred in 28,291 person-years of follow up in vaccinated subjects compared to 12,958 HZ cases in 1,291,829 person-years of follow up in unvaccinated subjects; the HZ incidence rate was equal to 5.4/1,000 person-year (95%CI: 4.6-6.4) and to 10/1,000 person-year (95%CI: 9.8-10.2) in vaccinated and unimmunized subjects, respectively.
Vaccine effectiveness against HZ in vaccinated subjects has been equal to 0.48 (95%CI: 0.39-0.56)
In immunocompromised subjects the vaccine effectiveness has been equal to 0.37 (95%CI: 0.06-0.58) (24 HZ cases in 1,981 immunosuppressed patients). The occurrence of PHN (30 days after HZ onset) has been equal to 16 PHN case in 71,457 immunized subjects and 1,665 PHN cases in 2,563,404 cases in unimmunized subjects; the effectiveness against PHN has been equal to 0.62 (95%CI: 0.37-0.77) and to 0.59 (95%CI: 0,21-0.79) at 30 and 90 days, respectively. Langan et al. have demonstrated a zoster vaccine effectiveness equal to 48%, 62% and 59% against HZ, PHN at 30 days and PHN at 90 days, respectively. The same study has confirmed the

zoster vaccine effectiveness in routine clinical use, even in immunosuppressed individuals [71].
A long-term effectiveness study has been planned in subjects ≥ 50 years of age included in the Kaiser Permanente Northen California health plan. The target is to immunize 15,000 subjects; a preliminary phase started in 2012, is already ongoing and two ad interim analysis are planned at the end of 2016 and 2020; the study will end in 2024 [68, 72].

Zoster vaccine: evaluation of safety

The studies SPS and ZEST has allowed to evaluated safety and tolerability of the new zoster vaccine. In detail, the SPS trial demonstrated an excellent tolerability and safety profile [63]. In this trial each enrolling site closely monitored adverse events in a subset of subjects (safety substudy). As a whole, the incidence of hospitalizations and deaths has been quite similar during the follow-up of both groups of subjects involved in the study. During the 42 days following immunization, a rash (usually mild) at the site of injection has been registered more frequently in immunized subjects than in those receiving placebo. Seven and 24 HZ cases has been registered in immunized and placebo-receiving subjects during the first 42 days after immunization. The Oka/Merck vaccinal strain has not been detected in any sample.
Five severe adverse events (SAEs) have been reported; only 2 have been observed in the immunized group.
The safety substudy pointed out a greater frequency of adverse events (AEs) involving the site of injection in the vaccine group than in the placebo; in immunized subjects the most frequent AEs have been erythema (35.8%), pain or tenderness (34.5%), swelling (26.2%), and pruritus (7.1%).
SAEs occurring during the first 42 days after immunization have been significantly higher in the vaccine group than in the placebo one (1.9% *vs.* 1.3%, p = 0.03). No significant differences in SAEs distribution accordingly to site or type of event has been demonstrated. No hospitalization was related to immunization [63].
The ZEST study confirmed the safety profile of zoster vaccine. The rate of at least one AEs was higher in immunized subjects than in those receiving placebo (73% *vs.* 42%), most of AEs were at the injection site. Few (0.7%) AEs have been reported as grade 3. Systemic AEs were reported in 35% of immunized subjects; 6.7% of these have been related to the vaccine. During the ZEST study the AEs incidence in immunized subjects has resulted higher than the one observed in the SPS study (63.9% *vs.* 48.3%); this fact could be possibly explained with a higher local reactogenicity in younger subjects [73]. The rate of subjects with SAEs during the first 42 days following immunization has been similar in immunized and in placebo group (0.6% *vs.* 0.5%). An anaphylactic reaction has been reported 15 minutes after vaccine administration with no sequelae. The molecular analysis of biological samples (n = 47) belonging to subjects with HZ-like rashes (n = 34) and varicella-like rashes (n = 124) identified wild-type virus in 11 cases; no Oka/Merck strain has been detected [64].
The safety profile of zoster vaccine has also been assessed in a study involving almost 12,000 subjects ≥ 60 years of age (5,983 immunized and 5,997 receiving placebo). During the first 42 days of follow up, a SAE was reported by 1.4% and 1.12% of immunized and placebo-receiving subjects, respectively (relative risk RR 1.26; 95%CI: 0.91-1.73; not statistically significant). During the follow up at 182 days, 5.7% (n = 340) and 5% (n = 300) subjects, immunized and placebo-receiving respectively, reported a SAE; the RR in this analysis was equal to 1.13 (95%CI: 0.98-1.32; not statistically significant). In conclusion, this study has demonstrated that the incidence of SAEs in the period 1-42 days and at 6 months was not statistically different comparing immunized and placebo-receiving subjects [74].
Zoster vaccine resulted well tolerated in a clinical trial involving subjects > 60 years of age on chronic/maintenance corticosteroids (5-20 mg of prednisone or equivalent daily/dose) for at least 2 week before enrollment and for > 6 weeks after immunization [62].
Two studies [75, 76] have shown that zoster vaccine is safe in subjects with a recent history of documented HZ in accordance to recommendations by CDC Advisory Committee on immunization practices already established in 2008 [77].
The good safety and tolerability profile of zoster vaccine has been confirmed in all effectiveness studies performed after licensure and commercial availability of the product. Generally, the most frequent AEs reported have been injection site reactions (redness, swelling and pain) (≥ 1/10) and headache (from ≥ 1/100 to < 1/10). No cases of secondary transmission of vaccinal strain have been reported; no age-related specific safety issues have been demonstrated.
Recently, a HZ case caused by VZV vaccine strain has been documented in an immunocompetent recipient of zoster vaccine [78]. The efficacy, effectiveness and safety profile of zoster vaccine has recently been confirmed in an European Health Technology pilot assessment [79].

Conclusions

The evaluation of the epidemiology, the frequent and debilitating complications (PHN), the sub-optimal available treatments and the costs related to the diagnosis and clinical/therapeutic management of HZ patients have been the rationale for the search of an adequate preventive measure against this important disease. The target of this specific intervention is to reduce the frequency and severity of HZ and related complications by stimulating CMI. High-antigen content vaccines elicit an effective CMI response, also in elderly subjects. Prevention has recently become possible with the live attenuated vaccine Oka/Merck, with an antigen content at least 10-fold higher than the antigen content of pediatric varicella vaccines. Clinical studies show a good level of efficacy and effectiveness, particularly against the burden of illness and PHN in all age classes. Protection seems to be long lasting and vaccine

safety matches registration requirements. Accordingly to the summary of the characteristics of the product the zoster vaccine is indicated for the prevention of HZ and HZ-related PHN of individuals 50 years of age or older and is effective and safe in subjects with a positive history of HZ. The evaluation of all the above mentioned points has already allowed some countries to recommend the use of zoster vaccine (e.g. USA, Canada, Austria, UK, Germany/Saxony, Sweden, Greece, France).

References

[1] Bonanni P, Breuer J, Gershon A, et al. *Varicella vaccination in Europe-taking the practical approach*. BMC Medicine 2009;7:26.

[2] Davison AJ. *Molecular evolution of alphaherpesviruses*. In: Arvin AM, Gershon AA, eds. *Varicella-Zoster Virus: Virology and Clinical Management*. Cambridge: Cambridge University Press, 2000, pp. 25-50.

[3] Mori I, NishiyamaY. *Herpes simplex virus and varicella-zoster virus: why do these human alphaherpesviruses behave so differently from one another?* Rev Med Virol 2005;15:393-406.

[4] Kim HK. *Herpes Zoster Vaccination*. Korean J Pain 2013;26:242-8.

[5] Arvin AM, Moffat JF, Redman R. *Varicella-zoster virus: aspects of pathogenesis and host response to natural infection and varicella vaccine*. Adv Virus Res 1996;46:263-309.

[6] Quinlivan M, Breuer J. *Molecular studies of varicella zoster virus*. Rev Med Virol 2006;16:225-50.

[7] Public Health Agency of Canada. *Varicella-zoster virus. Pathogen safety data sheet- infectious substances*. Available at http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/var-zo-eng.php; (accessed June 2014).

[8] Volpi A, Gross G, Hercogova J, et al. *Current management of herpes zoster. The European view*. Am J Clin Dermatol 2005;6:317-25.

[9] Coplan PM, Schmader K, Nikas A, et al. *Development of a measure of the burden of pain due to herpes zoster and postherpetic neuralgia for prevention trials: adaptation of the brief pain inventory*. J Pain 2004;5:344-56.

[10] Kawai K, Gebremeskei BG, Acosta CJ. *Systematic review of incidence and complications of herpes zoster: towards a global perspective*. BMJ Open 2014;4:e004833.

[11] Arvin AM, Koropchak CM, Williams BR, et al. *Early immune response in healthy and immunocompromised subjects with primary varicella-zoster virus infection*. J Infect Dis 1986;154:422-9.

[12] Gershon AA, Gershon MD, Breuer J, et al. *Advances in the understanding of the pathogenesis and epidemiology of herpes zoster*. J Clin Virol 2010;48 (S1):S2-7.

[13] Ihara T, Kato T, Torigoe S, et al. *Antibody response determined with antibody-dependent cellmediated cytotoxicity (ADCC), neutralizing antibody, and varicella skin test in children with natural varicella and after varicella immunization*. Acta Paediatr Jpn 1991;33:43-9.

[14] Asada H, Nagayama K, Okazaki A, et al. *An inverse correlation of VZV skin-test reaction, but not antibody, with severity of herpes zoster skin symptoms and zoster-associated pain*. J Dermatol Sci 2013;69:243-9.

[15] Levin MJ, Gershon AA, Dworkin RH, et al. *Prevention strategies for herpes zoster and post-herpetic neuralgia*. J Clin Virol 2010;48(Suppl 1):S14-9.

[16] Hope-Simpson RE. *The nature of herpes zoster: a long-term study and a new hypothesis*. Proc R Soc Med 1965;58:9-20.

[17] Ogunjimi B, Van Damme P, Beutels P. *Herpes zoster risk reduction through exposure to chickenpox patients: a systematic multidisciplinary review*. PLoS One 2013;8:e66485.

[18] Salleras M, Dominguez A, Soldevila N, et al. *Contacts with children and young people and adult risk of suffering herpes zoster*. Vaccine 2011;29:7602-5.

[19] Gaillat J, Gajdos V, Launay O, et al. *Does monastic life predispose to the risk of Saint Anthony'sfire (herpes zoster)?* Clin Infect Dis 2011;53:405-10.

[20] Tseng HF, Chi M, Smith N, et al. *Herpes zoster vaccine and the incidence of recurrent herpes zoster in an immunocompetent elderly population*. J Infect Dis 2012 Jul 15;206:190-6.

[21] Volpi A, Gross G, Hercogova J, et al. *Current management of herpes zoster: the European view*. Am J Clin Dermatol 2005;6:317-25.

[22] Gnann JW Jr, Whitley RJ. *Clinical practice: herpes zoster*. N Engl J Med 2002;347:340-6.

[23] Johnson R, McElhaney J, Pedalino B, et al. *Prevention of herpes zoster and its painful and debilitating complications*. Int J Infect Dis 2007;11(Suppl 2):S43-8.

[24] Franco E, Gabutti G, Bonanni P, et al. *Herpes Zoster and its prevention in Italy. Scientific consensus statement*. Ig Sanita Pubbl 2014;70:111-27.

[25] Arvin AM. *Varricella-zoster virus*. Clin Microbiol Rev 1996;9:361-81.

[26] Dworkin RH, Gnann JW Jr, Oaklander AL, et al. *Diagnosis and assessment of pain associated with herpes zoster and postherpetic neuralgia*. J Pain 2008;9(Suppl 1):S37-44.

[27] Opstelten W, Mauritz JW, de Wit NJ, et al. *Herpes zoster and postherpetic neuralgia: incidence and risk indicators using a general practice research database*. Fam Pract 2002;19:471-5.

[28] van Seventer R, Sadosky A, Lucero M, et al. *A cross-sectional survey of health state impairment and treatment patterns in patients with postherpetic neuralgia*. Age Ageing 2006;35:132-7.

[29] Johnson RW, Wasner G, Saddier P, et al. *Herpes zoster and postherpetic neuralgia: optimizing management in the elderly patient*. Drugs Aging 2008;25:991-1006.

[30] Cunningham AL, Breuer J, Dwyer DE, et al. *The prevention and management of herpes zoster*. Med J Aust 2008;188:171-6.

[31] Zaal MJ, Volker-Dieben HJ, D'Amaro J. *Prognostic value of Hutchinson's sign in acute herpes zoster ophthalmicus*. Graefes Arch Clin Exp Ophthalmol 2003;241:187-91.

[32] Tojo K, Onozawa T, Toyohara K, et al. *Herpes zoster ophthalmicus with delayed contralateral hemiparesis*. Jpn J Med 1990;29:99-103.

[33] Carneiro AV, Ferro J, Figueiredo C, et al. *Herpes zoster and contralateral hemiplegia in an African patient infected with HIV-1*. Acta Med Port 1991;4:91-2.

[34] Lin HC, Chien CW, Ho JD. *Herpes zoster ophthalmicus and the risk of stroke: a population-based follow-up study*. Neurology 2010;74:792-7.

[35] Breuer J, Pacou M, Gauthier A, et al. *Herpes zoster as a risk factor for stroke and TIA: a retrospective cohort study in the UK*. Neurology 2014;82:206-12.

[36] Langan SM, Minassian C, Smeeth L, et al. *Risk of stroke following Herpes Zoster: a self-controlled case-series study*. Clin Infect Dis 2014;58:1497-503.

[37] Dworkin RH. *Post-herpetic neuralgia*. Herpes 2006;13(Suppl 1):21A-7.

[38] Gnann JW Jr, Whitley RJ. *Clinical practice: herpes zoster*. N Engl J Med 2002;347:340-6.

[39] Galluzzi KE. *Management strategies for herpes zoster and postherpetic neuralgia*. J Am Osteopath Assoc 2007;107(3 Suppl 1):S8-13.

[40] Li Q, Chen N, Yang J, et al. *Antiviral treatment for preventing postherpetic neuralgia*. Cochrane Database Syst Rev 2009;15;(2):CD006866.

[41] Chen N, Li Q, Yang J, et al. *Antiviral treatment for preventing Post-Herpetic Neuralgia*. Cochrane database Syst Rev 2014;2:CD006866.

[42] Oxman MN. *Clinical manifestation of herpes zoster*. In: Arvin AM, Gerson AA, eds. *Varicella zoster virus: virology and clini-*

cal management. Cambridge, UK: Cambridge University press; 2000, pp. 246-75.

[43] Drolet M, Brisson M, Schmader K, et al. *Predictors of postherpetic neuralgia among patients with herpes zoster: a prospective study*. J Pain 2010;11:1211-21.

[44] Yawn BP, Saddier P, Wollan PC, et al. *A population-based study of the incidence and complication rates of herpes zoster before zoster vaccine introduction*. Mayo Clin Proc 2007;82:1341-9.

[45] Weaver BA. *The Burden of Herpes Zoster and Postherpetic Neuralgia in the United States*. J Am Osteopath Assoc 2007;107(3 Suppl 1):S2-7.

[46] Insinga RP, Itzler RF, Pellissier JM, et al. *The incidence of herpes zoster in a United States administrative database*. J Gen Intern Med 2005;20:748-53.

[47] Joon Lee T, Hayes S, Cummings DM, et al. *Herpes zoster knowledge, prevalence, and vaccination rate by race*. J Am Board Fam Med 2013;26:45-51.

[48] Pinchinat S, Ana M Cebrián-Cuenca, HélèneBricout, et al. *Similar herpes zoster incidence across Europe: results from a systematic literature review*. BMC Infectious Diseases 2013;13:170.

[49] Yawn BP, Wollan P, St Sauver J. *Comparing shingles incidence and complication rates from medical record review and administrative database estimates: how close are they?* Am J Epidemiol 2011;174:1054-61.

