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INFLUENZA IN CHILDREN:
Diagnosis, treatment and prevention

by

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To my Family

ABSTRACT

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Influenza in children – Diagnosis, treatment and prevention
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Background: The burden of influenza on children is substantial. Although mortality rates are low, the incidence of influenza is highest in children, among whom also complications are frequent. A more accurate recognition of influenza in children could enable the rational use of antiviral drugs and help to avoid unnecessary courses of antibiotics. Limited data exists on the efficacy of oseltamivir treatment and the trivalent inactivated influenza vaccine (TIV) in children.

Aims and methods: We sought for signs and symptoms that could help clinicians to diagnose influenza on clinical grounds in a case-control study in children <13 years of age. We further assessed the feasibility of different diagnostics methods during the early stage of the illness in children aged 1-3 years. The efficacy of early oseltamivir treatment (started <24h from the onset of symptoms) was evaluated in a randomized controlled trial (RCT) conducted in children 1-3 years of age, and the effectiveness of TIV to prevent laboratory-confirmed influenza was determined in a prospective, observational cohort study conducted among children aged 9 months to 3 years of age.

Results: Fever was the only symptom predicting influenza in children. The sensitivity of conventionally used laboratory methods to detect influenza during the first 24h of illness was 92%. The sensitivity of the influenza rapid test in the same setting was 90% for influenza A and 25% for influenza B. Early oseltamivir treatment shortened the duration of the illness in children with influenza A by 3.5-4.0 days, but no efficacy was observed against influenza B. The effectiveness of TIV was 84% against the well-matched influenza A, while no effectiveness against the mismatched influenza B was observed.

Conclusions: Laboratory diagnostics are needed for a reliable diagnosis of influenza in children and were found sensitive already during the early stage of the illness. Early oseltamivir treatment was highly effective against influenza A, but no efficacy was seen against influenza B. TIV is effective also in young children if a good match between the vaccine and circulating strain is achieved.

Keywords: Influenza, children, clinical diagnosis, rapid test, antivirals, neuraminidase inhibitors, oseltamivir, inactivated influenza vaccine

TIIVISTELMÄ

Santtu Heinonen
Lasten influenssa: diagnostiikka, hoito ja ehkäisy
Lastentautioppi, kliininen laitos, Turun yliopisto

Tausta: Influenssa aiheuttaa lapsille merkittävän tautitaakan. Vaikka influenssan aiheuttama kuolleisuus lapsilla on vähäistä, ovat sairastuvuusluvut toistuvasti korkeimpia lapsilla. Myös influenssan aiheuttamat komplikaatiot ovat lapsilla yleisiä. Influenssan luotettava tunnistaminen jo oireiden perusteella voisi auttaa vähentämään turhia antibioottikuureja. Inaktivoidun influenssarokotteen ja oseltamiviirihoidon tehosta pienillä lapsilla on olemassa niukasti tutkimustietoa.

Tavoitteet ja menetelmät: Selvitimme tapaus-verrokki asetelmassa, onko löydettävissä oireita, joita voitaisiin käyttää apuna influenssan kliinisessä diagnostiikassa lapsipotilailla. Arvioimme lisäksi erilaisten diagnostisten tutkimusmenetelmien käyttökelpoisuutta taudin alkuvaiheessa. Tutkimme varhain (<24 h oireiden puhkeamisesta) aloitetun oseltamiviirihoidon tehoa satunnaistetussa, lumekontrolloidussa kaksoissokkotutkimuksessa 1 – 3-vuotiailla lapsilla sekä arvioimme inaktivoidun influenssarokotteen tehoa havainnoivassa kohorttitutkimuksessa 9 kk – 3 v ikäisillä lapsilla.