[50] Thomas SL, Hall AJ. *What does epidemiology tell us about risk factors for herpes zoster?* Lancet Infect Dis 2004;4:26-33.

[51] Gialloreti LE, Merito M, Pezzotti P, et al. *Epidemiology and economic burden of Herpes Zoster and Post-Herpetic Neuralgia in Italy: a retrospective population-based study*. BMC Infect Dis 2010;10:230.

[52] Franco E, Perinetti E, Marchettini P, et al. *Proportion of Post-Herpetic Neuralgia among patients with Herpes Zoster in Italy - a multicenter prospective observational study (Heroes study)*. European Geriatric Medicine 2013;4:S115-6.

[53] Gabutti G, Serenelli C, Cavallaro A, et al. *Herpes zoster associated hospital admissions in italy: review of the hospital discharge forms*. Int J Eniron Res Public Health 2009;6:2344-53.

[54] Schmader K, Gnann JW Jr, Watson CP. *The epidemiological, clinical, and pathological rationale for the herpes zoster vaccine*. J Infect Dis 2008;197(Suppl 2):S207-15.

[55] Chidiac C, Bruxelle J, Daures JP, et al. *Characteristics of patients with herpes zoster on presentation to practitioners in France*. Clin Infect Dis 2001;33:62-9.

[56] Oster G, Harding G, Dukes E, et al. *Pain, medication use, and health-related quality of life in older persons with postherpetic neuralgia: results from a population-based survey*. J Pain 2005;6:356-63.

[57] Oxman MN. I*mmunization to reduce the frequency and severity of Herpes Zoster and its complications*. Neurology 1995;45(12 Suppl 8):S41-6.

[58] Levin MJ, Barber D, Goldblatt E, et al. *Use of a live attenuated varicella vaccine to boost varicella-specific immune responses in seropositive people 55 years of age and older: duration of booster effect*. J Infect Dis 1998;178(Suppl 1):S109-12.

[59] Trannoy E, Berger R, Holländer G, et al. *Vaccination of immunocompetent elderly subjects with a live attenuated Oka strain of Varicella Zoster Virus: a randomized, controlled, dose-response trial*. Vaccine 2000;18:1700-6.

[60] Breuer J. *Vaccination to prevent varicella and shingles*. J Clin Pathol 2001;54:743-7.

[61] Levin MJ, Smith JG, Kaufhold RM, et al. *Decline in Varicella-Zoster Virus (VZV)-specific cell-mediated immunity with increasing age and boosting with a high-dose VZV vaccine*. J Infect Dis 2003;188:1336-44.

[62] *Herpes Zoster vaccine* (live). Zostavax Summary of product characteristics 2013. Revision 18 December 2013.

[63] Oxman MN1, Levin MJ, Johnson GR, et al. *A vaccine to prevent Herpes Zoster and Post-Herpetic Neuralgias in older adults*. N Engl J Med 2005;352:2271-84.

[64] Schmader KE, Levin MJ, Gnann JW jr, et al. *Efficacy, safety and tolerability of Herpes Zoster vaccine in persons aged 50-59 years*. Clin Infect Dis 2012;54:922-8.

[65] Hope-Simpson RE. T*he nature of herpes zoster: a long-term stud and a new hypothesis*. Proc R Soc Med 1965;58:9-20.

[66] Coplan PM, Schmader K, Nikas A, et al. *Development of a measure of the burden of pain due to herpes zoster and postherpetic neuralgia for prevention trials: adaptation of the brief pain inventory*. J Pain 2004;5:344-56.

[67] Schmader KE, Oxman MN, Levin MJ, et al. *Persistence of the efficacy of Zoster vaccine in the Shingles Prevention Study and the short-term persistence sub-study*. Clin Infect Dis 2012;55:1320-8.

[68] EMA SPC Zostavax available at: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR__Product_Informaton/human/000674/WC500053462.pdf

[69] Tseng HF, Smith N, Harpaz R, et al. *Herpes zoster vaccine in older adults and the risk of subsequent herpes zoster disease*. Jama 2011;305:160-6.

[70] Zhang J, Xie F, Delzell E, et al. *Association between vaccination for herpes zoster and risk of herpes zoster infection among older patients with selected immune-mediated diseases*. JAMA 2012;308:43-9.

[71] Langan SM, Smeeth L, Margolis DJ, et al. *Herpes zoster vaccine effectiveness against incident herpes zoster and post-herpetic neuralgia in an older US population: a cohort study*. PLoS Medicine 2013;10(4):e1001420.

[72] https://clinicaltrials.gov/ct2/show/NCT01600079?term=NCT01600079&rank=1)

[73] Katz J, Cooper EM, Walther RR, et al. *Acute pain in herpes zoster and its impact on health-related quality of life*. Clin Infect Dis 2004;39:342-8.

[74] Murray AV, Reisinger KS, Kerzner B, et al. *Safety and tolerability of zoster vaccine in adults ≥ 60 years old.* Hum Vac 2011;7:1130-6.

[75] Mills R, Tyring SK, Levin MJ, et al. *Safety, tolerability, and immunogenicity of zoster vaccine in subjects with a history of Herpes Zoster*. Vaccine 2010;28:4204-9.

[76] Morrison VA, Oxman MN, Levin MJ, et al. *Safety of zoster vaccine in elderly adults following documented herpes zoster*. J Infect Dis. 2013;208:559-63.

[77] CDC. *Prevention of Herpes Zoster. Recommendations of the Advisory Committee on immunization practices (ACIP)*. MMWR 2008;57:1-29.

[78] Tseng HF, Schmid DS, Harpaz R, et al. *Herpes zoster caused by vaccine-strain varicella zoster virus in an immunocompetent recipient of zoster vaccine*. Clin Infect Dis 2014;58:1125-8.

[79] EUnetHTA. *Zostavax for the prevention of herpes zoster and post-herpetic neuralgia. Pilot assessment using the draft HTA Core Model for rapid relative effectiveness assessment*. Version 4.0 September 2013 http://www.eunethta.eu/sites/5026.fedimbo.belgium.be/files/Zostavax_main%20report%20including%20appendices_20130922.pdf (last accessed June 2014).

■ Received on October 20, 2014. Accepted on November 27, 2014.

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REVIEW

Staphylococcus aureus with reduced susceptibility to vancomycin in healthcare settings

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Key words

Staphylococcus aureus • MRSA, GISA• h-GISA • Glycopeptide • Vancomycin

Summary

Glycopeptide resistance in Staphylococcus aureus is a source of great concern because, especially in hospitals, this class of antibiotics, particularly vancomycin, is one of the main resources for combating infections caused by methicillin-resistant Staphylococcus aureus strains (MRSA).
Reduced susceptibility to vancomycin (VISA) was first described in 1996 in Japan; since then, a phenotype with heterogeneous resistance to vancomycin (h-VISA) has emerged.
H-VISA isolates are characterised by the presence of a resistant subpopulation, typically at a rate of 1 in 10^5 organisms, which constitutes the intermediate stage between fully vancomycin-susceptible S. aureus (VSSA) and VISA isolates. As VISA phenotypes are almost uniformly cross-resistant to teicoplanin, they are also called Glycopeptides-intermediate Staphylococcus aureus strains (GISA) and, in the case of heterogeneous resistance to glycopeptides, h-GISA.
The overall prevalence of h-VISA is low, accounting for approximately 1.3% of all MRSA isolates tested.
Mortality due to h-GISA infections is very high (about 70%), especially among patients hospitalised in high-risk departments, such as intensive care units (ICU).
Given the great clinical relevance of strains that are heteroresistant to glycopeptides and the possible negative impact on treatment choices, it is important to draw up and implement infection control practices, including surveillance, the appropriate use of isolation precautions, staff training, hand hygiene, environmental cleansing and good antibiotic stewardship.

Introduction

Since the 1970s, the selective pressure exerted by antibiotics has given rise to increasingly resistant bacterial species and the last 20 years have seen a marked increase in multi-resistant pathogenic strains [1].
Staphylococcus aureus (S. aureus), human commensal bacterium involved in an array of pathologies, from minor dermatological diseases to severe disorders, such as pneumonia, endocarditis, meningitis or sepsis, continues to be one of the main causes of hospital and community infections worldwide [2]. The emergence of resistance to penicillin, followed by the spread of strains resistant penicillins penicillinases resistant (headed by methicillin, macrolides, tetracyclines, aminoglycosides and, recently, glycopeptides has turned the therapy of staphylococcal infections into a global challenge.
Glycopeptide resistance in *S. aureus* is a source of great concern because, especially in hospitals, this class of antibiotics, particularly vancomycin, is one of the main resources for combating infections caused by methicillin-resistant *S. aureus* strains (MRSA).

Methicillin-resistant *S. aureus*

The rate of mortality due to *S. aureus* infections was drastically reduced by the introduction of penicillin in the early 1940s. A few years later, however, strains of *S. aureus* that had developed plasmid-mediated resistance to penicillin appeared; this resistance was due to the production of penicillinase, a ß-lactamase capable of breaking down the drug before it could reach its target.
Methicillin, the first semisynthetic penicillin resistant to penicillinases, was introduced into clinical practice in 1959. This antibiotic proved efficacious in combating infections due to ß-Lactam antibiotic-resistant *S. aureus* strains until the appearance of methicillin-resistant strains of *S. aureus*, which soon became one of the main causes of infection in hospitals.
The first report of MRSA strains was made in England in 1961 [3], not long after the introduction of methicillin, and epidemics caused by MRSA were already being recorded in the early 1960s [4, 5]. Since then, MRSA strains have spread throughout the world and their prevalence has increased in both hospital and community settings. The epidemiology of MRSA has therefore changed in recent years, in that infections are no longer confined to the hospital environment, but also involve healthy subjects without particular risk factors in the community setting [6].
In the USA, MRSA account for more than 60% of all *S. aureus* isolates in intensive care units (ICU) [7]. In Europe, it has been estimated that MRSA cause 171,200 nosocomial infections each year, corresponding to 44% of all hospital infections [8]. In Italy, the percentage of MRSA strains isolated in hospitals is around 40%, with peaks of up to 80% in some hospitals [9].

These strains generally display multi-resistance, which considerably limits therapeutic options. A study conducted in Canada revealed that the mortality associated with bacteraemia due to MRSA was 39%, as opposed to 24% due to strains of Methicillin-sensitive Staphylococcus aureus (MSSA) [6].

Mechanism of methicillin resistance

All strains of *S. aureus* produce 4 main membrane proteins capable of binding penicillin and other ß-Lactam antibiotics (penicillin-binding proteins, PBP). ß-Lactam antibiotics are substrate analogues, which covalently bind to the serine-active sites of the penicillin-binding proteins (PBPs), inactivating the enzyme at concentrations roughly comparable to the minimum inhibiting concentrations (MIC). PBPs 1, 2 and 3, which have a high affinity for most ß-Lactam antibiotics, are essential to the development of the cell and to the survival of sensitive strains; the binding of ß-Lactam antibiotics to these PBP can kill the bacterial cell [4, 10].
The *mec*A gene, the expression of which is generally regulated by the *mecI* and *mecR1* genes, codifies for PBP type 2a (PBP2a), a low-affinity PBP on which resistance itself depends. PBP2a is a 78 kDa protein which, in methicillin-resistant strains, owing precisely to its low affinity for most ß-Lactam antibiotics, is not saturated (and thus functionally blocked) by otherwise lethal concentrations of these antibiotics. In such conditions, not only does it continue to function, it is also able to vicariously carry out the functions normally performed by the other (functionally blocked) high-affinity PBPs [11].
The *mec*A gene (2.1 kb) participates in a broader block of DNA (up to 60 kb), called staphylococcal chromosomal cassette (SCC*mec*), containing the determinants of resistance to the various non- ß-Lactam antibiotics. *MecA* is normally regulated by the genes *mecI* (repression) and *mecR1* (induction) [4, 10].

Resistance to glycopeptides and epidemiology of h- glycopeptide intermediate-resistant *S. aureus* (GISA) strains

Following the global rise in infections caused by multi-resistant MRSA strains, glycopeptides have become the antibiotics of choice for the therapy of nosocomial staphylococcal infections in the last 20 years. The glycopeptides in clinical use are vancomycin, the co-founder drug that came onto the market at the end of the 1950s, and teicoplanin, which was introduced into clinical practice in the second half of the 1980s.
The glycopeptide antibiotics are large rigid molecules, which inhibit the last stages of peptidoglycan biosynthesis. Their antimicrobial activity, which is limited to Gram-positive bacteria owing to their inability to penetrate the external membrane, is due to their particular affinity for the D-alanyl-D-alanine (D-Ala-D-Ala) dimer of the lateral chain of the peptidoglycan precursor, to which they strongly bind, albeit non-covalently [10]. Although this antibiotic has been widely used in the last two decades, most MRSA strains are still sensitive to vancomycin. Indeed, the first MRSA isolates with reduced sensitivity to glycopeptides took about 40 years to emerge [12].
The first MRSA isolates displaying reduced sensitivity to vancomycin (VISA) were reported in Japan in 1996 [13]; soon afterwards, a phenotype of *S. aureus* with acquired heterogeneous resistance to vancomycin (h-VISA) emerged [14, 15]. h-VISA isolates are characterised by the presence of a subpopulation (1 per 10^5 bacterial cells) resistant to vancomycin and represent the intermediate stage between total sensitivity to vancomycin (VSSA) and VISA isolates [10, 16-18]. Following the appearance of the first VISA (Mu50) and h-VISA (Mu3) strains reported in Japan [13, 14], both phenotypes were described worldwide. However, the exact prevalence of h-VISA strains is difficult to determine, owing to the wide range of methodological tests used, of definitions and of modifications in the breakpoints of susceptibility to vancomycin. This might explain the considerable variability in the prevalence of h-VISA strains in the various institutions, geographic regions and patient populations.
Very recently, a further phenotype was found and characterized in Mu3-6R-P strain: slow vancomycin-intermediate S. aureus (s-VISA) strains [19]. h-VISA may escape vancomycin therapy temporarily converting into s-VISA and later returning to the previous stage as soon as therapy is suspended. Therefore, the passage from h-VISA to s-VISA and viceversa can be interpreted as an oscillating, reversible switch mechanism.
Nevertheless, the overall prevalence of h-VISA remains low: about 1.3% of all methicillin-resistant Staphylococcus aureus (MRSA) isolates tested [16]. Di Gregorio et al. computed h-VISA to be 4.5% of MRSA strains [20]. Hanaki and coauthors estimated that h-VISA represent 6.5% of MRSA strains [21], while Chaudhari and collaborators estimated h-VISA to represent 6.9% of 58 clinical isolates of MRSA [22]. Monaco and coworkers carried out a study in order to assess the presence of h-VISA strains in Italy: they found h-VISA to be 13.6% of MRSA strains and 6.1% of all the studied S. aureus strains [23].
As VISA strains generally display cross-resistance to teicoplanin, they are also called glycopeptide intermediate-resistant *S. aureus* (GISA) [24] and, in the case of heteroresistance, h-GISA. In the USA, however, where teicoplanin is not available, the terms VISA and h-VISA are currently used.
International data from the Tigecycline Evaluation and Surveillance Trial (T.E.S.T.) involving 20,004 *S. aureus* isolates show that the proportion of MRSA with vancomycin MICs ≥ 2 mg/L increased from 5.6% in 2004 to 11.1% in 2009 ($P < 0.001$) [8].
A study conducted in the metropolitan area of Detroit in the USA documented a significant increase in the prev-

alence of h-VISA over 20 years: from 2.27% between 1986 and 1993 to 8.2% between 2003 and 2006 [25].
VISA strains tend to develop multi-resistance to a large number of commonly used antibiotics, thereby determining a reduction in possible therapeutic options and increasing the risk of administering inadequate antibiotic therapy [26]. An increase in the resistance of MRSA strains leads to increased morbidity and mortality due to severe infections such as bacteraemia, endocarditis and osteomyelitis [27, 28].
Concern over the development of vancomycin resistance in staphylococci is destined to grow dramatically following reports of vancomycin-resistant strains of MRSA (VRSA). The first strain was reported in the United States in 2002, isolated from a haemodialysis patient; this strain proved to be highly resistant to vancomycin and was also resistant to teicoplanin. It was isolated from the patient together with an enterococcus, VanA, and was found to contain in its genome not only the *mecA* gene of methicillin-resistance, but also the vanA gene, which is responsible for the most widespread form of vancomycin-resistance in enterococci. The DNA sequence of the *vanA* gene of the Staphylococcus was identical to that of the vanA gene of the *E. faecalis* isolated from an infected ulcer in the same patient. This strain, the first clinical isolate of *S. aureus* highly resistant to vancomycin, therefore seems to be the result of the spread of VanA resistance from the enterococcus to the *S. aureus* [10, 29]. To date, strains displaying high levels of resistance to vancomycin (acquired through the *vanA* gene) are rare, though cases have been reported in the USA, India and Iran [8].
The results of a study conducted by Maor [30] revealed that 6% of patients affected by MRSA presented h-VISA strains and that the mortality rate among all the h-VISA patients was 75%. This study suggests that h-VISA infection is associated with unsatisfactory clinical outcomes despite the adequate administration of vancomycin.
A study conducted on 86 patients from whom MRSA strains with reduced susceptibility to teicoplanin were isolated revealed that 3.4% of patients were colonised by h-GISA and that 2.5% had bacteraemia caused by h-GISA. The results of this study suggest that recurrent bacteraemia in a patient who has previously undergone antibiotic therapy with glycopeptides is an important indicator of the presence of h-GISA [31].
Mortality due to h-GISA infections is very high (about 70%), especially among patients hospitalised in high-risk wards, such as intensive care units (ICU), where the vulnerability of the patient is exacerbated by such contingencies as invasive medical procedures, the insertion of prosthetic devices or of central venous catheters, the high frequency of nursing procedures, and the ample use of broad-spectrum antibiotic therapy [32].
The hospital environment can play an essential role in the transmission of multidrug-resistant pathogens, and environmental monitoring can reveal the degree of microbial contamination [33]. Environmental contamination by MRSA strains tends to be very persistent (up to 38 weeks) [34], which means that surfaces in wards can become veritable reservoirs and vehicles for the spread of infection [35, 36]. h-GISA strains are characterised by thickening of the peptidoglycan wall [14, 15], which is proportional to the degree of resistance to glycopeptides; this ultrastructural feature may favour adhesion to surfaces, with important implications for the type of sanitation measures that need to be implemented.
A study conducted by Perdelli et al. [37] evaluated the percentage of MRSA with reduced susceptibility to glycopeptides in four ICU by means of environmental sampling of air and representative surfaces. The antibiogram performed on the colonies of *S. aureus* revealed that, in the air of the four ICU sampled, 88.8% of the strains proved to be resistant to methicillin and that 91.9% of these displayed reduced susceptibility to glycopeptides. A similar situation emerged with regard to the surfaces sampled (72.0% MRSA, 81.1% of which h-GISA).
The prevalence of notified infections due to h-GISA strains is low. However, as mentioned previously, this might be due to the routine use of laboratory screening techniques that have low sensitivity and specificity. It would therefore be useful to implement quality controls in order to verify the reliability of results and to unmask any possible underestimation of the phenomenon [38].
Given the great clinical relevance of strains that are heteroresistant to glycopeptides, and their possible negative impact on therapeutic choices, measures for prevention and control should be implemented both on the clinical front and with regard to hygiene/behavior.

Treatment and management

As vancomycin and other glycopeptides, such as teicoplanin, have constituted the treatment of choice for infections due to MRSA, their excessive use may have led to the appearance of h-VISA, VISA and VRSA strains. Moreover, it is likely that the true magnitude of the problem has been underestimated and that many cases of h-VISA, VISA and perhaps VRSA have gone undetected owing to the implementation of suboptimal screening programs and the shortcomings of current diagnostic techniques [30]. As yet, the proportion of MRSA strains with reduced susceptibility to vancomycin and teicoplanin in the hospital setting is not known [26]. Such knowledge, however, would be extremely important for the purposes of prevention and control [39], in that strains heteroresistant to glycopeptides (h-GISA) are the direct precursors of vancomycin-resistant *S. aureus* (VRSA) strains and seem to be directly implicated in the failure of antibiotic therapy in MRSA infections that spread to deep layers [40, 41].
An alternative to vancomycin is daptomycin, an antibiotic belonging to the class of lipopeptides, which disrupts the functioning of the cell membrane through a calcium-dependent bond. Its bactericidal activity depends on the concentration. The breakpoint of sensitivity to daptomycin for *S. aureus* is ≤ 1 μg/ml. Non-susceptible strains have appeared during treatment with this antibi-

otic. Although the mechanism of resistance has not been clarified, these strains often display point mutations of mprF, the gene for lysophosphatidylglycerol synthetase. Previous exposure to vancomycin and a high MIC of vancomycin have been associated to the increase in the MIC of daptomycin, an observation that seems to indicate possible cross-resistance [42].

Prevention and control

In recent years, several international scientific associations and institutions have drawn up recommendations aimed at reducing the spread of MRSA infections in the healthcare setting [43-47]. These recommendations are concordant with regard to some essential aspects, such as the use of specific surveillance tools, the adoption of contact precautions (hand hygiene, use of barrier measures) to limit the spread of any cross-infection, and policies aimed at promoting the proper use of antibiotics. With regard to this last aspect, it is important to rationalise the administration and use of glycopeptides in relation not only to therapeutic results but also to phenomena of resistance.

However, antibiotic policy must not be limited only to this class of antibiotics; it must also involve cephalosporins and carbapenems, since the heterogeneous expression of glycopeptide resistance is also influenced by exposure to almost all ß-Lactam antibiotics, even when administered at optimal concentrations [15]. The issues of the active detection of colonised patients and their decolonisation are more controversial [48-53]. This latter question has been the subject of recently published systematic reviews [54, 55]. In 1997, the Centers for Disease Control and Prevention (CDC) in Atlanta drew up a document containing recommendations for preventing the spread of vancomycin resistance [56]. Further considerations on the control of infections due to vancomycin-resistant *S. aureus* strains were made by Wenzel and Edmond [57], particularly with regard to the utility of conducting studies on the prevalence of antibiotic resistance, implementing control strategies and, especially, contact precautions (hand-washing, use of gloves, isolation, etc.), and immediate notification to the Infections Committee of the hospital.

It is also important to utilise appropriate diagnostic techniques in order to minimise recourse to prolonged empirical therapy; for example to use venous catheters only for the time strictly necessary, and to remove prosthetic materials infected by *S. aureus*. It is well known that MRSA can spread easily in the hospital environment, and it is reasonable to suppose that VISA strains have the same potential for transmission [10]. Measures for the prevention and control of the spread of these microorganisms have recently been revised in a document endorsed by several European countries. This underscores a few key points: proper hand hygiene and routine cleansing and decontamination of environments; the use of personal protection devices by healthcare personnel when attending to MRSA-positive patients; the implementation of MRSA surveillance programs, and the screening of patients at risk [58]. It has been demonstrated that controlling the spread of MRSA in hospitals requires the simultaneous implementation of both "horizontal" and "vertical" strategies. Horizontal strategies are those aimed at preventing the spread of infections due to all possible pathogens [37, 59-62] through interventions such as hand hygiene, environmental cleansing, antibiotic stewardship and proper management of vascular catheters; vertical strategies are those aimed at controlling a specific pathogen (MRSA) [63]. An approach that combines these two strategies – horizontal and vertical – can optimise the results [57]. In Italy, the Ministry of Health has recently drawn up a document which identifies the priority measures to be adopted in order to reduce the risk of healthcare-related infections (HAIs) caused by MRSA, as indicated in the most recent international scientific literature [63].

The main measures are listed below:

Surveillance

Organising a system of surveillance is useful only if data analysis leads to the adoption of suitable provisions. Thus, identifying patients infected/colonised by MRSA is useful if the system prescribes the subsequent isolation of the positive patient and the implementation of contact precautions.

Surveillance can allow the spread of MRSA inside health facilities to be detected and monitored over time, in order to plan adequate intervention. To ensure optimal cooperation on the part of the various departments, surveillance data must be provided periodically.

Handling information on MRSA positivity

The correct and timely transmission of information on MRSA positivity is important in order to ensure that the necessary interventions and/or decisions be taken to address the problem.

At the moment of hospitalisation, the availability of information on previous colonisation by MRSA can enable the patient to be placed pre-emptively in isolation, thereby reducing the spread of the microorganism in the hospital.

Hand hygiene

Proper hand hygiene is deemed to be the main means of reducing HAIs. Compliance with this measure on the part of healthcare personnel is generally less than 40%; this low percentage has been associated with the use of gloves, a practice erroneously regarded as a substitute for hand hygiene.

Kapil and collaborators carried out a survey among health-care workers (HCWs) and found that 70% had bacterial counts ≥ 100 CFUs. Hand hygiene reduced the count of 95-99% among doctors and nurses, 70% among hospital attendants and 50% among sanitary attendants. *S. aureus* was present on the hands of 8 HCWs of which three were MRSA [64]. Similar findings were obtained by Monistrol and coworkers who found that *S. aureus* is the most common contaminant in health settings and

that, isolated from the hands of healthcare workers, after an educational intervention, the MRSA count decreased from 1.96 ± 1.2 log10 CFU/ml to 0.89 ± 1.2 log10 CFU/ml [65]. Al-Tawfiq and coauthors observed a marked decrease in the rate of MRSA cases per 1,000 patient-days from 0.42 to 0.08, with an increase in the hand hygiene compliance [66].
82% of patients colonized by MRSA had positive hand cultures for MRSA, which reduced after a single application of alcohol gel [67]. Besides HCWs hand hygiene compliance, also patient hand disinfection plays a major role [68].
The use of alcohol gels and solutions for hand hygiene has overcome many of the problems of non-compliance, especially when time is short owing to heavy workloads.

Contact precautions

The spread of infections in healthcare facilities is made possible by the interaction of three principal elements: a source (reservoir) of pathogenic microorganisms; a susceptible host and a suitable means of entry for that specific microorganism.
The main reservoir of infection is constituted by persons (patients, healthcare workers, visitors and family members). Human reservoirs may be subjects who are colonised or have active infections. The environment may also be involved in the spread of microorganisms, through contaminated environmental sources or vehicles (equipment, instruments, medical devices, solutions for infusion, etc.).
As MRSA is chiefly spread through contact (direct or indirect), contact precautions must be taken in order to reduce the risk of transmission to a susceptible patient. These precautions include:

- isolation in a single room or, if this is not possible, isolation by cohort;
- the use of dedicated materials;
- hand hygiene;
- the use of disposable gloves and overalls;
- the use of protective barriers;
- proper management of equipment;
- environmental hygiene;
- proper handling of bedding and crockery;
- healthcare education, and staff training.

Environmental hygiene

Healthcare facilities need to draw up regulations for environmental cleansing (frequency, methods) and to appoint a person to be responsible for ensuring that these regulations are respected.
The environmental surfaces in healthcare facilities can contribute to the spread of cross-infections, in that they constitute a possible site for the accumulation of microorganisms [69]. Like medical devices, surfaces must therefore be thoroughly cleaned and disinfected regularly; disinfectants must be appropriate and used in conformity with the manufacturers' recommendations and the indications of the Hospital Infections Committee, and particular attention should be paid to surfaces that are touched frequently.

Screening

In departments with a high incidence of MRSA or in those accommodating patients at risk of severe MRSA infections, it is advisable to carry out active screening of high-risk patients. However, the implementation of an MRSA screening system is meaningful only if the results of screening are used to enact infection control measures.

Decolonisation

Care bundles recommend that nasal decolonisation be carried out with mupirocin in all patients identified as MRSA-positive, according to the screening strategies identified, and skin decolonisation with 4% chlorhexidine, 7.5% iodopovidone or 2% triclosan.
Universal decolonization is cost-saving [70] in that prevents 44% of MRSA colonizations and 45% of MRSA infections. Also the REDUCE MRSA trial confirmed this finding, showing that compared with screening and isolation, universal decolonization could save $171,000 and prevent 9 additional bloodstream infections for every 1,000 ICU admissions [71].

Personnel

The screening of personal is recommended only when there is a strong suspicion that staff may be a source of transmission, as in the case of an uncontrolled epidemic.

Antibiotic stewardship

According to the international recommendations, in order to reduce or at least contain the problem of antibiotic resistance, antibiotic policies, such as the following, should be implemented:

1. Avoid inappropriate or excessive antibiotic therapies and prophylaxes.

Pay attention to the diagnosis and ensure that the therapy is appropriate.

2. Ensure that the dose and duration of antibiotic therapy are correct.
3. Reduce as far as possible the use of broad-spectrum antibiotics, in particular third-generation cephalosporins and quinolones.
4. Limit the use of glycopeptides and check therapeutic levels.

It is also important to check that preoperative antibiotic prophylaxis is appropriate in terms of indication, choice of drug, dose and duration of prophylaxis, and to monitor the consumption of antibiotics, at least in critical departments at high risk of MRSA.
Antibiotic stewardship is particularly helpful in reducing MRSA cases and has long-term effect, as shown by studies carried out in a secondary-care hospital in Germany [72], and in a tertiary-care teaching hospital in the USA [73].
It is also important to educate junior doctors about the importance of preserving the effectiveness of the available armamentarium against S. aureus, as demonstrated by an interventional study performed at two teaching hospitals in France and Scotland [74].

New strategies and forms of antibiotic stewardship have been recently implemented for raising awareness of the importance of a correct and proper antibiotic policy among the HCWs.

New technologies can help in making antibiotic stewardship highly sustainable, strengthening its impact and preserving high quality care while reducing the costs [75].

In conclusion, given the great clinical relevance of strains that are heteroresistant to glycopeptides and the possible negative impact on treatment choices, it is important to draw up and implement infection control practices, including surveillance, the appropriate use of isolation precautions, staff training, hand hygiene, environmental cleansing and good antibiotic stewardship.

References

[1] Acar JF, Kaplan EL, O'Brien TF. *Monitoring and management of bacterial resistance to antimicrobial agents, a World Health Organization symposium.* Clin Infect Dis 1997;24:S1-176.

[2] Cristina ML, Spagnolo AM, Orlando P, et al. *The role of the environment in the spread of emerging pathogens in at-risk hospital wards*. Rev Med Microbiol 2013;24:104-12.

[3] Jevons MP, Coe AW, Parker MT. *Methicillin resistance in staphylococci*. Lancet 1963;1:904-7.

[4] Manso E, Varaldo PE. *La resistenza agli antibiotici negli stafilococchi*. Website: http://www.masciabrunelli.it/biolife/upload/file/Documenti/Documenta_imp_2002.PDF

[5] *Epidemiology of methicillin-resistant Staphylococcus aureus infection in adults*. Website: http://www.uptodate.com/contents/epidemiology-of-methicillin-resistant-Staphylococcus-aureus-infection-in-adults.

[6] Kluytmans J, Struelens M. *Meticillin resistant Staphylococcus aureus in the hospital*. BMJ 2009;338:b364.

[7] Boucher HW, Corey GR. *Epidemiology of methicillin-resistant Staphylococcus aureus*. Clin Infect Dis 2008;46: S344-9.

[8] Gould IM, David MZ, Esposito S, et al. *New insights into methicillin-resistant Staphylococcus aureus (MRSA) pathogenesis, treatment and resistance.* Int J Antimicrob Agents 2012;39:96-104.

[9] *Staphylococcus aureus*. Website: http://www.iss.it/chem/index.php?lang=1&id=70&tipo=11.

[10] Howden BP, Davies JK, Johnson PD, et al. *Reduced vancomycin susceptibility in Staphylococcus aureus, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications.* Clin Microbiol Rev 2010;23:99-139.

[11] Tonin E, Tomasz A. *Beta-lactam-specific resistant mutants of Staphylococcus aureus.* Antimicrob Agents Chemother 1986;30:577-83.

[12] van Hal SJ, Lodise TP, Paterson DL. *The clinical significance of vancomycin minimum inhibitory concentration in Staphylococcus aureus infections: a systematic review and meta-analysis.* Clin Infect Dis 2012;54:755-71.

[13] Hiramatsu K, Hanaki H, Ino T, et al. *Methicillin-resistant Staphylococcus aureus clinical strain with reduced vancomycin susceptibility.* J Antimicrob Chemother 1997;40:135-6.

[14] Hiramatsu K, Aritaka N, Hanaki H, et al. *Dissemination in Japanese hospitals of strains of Staphylococcus aureus heterogeneously resistant to vancomycin.* Lancet 1997;350:1670-3.

[15] Hiramatsu K. *Vancomycin–resistant Staphylococcus aureus: a new model of antibiotic resistance.* Lancet Infect Dis 2001;1:147-55.

[16] van Hal SJ, Paterson DL. S*ystematic review and meta-analysis of the significance of heterogeneous vancomycin-intermediate Staphylococcus aureus isolates.* Antimicrob Agents Chemother 2011;55:405-10.

[17] van Hal SJ, Wehrhahn MC, Barbagiannakos T, et al. *Performance of various testing methodologies for detection of heteroresistant vancomycin-intermediate Staphylococcus aureus in bloodstream isolates.* J Clin Microbiol 2011;49:1489-94.

[18] van Hal SJ, Jones M, Gosbell IB, et al. *Vancomycin heteroresistance is associated with reduced mortality in ST239 methicillin-resistant Staphylococcus aureus blood stream infections.* PLoS One 2011;6:e21217.

[19] Saito M, Katayama Y, Hishinuma T, et al. "*Slow VISA," a novel phenotype of vancomycin resistance, found in vitro in heterogeneous vancomycin-intermediate Staphylococcus aureus strain Mu3*. Antimicrob Agents Chemother 2014;58:5024-35.

[20] Di Gregorio S, Perazzi B, Ordoñez AM, et al. *Clinical, microbiological, and genetic characteristics of heteroresistant vancomycin-intermediate Staphylococcus aureus bacteremia in a teaching hospital*. Microb Drug Resist 2015;21:25-34.

[21] Hanaki H, Cui L, Ikeda-Dantsuji Y, et al. *Antibiotic susceptibility survey of blood-borne MRSA isolates in Japan from 2008 through 2011*. J Infect Chemother 2014;20:527-34.

[22] Chaudhari CN, Tandel K, Grover N, et al. *Heterogeneous vancomycin-intermediate among methicillin resistant Staphylococcus aureus.* Med J Armed Forces India 2015;71:15-8.

[23] Monaco M, Sanchini A, Grundmann H, et al. *Vancomycin-heteroresistant phenotype in invasive methicillin-resistant Staphylococcus aureus isolates belonging to spa type 041*. Eur J Clin Microbiol Infect Dis 2010;29:771-7.

[24] Renzoni A, Kelley WL, Vaudaux P, et al. *Exploring innate glycopeptide resistance mechanisms in Staphylococcus aureus.* Trends Microbiol 2010;18:55-6.

[25] Rybak M, Chin JN, Lau K, et al. *Increasing prevalence of glycopeptide hetero-resistant S. aureus from the Detroit metropolitan area over a 20-year period (1986–2006).* In: *17th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) and the 25th International Congress of Chemotherapy (ICC).* 2007 [Abstract O32].

[26] Appelbaum PC. *MRSA-the tip of the iceberg.* Clin Microbiol Infect 2006;12:3-10.

[27] Blot SI, Vandewoude KH, Hoste EA, et al. *Outcome and attributable mortality in critically ill patients with bacteremia involving methicillin-susceptible and methicillin-resistant Staphylococcus aureus.* Arch Intern Med 2002;162:2229-35.

[28] Chang FY, MacDonald BB, Peacock Jr JE, et al. *A prospective multicenter study of Staphylococcus aureus bacteremia: incidence of endocarditis, risk factors for mortality, and clinical impact of methicillin resistance.* Medicine (Baltimore) 2003;82:322-32.

[29] Chang S, Sievert DM, Hageman JC, et al. *Infection with vancomycin-resistant Staphylococcus aureus containing the vanA resistance gene.* N Engl J Med 2003;348:1342-7.

[30] Maor Y, Rahav G, Belausov N, et al. *Prevalence and characteristics of heteroresistant vancomycin intermediate Staphylococcus aureus bacteremia in a tertiary care center.* J Clin Microbiol 2007;45:1511-4.

[31] Kirby A, Graham R, Williams NJ, et al. *Staphylococcus aureus with reduced glycopeptide susceptibility in Liverpool, UK*. J Antimicrob Chemother 2010;65:721-4.

[32] Linares J. *VISA/GISA problem: therapeutic implications.* Clin Microbiol Infect 2001;7:8-15.

[33] Ottria G, Dallera M, Aresu O, et al. *Environmental monitoring programme in the cell therapy facility of a research centre: Preliminary investigation.* J Prev Med Hyg 2010;51:133-8.

[34] Dietz B, Raht A, Wendt C, et al. *Survival of MRSA on sterile goods packaging.* J Hosp Infect 2001;49:255-61.

[35] Centers for Disease Control and Prevention/Healthcare Infection Control Practices Advisory Committee (HICPAC). 2001. *Draft Guidelines for Environmental Infection Control in Healthcare Facilities. Atlanta, GA: Centers for disease Control and Prevention.* Available at www.cdc.gov/ncidod/hip/enviro/guide.htm.

[36] Farr BM, Salgado CD, Karchmer TB, et al. *Can antibiotic-resistant nosocomial infections be controlled?* Lancet Infect Dis 2001;1:38-45.

[37] Perdelli F, Dallera M, Cristina ML, et al. *A new microbiological problem in intensive care units: environmental contamination by MRSA with reduced susceptibility to glycopeptides*. Int J Hyg Environ Health 2008;211:213-8.

[38] Boccia D, D'Ancona F, Pantosti A. *Resistenza alla Vancomicina in ceppi invasivi di Enterococcus faecalis ed enterococcus faecium.* Dati preliminari Bollettino Epidemiologico Nazionale – Notiziario ISS – 2002;15(5).

[39] Walsh T R, Howe R A, Wootton M, et al. *Detection of glycopeptide resistance in Staphylococcus aureus.* J Antimicrob Chemother 2001;47:357-8.

[40] Ariza J, Pujol M, Cabo J, et al. *Vancomycin in surgical infections due to methicillin-resistant Staphylococcus aureus with heterogeneous resistance to vancomycin.* Lancet 1999;353:1587-8.

[41] Moore M R, Perdreau-Remington F, Chambers HF. *Vancomycin treatment failure associated with heterogeneous vancomycin-intermediate Staphylococcus aureus in patient with endocarditis and in the rabbit model of endocarditis.* Antimicrob Agents Chemother 2003;47:1262-6.

[42] Liu C, Bayer A, Cosgrove SE, et al. *Clinical practice guidelines by the Infectious Diseases Society of America for the treatment of methicillin-resistant staphylococcus aureus infections in adults and children.* Clin Infect Dis 2011;52:e18-55.

[43] Infection Prevention Working Party. *MRSA Hospital.* The Netherlands, 2007.

[44] Rodríguez-Baño J, Bischofberger C, Álvarez-Lerma F, et al. *Vigilancia y el control de Staphylococcus aureus resistente a meticilina en hospitales españoles. Documento de consenso GEIH-SEIMC y SEMPSPH.* Enferm Infecc Microbiol Clin 2008;26:285-98.

[45] Calfee DP, Salgado CD, Classen D, et al. SHEA/IDSA. *Strategies to prevent transmission of methicillin-resistant Staphylococcus aureus in acute care hospitals.* Infect Control Hosp Epidemiol 2008;29:62-80.

[46] Cystic Fibrosis Trust. *Methicillin-resistant Staphylococcus aureus (MRSA).* Report of the UK Cystic Fibrosis Trust Infection Control Working Group 2008.

[47] Borgman S, Stark M, Kaiser P, et al. *MRSA in praxis, pflegeheim und häuslichem umfeld.* Bayerisches Ärzteblatt 2008;3:176-81.

[48] Tacconelli E. *Screening and isolation for infection control.* J Hosp Infect 2009;73:371-7.

[49] Harbarth S, Fankhauser C, Schrenzel J, et al. *Universal screening for methicillin-resistant Staphylococcus aureus at hospital admission and nosocomial infection in surgical patients.* JAMA 2008;299:1149-57.

[50] Robicsek A, Beaumont JL, Paule SM, et al. *Universal surveillance for methicillin-resistant Staphylococcus aureus in 3 affiliated hospitals.* Ann Intern Med 2008;148:409-18.

[51] Jeyaratnam D, Whitty CJ, Phillips K, et al. *Impact of rapid screening tests on acquisition of methicillin resistant Staphylococcus aureus: cluster randomised crossover trial.* BMJ 2008;336:927-30.

[52] Raineri E, Crema L, De Silvestri A, et al. *Methicillin-resistant Staphylococcus aureus control in an intensive care unit: a 10 year analysis.* J Hosp Infect 2007;67:308-15.

[53] Keshtgar MR, Khalili A, Coen PG, et al. *Impact of rapid molecular screening for methicillin-resistant Staphylococcus aureus in surgical wards.* Br J Surg 2008;95:381-6.

[54] Tacconelli E, De Angelis G, de Waure C, et al. *Rapid screening tests for methicillin-resistant Staphylococcus aureus at hospital admission: systematic review and meta-analysis.* Lancet Infect Dis 2009;9:546-54.

[55] van Rijen M, Bonten M, Wenzel R, Kluytmans J. *Mupirocin ointment for preventing Staphylococcus aureus infections in nasal carriers.* Cochrane Database Syst Rev 2008;4:CD006216.

[56] Centers for Disease Control and Prevention. *Interim guidelines for prevention and control of staphylococcal infection associated with reduced susceptibility to vancomycin.* MMWR Morb Mortal Weekly Rep 1997;46:626-35.

[57] Wenzel RP, Edmond MB. *Vancomycin-resistant Staphylococcus aureus: infection control considerations.* Clin Infect Dis 1998;27:245-51.

[58] Kalenic S, Cookson B, Gallagher R, et al. *Comparison of recommendations in national/regional Guidelines for the prevention and control of MRSA in thirteen European countries.* Int J Infect Control 2010;v6:i2.

[59] Spagnolo AM, Orlando P, Panatto D, et al. *An overview of carbapenem-resistant Klebsiella pneumoniae: Epidemiology and control measures.* Rev Med Microbiol 2014;25:7-14.

[60] Cristina ML, Spagnolo AM, Cenderello N, et al. *Multidrug-resistant Acinetobacter baumannii outbreak: an investigation of the possible routes of transmission.* Public Health 2013;127:386-91.

[61] Cristina ML, Spagnolo AM, Ottria G, et al. *Spread of multidrug carbapenem-resistant Acinetobacter baumannii in different wards of an Italian hospital.* Am J Infect Control 2011;39:790-4.

[62] Cristina ML, Spagnolo AM, Sartini M, et al. *Clostridium difficile infections: an emerging problem in healthcare facilities.* Rev Med Microbiol 2012;23:67-75.

[63] *Raccomandazioni sul controllo della diffusione nosocomiale dello Staphylococcus aureus resistente alla meticillina (MRSA) 2011.* A cura di: Istituto Superiore di Sanità, Agenzia sanitaria e sociale regionale dell'Emilia-Romagna, Ministero della salute – Ufficio V Malattie infettive, Direzione generale della prevenzione sanitaria. Disponibile sul sito: http://asr.regione.emilia-romagna.it/wcm/asr/aree_di_programma/rischioinfettivo/gr_ist/pr_staph/pubblicazioni/report_mrsa/link/mrsa.pdf.

[64] Kapil R, Bhavsar HK, Madan M. *Hand hygiene in reducing transient flora on the hands of healthcare workers: an educational intervention.* Indian J Med Microbiol 2015;33:125-8.

[65] Monistrol O, López ML, Riera M, et al. *Hand contamination during routine care in medical wards: the role of hand hygiene compliance.* J Med Microbiol 2013;62:623-9.

[66] Al-Tawfiq JA, Abed MS, Al-Yami N, et al. *Promoting and sustaining a hospital-wide, multifaceted hand hygiene program resulted in significant reduction in health care-associated infections.* Am J Infect Control 2013;41:482-6.

[67] Sunkesula V, Kundrapu S, Macinga DR, et al. *Efficacy of alcohol gel for removal of methicillin-resistant Staphylococcus aureus from hands of colonized patients*. Infect Control Hosp Epidemiol 2015;36:229-31.

[68] Hedin G, Blomkvist A, Janson M, Lindblom A. *Occurrence of potentially pathogenic bacteria on the hands of hospital patients before and after the introduction of patient hand disinfection*. APMIS 2012;120:802-7.

[69] Orlando P, Cristina ML, Dallera M, et al. *Surface disinfection: evaluation of the efficacy of a nebulization system spraying hydrogen peroxide.* J Prev Med Hyg 2008;49:116-9.

[70] Gidengil CA, Gay C, Huang SS, et al. *Cost-effectiveness of strategies to prevent methicillin-resistant Staphylococcus aureus transmission and infection in an intensive care unit.* Infect Control Hosp Epidemiol 2015;36:17-27.

[71] Huang SS, Septimus E, Avery TR, et al. *Cost savings of universal decolonization to prevent intensive care unit infection: implications of the REDUCE MRSA trial*. Infect Control Hosp Epidemiol 2014;35(Suppl 3):S23-31.

[72] Borde JP, Batin N, Rieg S, et al. *Adherence to an antibiotic stewardship bundle targeting Staphylococcus aureus blood stream infections at a 200-bed community hospital*. Infection 2014;42:713-9.

[73] Cook PP, Gooch M. *Long-term effects of an antimicrobial stewardship programme at a tertiary-care teaching hospital*. Int J Antimicrob Agents 2015;45:262-7.

[74] Pulcini C, Williams F, Molinari N, Davey P, Nathwani D. *Junior doctors' knowledge and perceptions of antibiotic resistance and prescribing: a survey in France and Scotland*. Clin Microbiol Infect 2011;17:80-7.

[75] Chan YY, Lin TY, Huang CT, et al. *Implementation and outcomes of a hospital-wide computerised antimicrobial stewardship programme in a large medical centre in Taiwan*. Int J Antimicrob Agents 2011;38:486-92.

Abbreviations

CDC: Centers for Disease Control and Prevention; D-ala-D-ala: D-alanyl-D-alanine; eDNA: extracellular DNA; GISA: glycopeptides-intermediate Staphylococcus aureus strains; HAIs: healthcare-associated infections; HCAAS: hospital-wide computerised antimicrobial approval system; h-GISA: heterogeneous glycopeptides-intermediate Staphylococcus aureus strains; h-VISA: heterogeneous vancomycin-intermediate Staphylococcus aureus strains; h-VRSA: heterogeneous vancomycin-resistant Staphylococcus aureus strains; ICU: intensive care unit; MGEs: mobile genetic elements; MIC: minimum inhibitory concentration; MRSA: methicillin-resistant Staphylococcus aureus strains; MSSA: Methicillin-sensitive Staphylococcus aureus; *PBPs: penicillin-binding proteins; PBP2a: PBP type 2a; PBP4: PBP type 4, a transpeptidase involved in crosslinking peptidoglycans; PRPs: penicillinase-resistant penicillins; SCCmec: staphylococcal chromosomal cassette; s-VISA: slow vancomycin-intermediate Staphylococcus aureus strains; T.E.S.T.: Tigecycline Evaluation and Surveillance Trial; TOMMs: thiazole/oxazole-modified microcins; VISA: vancomycin-intermdiate Staphylococcus aureus strains; VRSA: vancomycin-resistant Staphylococcus aureus strains; VSSA: vancomycin-susceptible Staphylococcus aureus strains.*

■ Received on November 13, 2014. Accepted on December 15, 2014.

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ORIGINAL ARTICLE

Surveillance of adverse events following immunization with meningococcal group C conjugate vaccine: Tuscany, 2005-2012

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Key words

Adverse events • Meningococcal C conjugate vaccine • Tuscany

Summary

Introduction. *Post-licensure vaccine safety studies are essential to identify uncommon events that may be difficult to assess during pre-licensure studies. The aim of our study was to evaluate the safety of serogroup C meningococcal conjugate (MCC) vaccine in Tuscany from 2005 to 2012.* **Methods**. *All adverse events (AEs) to MCC vaccine notified from 2005 to 2012 were obtained from the regional health authority.*
Results. *Following 451,570 doses administered, 110 suspected AEs were notified (mean annual reporting rate: 2.8/10,000 doses). The most frequently AE reported was fever (60%), followed by swelling at the injection site (11%) and febrile seizures (10%). Overall, 77.3% of cases were not severe, while 21.8% required hospitalization. Almost four months after the receipt of the vaccine, a one-year-old infant was diagnosed with a pervasive developmental disorder with disturbance of speech, but any link with the vaccinations received was refuted. Most AEs (80.9%) occurred after co-administration with other vaccines, especially with MMR or MMRV vaccines (42.7%) or the DTPa-HBV-IPV/Hib vaccine (33.7%).*
Discussion. *Our study confirmed the high level of safety of MCC vaccine in Tuscany: AEs proved rare and all cases had only temporary and self-resolving consequences. As usually only the most severe suspected AEs are reported, the true proportion of AEs requiring hospitalization was most probably overestimated, and it is plausible that most of these cases were in fact only temporally related.*

Introduction

Invasive meningococcal disease (IMD), a potentially life-threatening acute disease with a rapid evolution caused by the gram-negative, encapsulated and coffee-bean shaped diplococcus *Neisseria meningitides,* still represents a global public health challenge, with around 500,000 cases and 50,000 deaths occurring every year worldwide [1]. IMD can be characterized by meningitis, bacteremia, sepsis, pneumonia, or, less commonly, by localized infections such as arthritis, myocarditis, pericarditis and endophtalmitis [2-4]. Prognosis considerably improved after the introduction of antibiotic therapy, but the case fatality rate is still between 5 and 10% in industrialized countries and up to 20% of survivors suffer from lifelong sequelae, such as mental retardation, seizures, bilateral hearing loss, low vision or loss of limbs caused by the tissue necrosis [5]. According to the bacterial capsular antigens, 12 serogroups of *N. meningitidis* have been identified (A, B, C, 29E, H, I, K, L, Y, W135, X and Z), but those most often associated with the disease are serogroups A, B, C, X, Y and W135 [6]. In Europe, most meningococcal disease is caused by B and C serogroups.
Effective vaccination programmes represent the most important tool to fight against the disease. Infections caused by serogroups A, C, Y and W135 can be prevented by polysaccharide vaccines, which, however, are poorly immunogenic in children aged under two years and fail to induce immunological memory in people of any age, or by two types of conjugate vaccines, which allow the induction of immune memory also in children aged under two years [7]. The first, the meningococcal C conjugate (MCC) vaccine, is directed only against type C meningococcus; the capsular polysaccharide antigens are conjugated to an immunogenic protein, either to diphtheria toxoid, or to CRM_{197}, a non-toxic mutant of diphtheria toxin, or to tetanus toxoid and may be used after the third month of age. Recently, tetravalent vaccines against the meningococcal groups A, C, W135 and Y, mainly recommended to travellers to Sub-Saharan Africa, have been made available.
In Italy, the previously increasing trend of serogroup C meningococcal disease dramatically declined after the introduction of a universal vaccination programme against *Neisseria meningitidis* serogroup C. Tuscany was the first Italian Region to approve, in 2005, a policy of active offer of MCC vaccine with three doses to all newborns at three, five and 13 months of age, and a catch-up until six years with a single dose. Immunization with MCC vaccine was also recommended for subjects of any age at risk for developing IMD [8]. In July 2008, the newborn schedule turned to a single dose after the first year of age, at around 13 months. Therefore,

presently, at 13-15 months four vaccines are administered: MCC, pneumococcal, hexavalent and MMR or MMRV vaccines [9]. The adoption of the new schedule was established in reason of the high herd immunity created by the vaccination programme, as a result of which the incidence of meningococcal disease was reduced by 80% in children under one year of age, not yet vaccinated [10]. Catch-up of children aged two to six years was maintained by offering a single dose, in order to create a solid immunity in the population. The vaccine is also offered to the 12-14 years age group. With the recognition that on-going post-marketing monitoring is essential in order for the general population to maintain confidence in vaccine safety, the aim of the present study was to evaluate the safety and tolerability of MCC vaccine in Tuscany between 2005 and 2012 through an analysis of the suspected adverse events (AEs) to the MCC vaccine notified to the regional health authority since the inclusion of the MCC vaccination in the recommended vaccination programme.

Materials and methods

The notification of a suspected AE following a vaccination is regulated by a Ministerial Decree issued in 2003 [11]: the same procedure and reporting form as in the case of suspected AEs following pharmacological treatments are used. Consistently with Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use, an AE is a noxious and unintended response to a medicinal product used at normal dosages. A serious adverse reaction is an AE "which results in death, is life-threatening, requires in-patient hospitalisation or prolongation of existing hospitalisation, results in persistent or significant disability or incapacity, or is a congenital anomaly/birth defect". The reporting form, filled in by a healthcare worker, is sent to the pharmacovigilance unit of the respective health service, data are registered through the national network of pharmacovigilance and sent to the regional health authority, as well as to the drug or vaccine manufacturer and to the Italian Medicines Agency, within seven days. The information regarding serious AEs are also made available to the European Medicines Agency and to the other EU Member States. The reporting form must include the patient's initials, date of birth, gender, the description and the severity of the event, the effects caused, the name of the suspected drug or vaccine, possible risk factors and information on other vaccines/drugs that may have been co-administered.
As for vaccinations, the time of administration and the dose number are also reported, with the specification of the batch number and expiration date [11]. In the reporting form it must be specified whether the AE i) was not severe; ii) was severe requiring hospitalization but followed by resolution; iii) was very severe, possibly with long-term consequences; iv) caused death.

Data regarding all AEs to MCC vaccine from 2005 to 2012, collected by the Regional Health Authority, were obtained after been made anonymous. For each suspected AE the following information were made available: the specific numeric code assigned to the individual; the reporting local health unit; the date of occurrence; the subject's age (due to privacy regulations the date of birth was not available) and gender of the subjects; type, severity and outcome of the reaction; the reporting source (hospital, general practice, primary care, drug store); the contact details of the person who reported the AE; the type of administration and data regarding other vaccines, drugs, herbal or homeopathic products or food supplements that may have been co-administered. Data analysis was performed using descriptive statistics in Microsoft Excel 2010.

Results

From 2005 to 2012, 451,570 doses of conjugate meningococcal C vaccine were administered in Tuscany and, during this period, 110 cases of suspected AEs to the MCC vaccine were notified, with an average annual reporting rate of 2.8/10,000 doses. In Figure 1, the number of doses administered each year and the annual reporting rates are shown. In 2005, the average reporting rate was 1.3/10,000 doses; it increased in the following years until 2008. In that year the schedule was amended to a single dose at 13 months. In 2009, the reporting rate dropped to 1.3/10,000, then an increase of the annual reporting rate, up to 8.0/10,000 doses in 2012, was observed. The vaccine coverage at 24 months progressively increased from 65.8% in 2005, when the policy of active offer of MCC vaccine was introduced, to 90.5% in 2011; in 2012 it was 89.4% (Fig. 2). Females and males were almost equally affected (51% males, 49% females). Given the recommended schedule, AEs mostly affected the youngest age groups: 58.2% of AEs were reported in children

Fig. 1. Number of doses administered per year and average annual reporting rates of suspected adverse events following immunization with MCC vaccine per 10,000 doses administered, Tuscany, 2005-2012.

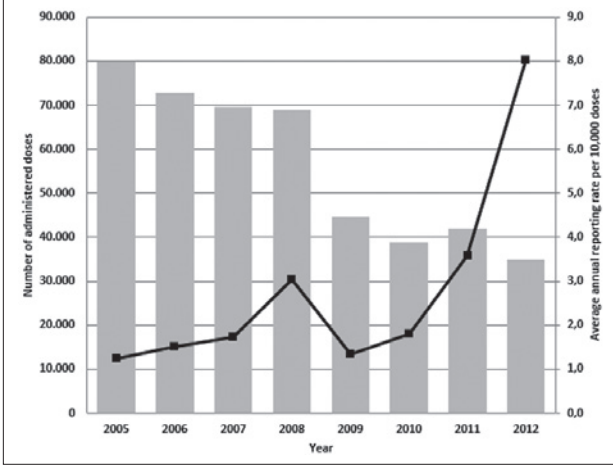


Fig. 2. Vaccination coverage with MCC vaccine at 24 months of age (%), Tuscany, 2006-2012.

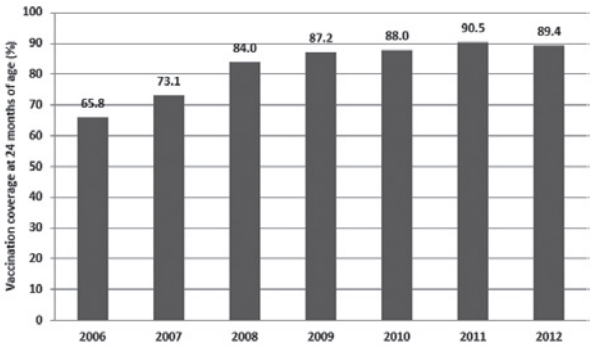
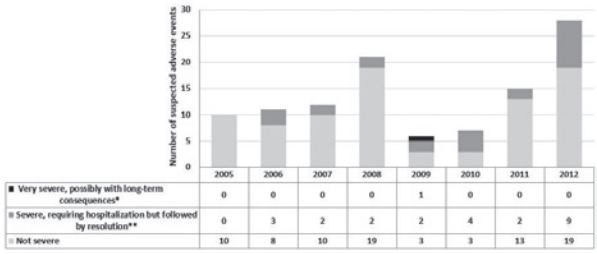


Fig. 3. Number of suspected adverse events following immunization with MCC vaccine by severity of the events, Tuscany, 2005-2012. * Lack of biological plausibility, ** Causal association not demonstrated.



	2005	2006	2007	2008	2009	2010	2011	2012
Very severe, possibly with long-term consequences*	0	0	0	0	1	0	0	0
Severe, requiring hospitalization but followed by resolution**	0	3	2	2	2	4	2	9
Not severe	10	8	10	19	3	3	13	19

aged one to two years, 15.5% in infants up to one year of age and 13.6% in children aged two to seven years (Tab. I). Most AEs, 25.5% and 19.1% respectively, were recorded in 2012 and 2008.

Overall, the most frequently reported AE was fever (60%), followed by swelling at the injection site (11%). Ten cases of febrile seizures (10%) were reported. Four cases of non-febrile seizures (3.6%) and three cases (2.7%) of unspecified convulsions were also notified. Rash was also common (10%). Other suspected AEs were vomiting (7.2%), diarrhoea, drowsiness, agitation/restlessness (4.5%), lymphadenopathy, persistent crying, pain at the injection site (3.6%). Two cases of thrombocytopenia purpura (1.8%), one of which classified as idiopathic, and one case of ataxia (1%) were also notified.

The majority of suspected AEs to MCC vaccine, 77.3%, were not severe, whereas approximately a fifth (21.8%) were severe and required patients' hospitalization, but were followed by resolution (Fig. 3). Almost half (49%) of total suspected AEs occurred the same day the vaccine was administered, most of these (87%) were not severe. Most febrile seizures (6/10) occurred between six and 11 days after vaccination.

Half of the 24 cases requiring hospitalization occurred after six days from the vaccination.

One third of hospitalized cases (8/24) was admitted to hospital due to convulsions (Fig. 4). Among these, 63% (N = 5) were febrile seizures. A fifth (5/24; 20.8%) were hospitalized for the onset of fever (all in children aged one). Another fifth was hospitalized due to disorders of the nervous system other than convulsions: two cases of hypotonia (one in a two-year-old; the other, followed by loss of consciousness, was reported in a two-month-old); sleepiness and irritability were notified for a one-year-old; an infant was admitted for absence seizure and hyperpyrexia; finally, a case of ataxia was reported in a one-year-old after concomitant administration with MMRV. The other causes of hospitalization were: development of acute dyspnea or apnea accompanied by fever (n = 2); thrombocytopenic purpura (n = 2; one after co-administration with the MMRV vaccine); giant urticaria (n = 1). In the case of an eight-year-old child, the cause of hospitalization was itchiness at the injection site.

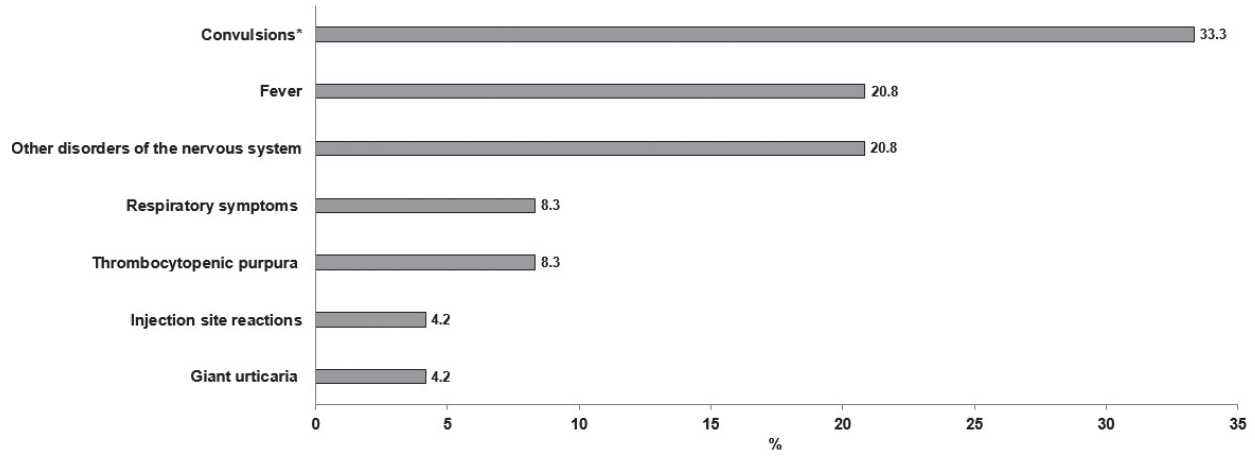
In 2009, one suspected AE was classified as "very severe, possibly with persistent consequences": it was the case of a one-year-old infant, for whom a pervasive developmental disorder with disturbance of speech was reported about four months after the administration of the MCC vaccine. According to the recommendations of the regional vaccination plan, the child was vaccinated with MMRV and MCC vaccines in March and with the 7-valent pneumococcal conjugate vaccine in April and in June. Any causal correlation with the MCC vaccine or with the other vaccines administered simultaneously or afterwards was refuted by the paediatrician, due to the lack of biological plausibility, and an autism spectrum disorder was hypothesized instead. The physician, however, was still not completely certain about the diagnosis as of July 2014.

Tab. I. Suspected AEs following immunization with MCC vaccine by age groups and year, Tuscany, 2005-2012.

Age groups	2005 N (%)	2006 N (%)	2007 N (%)	2008 N (%)	2009 N (%)	2010 N (%)	2011 N (%)	2012 N (%)	Suspected AEs 2005-2012 N (%)
< 1	2 (20)	5 (45.5)	4 (33.3)	4 (19.0)	0 (0)	0 (0)	1 (6.7)	1 (3.6)	17 (15.5)
1-2	3 (30)	2 (18.2)	6 (50.0)	11 (52.4)	5 (83.3)	2 (28.6)	12 (80)	23 (82.1)	64 (58.2)
2-7	4 (40)	3 (27.3)	2 (16.7)	1 (4.8)	0 (0)	2 (28.6)	1 (6.7)	2 (7.1)	15 (13.6)
7-14	1 (10)	1 (9.1)	0 (0)	1 (4.8)	0 (0)	0 (0)	0 (0)	1 (3.6)	4 (3.6)
> 14	0 (0)	0 (0)	0 (0)	4 (19.0)	1 (16.7)	3 (42.9)	1 (6.7)	1 (3.6)	10 (9.1)
TOTAL N (%) *	10 (9.1)	11 (10.0)	12 (10.9)	21 (19.1)	6 (5.4)	7 (6.4)	15 (13.6)	28 (25.5)	110 (100)

* % on the total suspected AEs reported between 2005 and 2012.

Fig. 4. Causes of hospitalization following MCC vaccine in Tuscany (2005-2012). * Five cases of convulsions out of eight were febrile seizures.



Most suspected AEs (89/110; 80.9%) occurred the same day of co-administration with other vaccines. Three vaccines, MCC included, were co-administered in 7.3% of AEs and a fourth vaccine was administered in one case (0.9%). The most common associations were those with the MMRV or the MMR vaccines (42.7%) and those with the hexavalent diphtheria-tetanus-acellular pertussis-hepatitis B virus-inactivated polio/Haemophilus influenzae b (DTPa-HBV-IPV/Hib) vaccine (33.7%) (Fig. 5). The majority of suspected AEs following co-administration with another vaccine (57%) occurred between 2009 and 2012, i.e. after the switch, in 2008, to a single dose at 13 months, age in which, according to the regional schedule, children are also immunized against MMR or MMRV. All ten cases of febrile seizures occurred after co-administration with other vaccines: five after MMR vaccine, two after MMRV vaccine, two after the DTPa-HBV-IPV/Hib vaccine, and one after the varicella virus vaccine. In Figure 6, the suspected AEs reported following co-administration of MCC with MMR or MMRV vaccines and the vaccination coverage at 24 months for measles and/or varicella containing vaccines are shown.

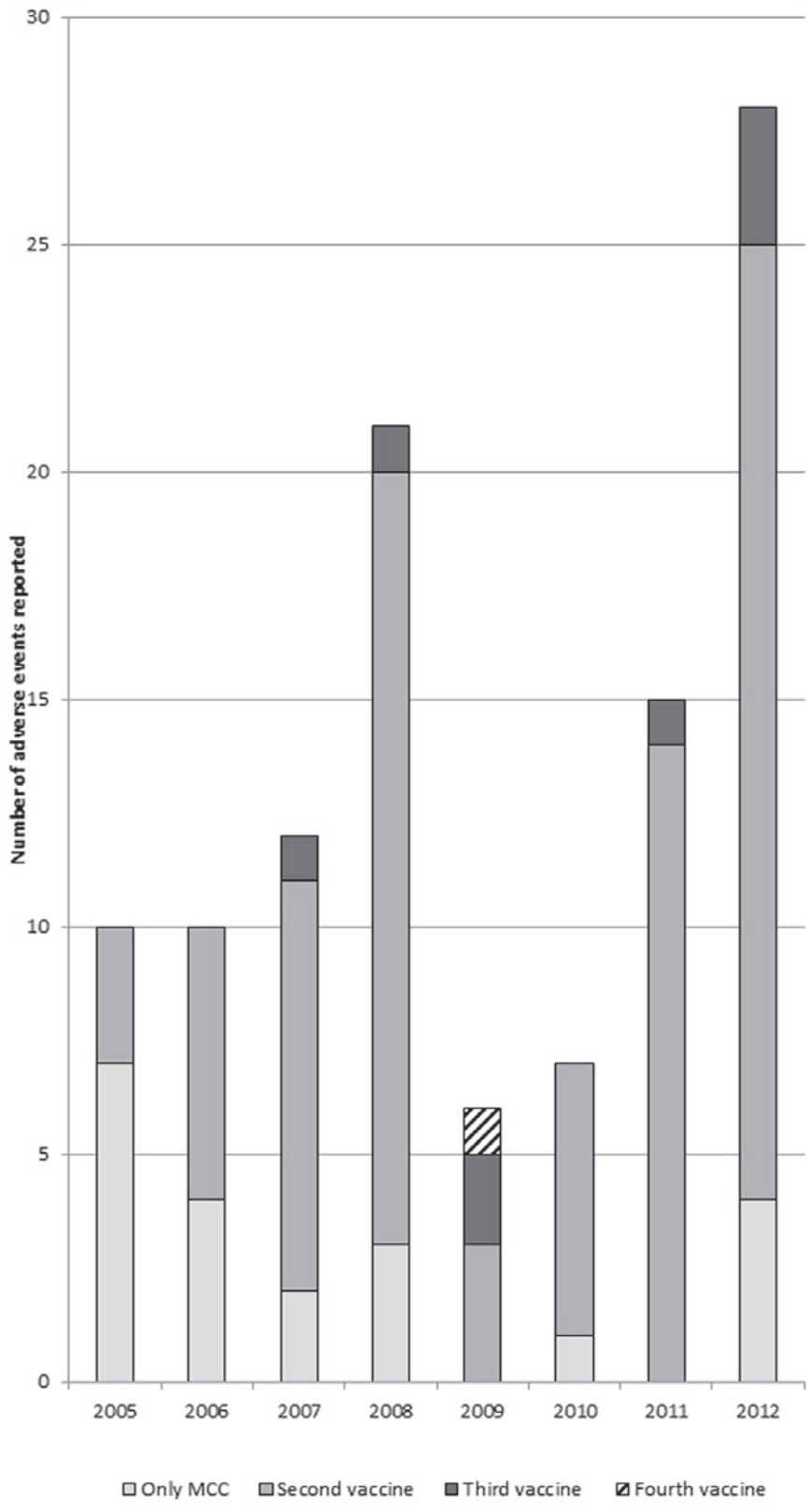
For seven severe cases that required hospitalization, data concerning the outcomes are missing. The causes of hospitalization for these cases were: febrile seizures (n = 3); unspecified convulsions (n = 1); hypotonia (n = 1); idiopathic thrombocytopenic purpura (n = 1); and, finally, hyperpyrexia (n = 1). All other cases were followed by improvement or complete resolution.

Discussion

The development of the meningococcal serogroup C conjugated vaccine was prompted by the increasing number of serogroup C infections in the 1990s, especially in children under two years: these were cases that could not be prevented on account of the poor immunogenicity granted for this age group by the already available polysaccharide vaccine. The safety and the immunogenicity of MCC vaccine had been clearly evaluated in several pre-licensure trials [12-14]. The first country to implement a national MCC immunization programme, in November 1999, was the UK, where, in less than one year and a half, each individual aged under 18 years was immunised. The Committee on Safety of Medicines Expert Working Group assessed the MCC vaccine safety profile during this immunisation campaign and concluded for its extremely favourable risks/benefits balance [15].

Post-licensure surveillance of vaccine safety is essential in order to identify uncommon events that may be difficult to assess during pre-licensure studies, when, usually, the small sample size and the relatively short period of observation only allow to describe the most common and expected AEs. Furthermore, the effects on susceptible individuals that might eventually become the target of vaccination strategies, such as subjects with medical conditions, are not commonly evaluated in pre-licensure studies [16]. Research in vaccine safety can help to maintain public confidence in immunizations and to prevent the decrease of vaccination coverage, the return of previously under control infectious diseases, as well as avoidable deaths [17]. As a matter of fact, at the present time, vaccinations are at risk to become victims of their own success, especially in Western Europe, where some illnesses against which vaccines offer protection (e.g. haemophilus influenzae infections or diphtheria) have become so sporadic that even health professionals sometimes fail to appreciate the potential of one of the most successful tools for protecting the public's health, and anti-vaccine movements have gained popularity in recent decades. When, very rarely, true severe adverse reactions to immunizations do arise, they are generally short-lived and can be treated under the circumstances in which vaccines are nowadays administered. However, although vaccines are recognized as the most effective and safest medical and public health interventions [18], second only to the development of safe water resources [19], yet, very rarely, they may cause severe AEs. It is therefore important to timely identify such events, so that regulatory actions can be promptly taken in order

Fig. 5. Suspected adverse events following immunization with MCC vaccine in co-administration with other vaccines, Tuscany, 2005-2012.



2005

Second vaccine:
- DTPa-HBV-IPV/Hib (n=1)
- MMR (n=1)
- Pneumococcal (n=1)

2006

Second vaccine:
- DTPa-HBV-IPV/Hib (n=3)
- MMR (n=2)
- Pneumococcal (n=1)
- DTPa (n=1)

2007

Second vaccine:
- DTPa-HBV-IPV/Hib (n=9)
- MMR (n=1)

Third vaccine:
- DTPa (n=1)

2008

Second vaccine:
- DTPa-HBV-IPV/Hib (n=13)
- DTPa (n=3)
- 5.5% MMR (n=1)
- 5.5% HBV (n=1)

Third vaccine:
- Pneumococcal (n=1)

2009

Second vaccine:
- MMRV (n=3)
- MMR (n=1)
- DTPa-HBV-IPV/Hib (n=1)
- Yellow fever (n=1)

Third vaccine:
- Pneumococcal (n=1)
- HAV (n=1)

Fourth vaccine:
- dT (n=1)

2010

Second vaccine:
- MMRV (n=3)
- DTPa (n=2)
- MMR (n=1)

2011

Second vaccine:
- MMRV (n=5)
- DTPa (n=4)
- DTPa-HBV-IPV/Hib (n=2)
- Pneumococcal (n=2)
- MMR (n=1)
- DTPa-IPV (n=1)

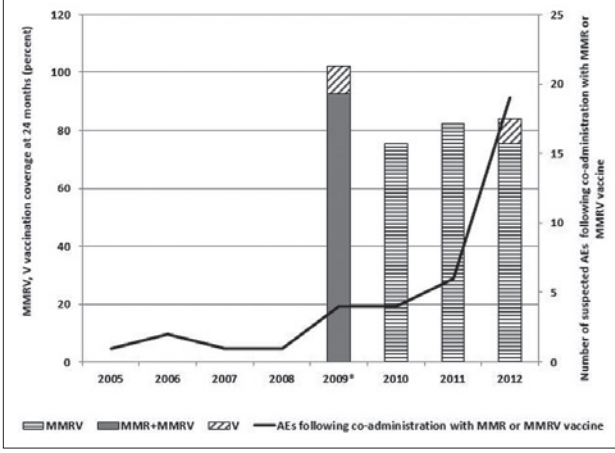
Third vaccine:
- Varicella (n=1)

2012

Second vaccine:
- MMR (n=11)
- MMRV (n=8)
- Pneumococcal (n=2)
- Varicella (n=1)
- DTPa-HBV-IPV/Hib (n=1)
- DTPa (n=1)

Third vaccine:
- Varicella (n=3)

Fig. 6. Suspected adverse events following MCC vaccination co-administered with MMR or MMRV vaccine, and MMRV, MMR, V vaccination coverage at 24 months of age, Tuscany, 2009-2012. * For 2009, the vaccine coverage at 24 months is beyond 100%, because the information obtained by the regional authority regarded the combined MMR/MMRV vaccine coverage (separate data were not available) and about the monovalent varicella (V) vaccine coverage: these two values partly overlap, as MMR and V vaccines could be administered simultaneously in the same day.



to ensure that vaccines continue to have the desirable safety and quality profiles.

The aim of the present study was to evaluate the safety and tolerability of MCC vaccine in Tuscany since its introduction into the regional immunization programme, through an analysis of the suspected AEs reported between 2005 and 2012. Due to privacy regulations, data were obtained anonymized, but we could assess the reporting rate per doses administered. Our findings confirmed the high level of safety and tolerability of the vaccine in Tuscany: AEs proved to be rare, the average annual reporting rate being 2.8/10,000 doses. The increase of the reporting rates after 2009 reflects the transition from a three-dose to a single-dose schedule and the subsequent decreased denominators. The events notified were not severe in nearly four-fifths of the cases. All suspected AEs for whom the information on the outcome was available proved to be temporary and self-resolving. For the one severe suspected AE with probable permanent disability, any causal relationship with the vaccines administered around the time of the onset of symptoms (pervasive developmental disorder with disturbance of speech) was conclusively ruled out by the paediatrician, due to the lack of biological plausibility. As for the most severe AEs registered, it is important to highlight that all febrile seizures registered occurred following co-administration with MMR, MMRV, DTPa-HBV-IPV/Hib or varicella vaccines. The risk of febrile seizures, generally occurring seven to 10 days after immunization, particularly increases with MMR or MMRV vaccines: up to 3.4 additional cases per 10,000 children [20, 21] and 5.8 additional cases per 10,000 doses [22], respectively, have been described in the literature. Results from our study indeed confirmed the post-vaccine "peak period" for febrile convulsions incidence.

One of the two cases of thrombocytopenic purpura that were registered can be put in causal correlation with the MMRV vaccine, since it occurred after co-administration with this vaccine, and while there is no evidence of an increased risk in children following immunization with MCC [23], idiopathic thrombocytopenic purpura is a recognized adverse event of measles-containing vaccines [24]: in the literature up to 1 case per 22,300 doses have been reported in association with these vaccines [25-30]. Also the case of ataxia in one-year-old infant, which resulted in complete resolution, could be related to MMRV vaccine: it occurred after co-administration with this vaccine and transient ataxia has been, very rarely, reported after MMRV vaccinations in post-marketing surveillance studies [31, 32].

Conclusions

Since usually only the most severe AEs are reported, the suspected AEs that required inpatient hospitalization (21.8%) in all likelihood overestimated the true proportion of severe AEs. Most of these observed cases may be unrelated to the immunization, but have a temporal association with it. The increase in the reporting rate in the last two years of our period of observation (2011-2012) is indeed noteworthy: it followed the publication of a study, in 2010, pointing at an increased risk for febrile seizures in subjects immunized with the MMRV vaccine [33], which contributed to focus widespread attention on the problem of adverse events following immunizations. The findings of the present study, which confirmed the high level of safety of the MCC vaccine, can contribute to support public health professionals in addressing parents' concerns regarding the safety profile of the vaccines recommended in our national and regional immunization programmes.

Acknowledgements

We would like to thank Maria Parrilli from the Regional Center of Pharmacovigilance – Local Health Unit of Florence for providing the Excel database used to carry out this study.

References

[1] Wilder-Smith A. *Meningococcal vaccine in travelers.* Curr Opin Infect Dis 2007;20:454-60.

[2] European Centre for Disease Prevention and Control. *Surveillance of invasive bacterial diseases in Europe, 2011.* Stockholm 2013.

[3] Jafri RZ, Ali A, Messonnier NE, et al. *Global epidemiology of invasive meningococcal disease.* Popul Health Metr 2013;11:17.

[4] World Health Organization. *Meningococcal vaccines: WHO position paper, November 2011.* Wkly Epidemiol Rec 2011;47:521-40.

[5] Bonanni P, Bechini A, Boccalini S, et al. *Epidemiologia e prevenzione delle malattie infettive.* In: *Igiene e Sanità Pubblica.* 2012. pp. 355-447.

[6] Stephens DS. *Biology and pathogenesis of the evolutionarily successful, obligate human bacterium Neisseria meningitidis.* Vaccine 2009;27(Suppl 2):B71-7.

[7] Trotter CL, Ramsay ME. *Vaccination against meningococcal disease in Europe: review and recommendations for the use of conjugate vaccines.* FEMS Microbiol Rev 2007;31:101-7.

[8] Giunta Regionale Regione Toscana. DGR n. 379 del 7/03/2005. *Direttive in materia di vaccinazioni e indirizzi per la stesura del nuovo calendario regionale delle vaccinazioni.* Modifiche alla delibera G.R. 24.11.2003 n°1249. 2005.

[9] Giunta Regionale Regione Toscana. DGR n. 1020 del 27-12-2007. *Aggiornamento direttive regionali in materia di vaccinazioni.* Revoca delibere n. 1249 del 24/11/2003, n.379 del 7/3/2005 e n.1060 del 10/10/2000. Modifica delibera n. 1386 del 17/12/2001. 2008.

[10] Regione Toscana DGR n. 1020 del 27/12/2007. *Aggiornamento direttive regionali in materia di vaccinazioni.* Revoca delibere n. 1249 del 24/11/2003, n.379 del 7/3/2005 e n.1060 del 10/10/2000. Modifica delibera n. 1386 del 17/12/2001. BURT n. 2 del; 9.01.200.

[11] Ministero della Salute. *Decreto 12 Dicembre 2003, Nuovo modello di segnalazione di reazione avversa a farmaci e vaccini.* 2003.

[12] Miller E, Salisbury D, Ramsay M. *Planning, registration, and implementation of an immunisation campaign against meningococcal serogroup C disease in the UK: a success story.* Vaccine 2001;20(Suppl 1):S58-67.

[13] Richmond P, Borrow R, Goldblatt D, et al. *Ability of 3 different meningococcal C conjugate vaccines to induce immunologic memory after a single dose in UK toddlers.* J Infect Dis 2001;183:160-3.

[14] MacLennan JM, Shackley F, Heath PT, et al. *Safety, immunogenicity, and induction of immunologic memory by a serogroup C meningococcal conjugate vaccine in infants: a randomized controlled trial.* JAMA 2000;283:2795-801.

[15] Medicines Control Agency. *Report of the Committee on Safety of Medicines Expert Working Group on Meningococcal Group C Conjugate Vaccines.* 2002.

[16] Australian Government. Deaprtment of Health. *Therapeutic Goods Administration. Reporting medicine and vaccine adverse events.*

[17] Lakshman R, Jones I, Walker D, et al. *Safety of a new conjugate meningococcal C vaccine in infants.* Arch Dis Child 2001;85:391-7.

[18] Andre FE, Booy R, Bock HL, et al. *Vaccination greatly reduces disease, disability, death and inequity worldwide.* Bull World Health Organ 2008;86:140-6.

[19] Plotkin S, Plotkin S. *A short history of vaccination.* In: Plotkin S, Orenstein W, Offit P, editors. *Vaccines.* Philadelphia: Elsevier-Saunders 2013, pp. 1-13.

[20] Barlow WE, Davis RL, Glasser JW, et al. *The risk of seizures after receipt of whole-cell pertussis or measles, mumps, and rubella vaccine.* N Engl J Med 2001;345:656-61.

[21] Farrington P, Pugh S, Colville A, et al. *A new method for active surveillance of adverse events from diphtheria/tetanus/pertussis and measles/mumps/rubella vaccines.* Lancet 1995;345:567-9.

[22] MacDonald SE, Dover DC, Simmonds KA, et al. *Risk of febrile seizures after first dose of measles-mumps-rubella-varicella vaccine: a population-based cohort study.* CMAJ 2014;186:824-9.

[23] Andrews N, Stowe J, Miller E, et al. *Post-licensure safety of the meningococcal group C conjugate vaccine.* Hum Vaccin 2007;3:59-63.

[24] Maglione MA, Das L, Raaen L, et al. *Safety of vaccines used for routine immunization of U.S. children: a systematic review.* Pediatrics 2014;134:325-37.

[25] France E, Glanz J, Xu S, et al. *Risk of immune thrombocytopenic purpura after measles-mumps-rubella immunization in children.* Pediatrics 2008;121:e687–92.

[26] Andrews N, Stowe J, Miller E, et al. *A collaborative approach to investigating the risk of thrombocytopenic purpura after measles-mumps-rubella vaccination in England and Denmark.* Vaccine 2012;30:3042-6.

[27] Rajantie J, Zeller B, Treutiger I, et al. *Vaccination associated thrombocytopenic purpura in children.* Vaccine 2007;25:1838-40.

[28] Black C, Kaye JA, Jick H. *MMR vaccine and idiopathic thrombocytopaenic purpura.* Br J Clin Pharmacol 2003;55:107-11.

[29] Miller E, Waight P, Farrington CP, et al. *Idiopathic thrombocytopenic purpura and MMR vaccine.* Arch Dis Child 2001;84:227-9.

[30] Jonville-Béra AP, Autret E, Galy-Eyraud C, et al. *Thrombocytopenic purpura after measles, mumps and rubella vaccination: a retrospective survey by the French regional pharmacovigilance centres and pasteur-mérieux sérums et vaccins.* Pediatr Infect Dis J 1996;15:44-8.

[31] GlaxoSmithKline. *PRIORIX-TETRA® Combined measles, mumps, rubella and varicella vaccine, live, attenuated. Product monograph.* Mississauga, Ontario: 2014.

[32] Knuf M, Zepp F, Meyer CU, et al. S*afety, immunogenicity and immediate pain of intramuscular versus subcutaneous administration of a measles-mumps-rubella-varicella vaccine to children aged 11-21 months.* Eur J Pediatr 2010;169:925-33.

[33] Klein NP, Lewis E, Baxter R, et al. *Measles-containing vaccines and febrile seizures in children age 4 to 6 years.* Pediatrics 2012;129:809-14.

■ Received on November 28, 2014. Accepted on December 15, 2014.

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ORIGINAL ARTICLE

Logistic regression of attitudes and coverage for influenza vaccination among Italian Public Health medical residents

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Key words

Medical Residents • Influenza vaccination • Risk perception

Summary

Introduction. *A few number of literature specifically addresses vaccination uptake among Public Health Residents (PHRs). Influenza vaccine attitudes and risk perceptions of PHRs across Italy were studied, contributing to literature on influenza vaccination uptake predictors, in particular among young physicians.*
Methods. *An online survey was conducted in 25 Schools of Public Health in Italy in 2011-2012. Results were analysed using prevalence and logistic regression methods.*
Results. *A total of 365 Italian public health residents were included in the study. Vaccination uptake was confirmed by 22.2 and 33.2% of PHRs in 2010-2011 and 2011-2012, respectively. For the 2010-2011 influenza season, vaccination was associated with male sex (adj-OR 3.43; 95%CI = 1.5-7.84) and vaccination history (adj-OR 29.44; 95%CI = 6.4-135.04). For the 2011-2012 season, vaccination was significantly associated with having had between one and three influenza vaccinations in the previous five years (adj-OR 11.56; 95%CI = 6.44-20.75) or more than three (adj-OR 136.43; 95%CI = 30.8-604.7) and with individual participation in general population vaccination campaigns (adj-OR 1.85; 95%CI = 1.01-3.41).*
Discussion. *Italian residents in public health have no confidence and a low personal risk perception about vaccinations therefore taking no measures to protect patients, general population and themselves. Annual influenza vaccination acceptance is associated with influenza vaccine uptake in the previous years and personal involvement in general population vaccination campaigns. These factors should be considered for the design of future campaigns targeting public health residents.*

Introduction

The bottom line health impact and the degree of success of influenza vaccination campaigns among health care workers (HCWs) has been largely discussed in literature [1-3].
Influenza vaccination is universally recognized as an essential intervention to minimize the risk for medical-care-acquired influenza illnesses among older patients and with comorbidities [4, 5].
Moreover, within HCW communities, this vaccination can reduce absence from work during annual epidemics [5, 6].
Nevertheless the communication inside the public is increasing. Influenza vaccination rates are always below the ECDC requirements. US data report 66.9% of adherence in 2012 but even European and Italian data for 2012 campaign were always below the threshold of 75% [7-9].
With regards to influenza vaccination, it is important to focus on the psychological factors that influence medical professionals regarding their vaccination behaviour [9, 10]. Attitudes and determinants associated with influenza vaccine uptake have been studied and theorized, using different models, to explain fears, complaints, disease complacency, and HCW worries and willingness to participate in annual influenza vaccination campaigns, both actively and passively [9-15].
Several studies from different European countries explored the link between HCW influenza vaccine coverage rates and their knowledge, attitudes and practice (KAPs) [14, 15].
Coverage among adults in Italy is uneasily traceable due to the non-mandatory policy on influenza vaccination in our country
It is also important to note, however, that self-reported surveys on influenza vaccination can be considered a good proxy for the real coverage rate and data reported [16].
HCWs have an important role in influencing, motivating and empowering patients, the general population and other health care workers to promote vaccination and to actively take action to reduce biological risk in sanitary settings [16, 17].
In particular, Public Health medical residents (PHRs) could be considered a particularly influential and important group, given that they act as public health advisors

for the general population and for other medical residents [18].
The main objective of our study was to investigate, through a multicenter survey, determinants for the uptake of influenza vaccination among Italian PHRs. This paper will also contribute to literature on influenza vaccination uptake predictors, in particular among young physicians.

Methods

Data were collected with an anonymous, self-administered questionnaire, sent by e-mail, previously tested in a pilot study presented at the XII Italian Public Health Conference held in Rome from 12 to 15 of October 2011 and partially based on a survey conducted among medical residents in the University of Palermo [18].
Preliminary data from two regional settings (Calabria and Sicily) were published in the past year [19].
Each questionnaire included nine sections with a total of 20 items as outlined below:

a) Demographic and academic characteristics: sex, age, year of graduation, speciality if already attended (categorized in clinical, surgical and diagnostic duties).
b) Episodes of influenza/like illness in the previous five years.
c) Considering themselves as part of a high risk group for contracting influenza
d) Personal experiences of seasonal influenza vaccination in the previous five years (categorized as "never vaccinated", "one to three times" and "more than three times"), for the 2009-2010 seasonal influenza vaccination, for pandemic A (H1N1) influenza vaccination, and for 2010-2011 seasonal influenza vaccination.
e) Reasons for getting vaccinated or not getting vaccinated for 2010-2011 and for 2011-2012 seasonal influenza.
f) Main sources of information on influenza/influenza vaccination were investigated as closed- end questions (categorized as "none", "recommendation of Health Minister", "scientific sources" and "mass media").
g) The influence of the Influenza A(H1N1) pandemic vaccination campaign on vaccination choice during the following influenza seasons.
h) Attitude to recommend influenza vaccination to patients: categorized as "Yes, according to the recommendations of the Health Minister", "Yes, according to my clinical experience", "No, leaving patients to their free will", "No".
i) Participation to influenza vaccination campaign among HCWs and the general population during his/her residency program.
j) Recommended public health strategy to implement low coverage rate of influenza vaccination among HCWs (multidisciplinary courses, mandatory vaccination, vaccination incentives, settled university training on influenza vaccination, other).

We piloted a multicentre study using data collected from November 2011 to February 2012 among Italian PHRs in Hygiene, Preventive Medicine and Public Health. In total, 25 out of the 32 Italian postgraduate Italian Public Health Schools participated in the study. The post-graduate public health schools involved in the study, were Torino, Milano Bicocca, Milano Statale, Brescia, Pavia, Verona and Padova in the North, Bologna, Parma, Perugia, Modena, Siena, L'Aquila, Roma Cattolica, Roma Tor Vergata, Roma Sapienza 2, Chieti and Ancona in the Center, Bari, Napoli Federico II, Napoli Seconda Università, Catanzaro, Palermo, Messina and Catania in the South of Italy.
We collected a mailing list of PHR whose schools had accepted to participate to the project and asked the residents to complete the questionnaire anonymously.
Information contained in the questionnaires was only available to, and only reviewed by, the research investigators, with stringent assurance of the confidentiality of the individual data. The study was approved by the Institutional Review Board of the Azienda Ospedaliera Universitaria "P. Giaccone" of Palermo, Italy.
We entered all the information in a database created within EpiInfo 3.5.1 software. All the data were analysed using the R statistical software package [20].
Absolute and relative frequencies were calculated for qualitative variables. Quantitative variables were normally distributed and summarized as means (standard deviation).
The associations between the potential determinants and the two different dichotomous outcomes were evaluated by the Fisher Exact Test (dichotomous variables) or Chi-square test (categorical variables).
Odds ratio (OR) and adjusted OR (adj-OR) with 95% confidence intervals (95%CIs) were also calculated. Differences in means were compared with the Student t-test.
All variables found to have a statistically significant association (two-tailed p-value < 0.05) with vaccine uptake in the univariate analysis were included in two different multivariable stepwise logistic-regression models, having the following dependent variables:

a) Italian PHRs's decision to get vaccinated against seasonal influenza (season 2010-2011).
b) Italian PHRs's decision to get vaccinated against seasonal influenza (season 2011-2012)

Measures of goodness of fit were calculated to compare logistic regression models by using Akaike's Information Criterion (AIC) and the model with the lowest AIC was considered the best fit. The significance level chosen for all analysis was $p < 0.05$ (two-tailed).

Results

The overall response rate among Italian Public Health residents from the participating schools was 80.1% (365/456). The general characteristics of the 365 PHRs included in the study are summarized in Table I.

Tab. I. Characteristics of the 365 Italian public health residents (PHRs) responding to the survey, collected from November 2011 to February 2012.

Response rate: 80.1%	n=365/456
Age, mean in years ± SD	31.4 ± 4.5
Age, median in years (interquartile range)	30 (28-33)
Gender, n (%)	
- male	145 (39.7)
- female	220 (60.3)
Age Class in years, n (%)	
- <29	99 (27.1)
- 29 to 31	123 (33.7)
- >31	143 (39.2)
Year of residency, n (%)	
- R1	106 (29.0)
- R2	105 (28.8)
- R3	88 (24.1)
- R4	66 (18.1)

In the component investigating knowledge, 64% of PHR reported that they recommended influenza vaccination to their patients as per guidelines from the Ministry of Health. An additional 19.5% declared they recommended influenza vaccination based on their clinical evaluation alone and 15.3% of medical residents did not recommend influenza vaccination, leaving patients free to decide. Only 1.4% did not recommend influenza vaccination at all.

Of the PHRs respondents in this study, 52% did not check any information sources about influenza vaccination, 28% report having read scientific reports (scientific literature, Center for Disease Control and Prevention, European Centre for Disease Control and Prevention, World Health Organization), and only 10% declaring they had read recommendations from the Italian Ministry of Health. In 2011-2012, the main reason for influenza vaccination uptake, as reported by the 123 PHRs who were vaccinated, was to avoid virus diffusion among relatives and the general population (69.9%). However, the main reason for not being vaccinated against influenza in 2011/2012 was "I do not consider myself in a high risk group for developing influenza and its complications" (data not shown in Table).

In the component investigating *attitudes,* 81 PHRs (22.2%) were vaccinated for seasonal influenza during the 2010-2011 influenza vaccine campaign. During the 2011-2012 influenza vaccine campaign, 123 PHRs (33.7%) were vaccinated for seasonal influenza (data not shown in Table).

Table II reports KAP (knowledge attitudes and practice) towards influenza vaccination. 61.1% of the sample was never vaccinated in the previous five years. For 80.8% of participants the occurrence of the Pandemic A (H1N1) influenza and the subsequent campaign did not impact their practice and attitudes towards the influenza vaccination.

Moreover, 48.2% (n = 176) of PHRs suggested that training and organisation of multidisciplinary courses on influenza vaccination, are the best strategy for increasing influenza immunization rate among Italian health care workers (HCW). The next most frequently recommended course of action was to improve University training (during degree and postgraduate medical schooling) on influenza and vaccinology (23.3%; n = 85) (data not shown in Table).

Factors associated with vaccine uptake during the 2010–2011 and the 2011-2012 influenza seasons are presented

Tab. II. Attitudes, behaviours and perception on influenza vaccination of the 365 Italian PHRs responding to the survey.

	n = 365
Personal experiences of influenza vaccination for the previous five years (2004-2008)	
- never	223 (61.1)
- for one to three years	103 (28.2)
- more than three years	39 (10.7)
Main information sources on influenza vaccination, n (%)	
- none	190 (52.0)
- recommendations of Health Minister	37 (10.1)
- mass media	31 (8.5)
- scientific reports (Literature, CDC, ECDC, WHO)	103 (28.3)
- other sources (blog, youtube, facebook, etc.)	4 (1.1)
Attitude to recommend influenza vaccination for patients, n(%)	
- Not recommended	5 (1.4)
- No, leaving patients to their free will	56 (15.3)
- Yes, according to the recommendations of the health minister	233 (63.8)
- Yes, according to my clinical evaluation	71 (19.5)
Pandemic A (H1N1) influenza modified attitudes on influenza vaccination, n(%)	
- No	295 (80.8)
- Yes, less predisposed to influenza vaccination	27 (7.4)
- Yes, less predisposed to vaccinate patients	14 (3.8)
- Yes, less predisposed to vaccinate myself and patients	27 (7.4)
- Yes, more prone to update on influenza vaccination	2 (0.5)

in Table III. In the multivariate analysis, 2010-2011 uptake of seasonal influenza vaccination was strongly associated with being male (adj-OR 3.43; 95%CI: 1.5-7.84) and with having received more than three vaccinations in the previous five years (adj-OR 29.44; 95%CI: 6.4-135.04). Vaccination against 2011-2012 seasonal influenza was significantly associated with having had one to three (adj-OR 11.56; 95%CI: 6.44-20.75) or more than three (adj-OR 136.43; 95%CI: 30.8-604.7) vaccinations against influenza in the previous five years and with the respondent's participation in vaccination campaigns targeting general population during the period of the participant's residency programme. (adj-OR 1.85; 95%CI: 1.01-3.41)

Discussion

As previously reported in literature, the uptake of seasonal influenza vaccine (22.2% in 2010-2011 season and 33.7% in 2011-2012 season) among PHRs has increased over the past few years, but remains below the national and European target (75% of minimum coverage recommended) [9, 13, 17].
The main reason for vaccine uptake among HCWs, as supported by other studies, is that vaccination protects family members, friends and patients from being infected [10, 11, 21].
Somewhat contradictory to this, due to the role of Italian PHRs, they consider the risk of transmitting influenza as being very low, insufficient to justify influenza vaccination (70.2%; an increase of 8.6% in 2011/2012 compared to 2010/2011). These findings support several studies conducted at local and regional level [10-12].
Furthermore, in comparing Italian findings with similar contexts, a decreasing trend in influenza vaccination coverage can be observed among the whole French Medical Residents (with a rate of 45.6% in 2008 and 65.6% in 2007). 19.6% of the French MRs declared they were not willing to receive influenza vaccination for the next seasonal campaign [21].
While our study's sample covers only a specific target of adults (the majority are over 30 years of age), we could extend results to our medical doctors population.
La Torre et al. stated that 30-49 years HCWs were less likely to get the vaccination compared to younger colleagues (adj-OR=0.66; 95% confidence interval, CI: 0.52-0.83) and females also are less likely to get vaccinated (adj-OR=0.64; 95%CI: 0.51-0.8) [10].
Previous studies focusing on the H1N1 campaign, showed that Italian medical doctors use different types of information sources, including Internet (41.5%) and hospital internal communication (33.1%) [11].
In our study the majority of interviewees declared they did not get any information on the seasonal vaccination campaign at all, nor received it from scientific reports.

Tab. III. Multivariable logistic regression analysis of factors involved in the decision to get vaccinated during the 2010-2011 (A) and 2011-2012 influenza season (B) of the 365 Italian PHRs responding to the survey.

	A: Vaccine uptake during the 2010-2011 season		B: Vaccine uptake during the 2011-2012 season	
	Crude OR (95%CIs)	Adj OR (95%CIs)	Crude OR (95%CIs)	Adj OR (95%CIs)
Gender				
- females	Referent	Referent	Referent	Referent
- males	1.66 (1.01-2.73)	3.43 (1.5-7.84)	0.95 (0.61-1.49)	0.91 (0.51-1.64)
Age, in years				
- < 29	Referent		Referent	-
- 29 to 31	0.86 (0.44-1.69)	-	0.89 (0.51-1.58)	-
- > 31	1.48 (0.8-2.74)	-	1.14 (0.67-1.96)	-
Year of residency				
- R1	Referent		Referent	
- R2	1.07 (0.55-2.1)	-	1.15 (0.65-2.06)	-
- R3	1.34 (0.68-2.66)	-	1.2 (0.65-2.19)	-
- R4	1.29 (0.62-2.71)	-	1.5 (0.79-2.86)	-
Influenza vaccination in the previous 5 years				
- never	NC	-	Referent	Referent
- yes, from one to three times	Referent	Referent	12.5 (7-22.4)	11.56 (6.44-20.75)
- yes, more than three times	24.81 (5.67-108.5)	29.44 (6.4-135.04)	153.4 (34.8-676.9)	136.43 (30.8-604.7)
Partecipation to vaccination campaigns among HCWs	1.9 (1.1-3.2)	1.88 (0.77-4.58)	1.5 (0.9-2.4)	1.08 (0.56-2.08)
Partecipation to vaccination campaigns among general population	3.19 (1.91-5.34)	1.87 (0.83-4.25)	2.9 (1.8-4.6)	1.85 (1.01-3.41)

Only a few respondents reported use of mass media or unofficial Internet sources. This result suggests that PHR are not active seekers of information on influenza or influenza vaccination and instead need to be treated as passive, with information *delivered* in the most easy-to-use and accessible manner. PHRs who took part in this study showed little interest in anti-immunization information sources or materials. Information use is relevant to understand subsequent attitude towards immunization: public trust is also risen by correct information through media and national campaign [22].

PHRs participating in this study do not accurately perceive threat or severity of influenza, and this directly translates to their lack of promoting vaccination to patients. Their behavioural intent is influenced by their perceived lack of threat and their variable evaluation of the benefits of vaccination. PHRs recognize the importance of the problem and acknowledge that there is a need for more information and awareness on the topic. Same attitude was retrieved in one example of public health intervention called Intervention Mapping. This was defined as an organisational theory for the planning of the health promotion regarding the Influenza campaign which the vaccination is a benefit in health to succeed and the audience is supposed to understand the reasons and methods which drive to it. Emotional and Impulsive reactions distinguish between a reflective system and an impulsive mechanism: the first generates decision and judgement which influences behaviour while the impulsive system seeks pleasure and avoids delusion. During the one's attitude determination, many elements of the organisational field were showing the representation of different behaviours: building and transmitting information actively, meetings, convenient access and timetable arrangements [23].

While many PHRs consider the risk of transmitting influenza as being very low, insufficient to justify influenza vaccination, the perceived benefits of accepting vaccination against influenza have to do with protecting family members, friends and patients from being infected. This suggests an understanding of perceived susceptibility and severity of influenza that is not extended to the individual themselves. In other words, PHRs may see themselves as carriers and transmitters of influenza in hospital but not in the community and not potential victims. Perception of risk can influence the vaccine attitude either for the fact that adverse events are more visible than benefits either because this decision can be amended later, when necessary. Mainly, the most important perception is the self perception of benefits instead of risk. (46% wanted to be a role model and between them, 80% received Pandemic vaccination) [24].

Moreover, PHRs attitudes about influenza vaccine uptake was related to a first-person involvement during post-graduate training programme in flu vaccination campaigns among general population. This evidence should result in a standardization and harmonization of European postgraduate medical school courses to promote positive influenza vaccination attitudes.

Nevertheless, vaccination history and behaviours already adopted are clearly the strongest factors associated with influenza vaccine uptake among PHRs, and future campaign should also consider using approaches such as positive deviance to motivate non-vaccinated to vaccinate and, in turn, promote vaccination [25].

Positive deviance and similar community-driven approaches permit PHRs to take part in the development of campaigns, drawing on their personal experiences with vaccination and jointly developing plans and strategies to motivate vaccination uptake and active HCW-parent vaccination promotion within their community.

The main limitation of this study was as follow. The questionnaire is not a highly reliable mean of anonymous investigation if administered by e-mail. Despite this, Llupia et al. compared self-reported data on influenza vaccination to real coverage and concluded that the former is a good proxy, although it might somewhat overestimate the actual uptake [15].

Another limit of this study was the possible economic and environmental influences that are less explored which could also account for differences in promoting vaccination. For example, study outcomes can also be explained by socioeconomic determinants, which show a relationship between higher socioeconomic background characteristic and lower uptake of influenza vaccination [26, 27].

In conclusion, the risk perception in HCW may need to be addressed in future campaign. Future behavioural communications direct to change management in the health care sector campaigns targeting PHRs and healthcare workers should consider emotional and social responsibility elements relevant to stress on.

Ethical approval

The study was approved by the Institutional Review Board of the AOUP "P. Giaccone" of Palermo, Italy.

Acknowledgements

The authors thank Elena Azzolini, Cesare Baldini, Angelo Battaglia, Ilaria Bernardini, Alessio Biafiore, Deris Boemo, Francesca Campanella, Lorenzo Capasso, Mario D'Asta, Edoardo D'Ippolito, Ferretti Valentina, Francesco Gilardi, Sara Gobbo, Alessandro Guaccero, Giuseppe La Maestra, Giuseppe Licitra, Nadia Mallamace, Pietro Manotti, Rocco Micò, Annalisa Montante, Marta Nobile, Anna Odone, Raffaele Palladino, Riccardo Papalia, Salvo Parisi, Caterina Pasqua, Marika Passaro, Paola Pelullo, Lucia Pennacchietti, Andrea Poscia, Alessandra Ricciardi, Alessandro Rinaldi, Gabriele Romani, Laura Rossi, Maria Rosaria Russo, Silvia Sanasi and Shilpi Sironi for their helpful local collection of the data.

References

[1] Fiore AE, Uyeki TM, Broder K, et al.; Centers for Disease Control and Prevention (CDC). *Prevention and control of influenza*

with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2010. MMWR Recomm Rep 2010;59(RR-8):1-62.

[2] Pearson ML, Bridges CB, Harper SA et al. *Influenza vaccination of health care personnel: recommendations of the HICPAC and the ACIP.* MMWR Rep 2006;55(RR-2):1-16.

[3] Abramson ZH. *What, in fact, is the evidence that vaccinating health care workers against seasonal influenza protects their patients? A critical review.* Int J Family Med 2012;(2012):2054-64.

[4] Simonsen L, Taylor RJ, Viboud C, et al. *Mortality benefits of influenza vaccination in elderly an ongoing controversy.* Lancet Infect Dis 2007;7:658-66.

[5] Music T. *Protecting patients, protecting health care workers: a review of the role of influenza vaccination.* Int Nurs Rev 2012;59:161-7.

[6] Amodio E, Anastasi G, Di Pasquale M, et al. *Influenza vaccination among health care workers and absenteeism from work due to influenza-like illness in a teaching hospital in Palermo.* Ital J Public Health 2010;357:30-44.

[7] European Centre for Disease Prevention and Control. *Annual Epidemiological Report 2013. Reporting on 2011 surveillance data and 2012 epidemic intelligence data.* Available at: http://www.ecdc.europa.eu/en/publications/Publications/annual-epidemiological-report- 2013.pdf (last accessed: 16/10/2014).

[8] CDC. *Surveillance of influenza vaccination coverage – United States, 2007-08 through 2011-12 influenza seasons.* MMWR 2013;62(ss04):1-29. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/ss6204a1.htm (last accessed: 16/10/2014).

[9] La Torre G, Di Thiene D, Cadeddu C, et al. *Behaviours regarding preventive measures against pandemic H1N1 influenza among Italian healthcare workers, October 2009.* Euro Surveill 2009;14(49). pii: 19432.

[10] La Torre G, Semyonov L, Mannocci A, et al. *Knowledge, attitude, and behaviour of public health doctors towards pandemic influenza compared to the general population in Italy.* Scand J Public Health. 2012;40:69-75.

[11] Prematunge C, Corace K, McCarthy A, et al. *Factors influencing pandemic influenza vaccination of health care workers: a systematic review.* Vaccine 2012;30:4733-43.

[12] Costantino C, Amodio E, Vitale F, et al. *Attitudes, behaviours and perceptions of Italian General Practitioner trainees towards influenza vaccination in Western Sicily (Italy).* Ital J Public Health 2012;9:33-9.

[13] WHO. *Influenza (seasonal) fact sheet number 211. 2009 April.* Available at: http://www.who.int/mediacentre/factsheets/fs211/en/ (last accessed: 16/10/2014).

[14] Toy WC, Janosky JE, Laird SB. *Influenza immunization of medical residents: knowledge, attitudes, and behaviors.* Am J Infect Control 2005;33:473-5.

[15] Llupia A, Garcia-Basteiro A, Mena G, et al. *Vaccination behaviour influences self-report of influenza vaccination status: a cross-sectional study among health care workers.* PloS one 2012,7(7)e39496.

[16] *National Vaccination Plan 2012-2014 Italian Minister of Health.* Available at: http://www.salute.gov.it/imgs/c_17_pubblicazioni_1721_allegato.pdf (last accessed: 16/10/2014).

[17] Centers for Disease Control and Prevention (CDC). *Prevention and control of influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP).* MMWR Morb Mortal Wkly Rep 2011;60:1128-32.

[18] Amodio E, Tramuto F, Maringhini G, et al. *Are medical residents a "core group" for future improvement of influenza vaccination coverage in health-care workers? A study among medical residents at the University Hospital of Palermo (Sicily).* Vaccine 2011;29:8113-7.

[19] Costantino C, Battaglia A, D'Asta M, et al. *Knowledge, attitudes and behaviors regarding influenza vaccination among Hygiene and Preventive Medicine residents in Calabria and Sicily.* Euromed Biomed J 2012;7:77-83.

[20] R Development Core Team. R statistical software package, version 2.2.0, 2005. Available at: http://www.r-project.org (last accessed: 16/10/2014).

[21] Mir O, Adam J, Gaillard R, et al. *Vaccination coverage among medical residents in Paris, France.* Clin Microbiol Infect 2012;18:E137-9.

[22] Dubé E, Laberge C, Guay M, et al. *Vaccine hesitancy. An overview.* Hum Vaccin Immunother 2013;9:1763-73.

[23] Kok G, Van Essen GA, Wicker S, et al. *Planning for influenza vaccination in health care workers: An Intervention Mapping Approach.* Vaccine 2011;29:8512-9.

[24] Bouadma L, Barbier F, Biard L, et al. *Personal decision making criteria related to seasonal and pandemic A(H1N1) Influenza vaccination acceptance among French Health Care workers.* Plos One 2012;7(7):e38646.

[25] Bradley EH, Curry LA, Ramanadhan S, et al. *Research in action: using positive deviance to improve quality of health care.* Implement Sci 2009;4:25.

[26] Chiatti C, Di Rosa M, Barbadoro P, et al. *Socioeconomic determinants of influenza vaccination among older adults in Italy.* Prev Med 2010;51:332-3.

[27] Bonito AJ, Lenfestey NF, Eicheldinger C, et al. *Disparities in immunizations among elderly Medicare beneficiaries, 2000 to 2002.* Am J Prev Med 2004;27:153-60.

■ Received on November 3, 2014. Accepted on November 30, 2014.

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Published online April 2015.

Journal registered at "Registro pubblico degli Operatori della Comunicazione" (Pacini Editore SpA registration n. 6269 - August 29, 2001).

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