Tulokset: Kuume oli ainoa oire, joka itsenäisesti ennusti influenssaa lapsipotilailla. Influenssa pystyttiin havaitsemaan perinteisesti käytetyillä laboratoriomenetelmillä jo 24 tunnin kuluessa taudin alusta 92 %:lla influenssaa sairastavista lapsista. Influenssapikatestin herkkyys taudin alkuvaiheessa oli 90 % influenssa A:n ja 25 % influenssa B:n diagnostiikassa. Varhainen oseltamiviirihoito lyhensi taudin kestoa influenssa A:ta sairastavilla lapsilla 3.5 – 4.0 vuorokautta, mutta lääke ei ollut tehokas influenssa B:tä vastaan. Inaktivoidun influenssarokotteen teho hyvin rokotekantaa vastannutta influenssa A:ta vastaan oli 84 %, mutta rokote ei antanut suojaa huonosti rokotekantaa vastannutta influenssa B:tä vastaan.

Johtopäätökset: Lapsilla influenssan diagnostiikassa tarvitaan apuna laboratoriotutkimuksia. Influenssavirus pystytään havaitsemaan yleisesti käytetyillä diagnostisilla tutkimusmenetelmillä herkästi jo taudin ensimmäisenä päivänä. Varhain aloitettu oseltamiviirihoito on tehokas influenssa A:ta vastaan, mutta teho on huono influenssa B:n kohdalla. Inaktivoitu influenssarokote on tehokas myös pikkulapsilla silloin, kun rokotteen sisältämät viruskannat vastaavat hyvin epidemian aiheuttavia viruskantoja.

Avainsanat: Influenssa, lapset, kliininen diagnoosi, pikatesti, viruslääke, neuraminidaasi-inhibiittori, oseltamiviiri, influenssarokote

TABLE OF CONTENTS

ABBREVIATIONS

aHR	Adjusted hazard ratio
AOM	Acute otitis media
ARDS	Acute respiratory distress syndrome
CDC	Centers for Disease Control and Prevention
CI	Confidence interval
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
cDNA	Complementary deoxyribonucleic acid
CSF	Cerebrospinal fluid
cRNA	Complementary ribonucleic acid
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EIA	Enzyme immunoassay
EIND	Emergency investigational new drug
EMA	European Medicines Agency
EUA	Emergency use authorization
FDA	U.S. Food and Drug Administration
FEV1	Forced expiratory volume in the first second
FIMEA	Finnish Medicines Agency
GBS	Guillain-Barré syndrome
GMT	Geometric mean titer
HA	Hemagglutinin
HAI	Hemagglutination inhibition
IF	Immunofluorescence
ILI	Influenza-like illness
IQR	Interquartile range
ITT	Intention-to-treat
LAIV	Live attenuated influenza vaccine
LR	Likelihood ratio
M1	Influenza matrix 1 protein
M2	Influenza matrix 2 protein
mRNA	Messenger ribonucleic acid
NA	Neuraminidase
NP	Nucleoprotein
OR	Odds ratio
PCR	Polymerase chain reaction
PEP	Post-exposure prophylaxis
POC	Point-of-care (test)
RCT	Randomized, controlled trial
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
TIV	Trivalent inactivated (influenza) vaccine
THL	Finnish National Institute for Health and Welfare
TR-FIA	Time-resolved fluoroimmunoassay
VE	Vaccine efficacy / effectiveness

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by the Roman numerals I-IV. The original publications have been reproduced with the kind permission of the copyright holders.

I Heinonen, S., Peltola, V., Silvennoinen, H., Vahlberg, T., Heikkinen, T. Signs and symptoms predicting influenza in children: a matched case-control analysis of prospectively collected clinical data. Eur J Clin Microbiol Infect Dis 2011 (e-pub ahead of print) DOI:10.1007/s10096-011-1479-4

II Heinonen, S., Silvennoinen, H., Lehtinen, P., Vainionpää, R., Heikkinen, T. Feasibility of diagnosing influenza within 24 hours of symptom onset in children 1-3 years of age. Eur J Clin Microbiol Infect Dis 2011:30:387–392

III Heinonen, S., Silvennoinen, H., Lehtinen, P., Vainionpää, R., Vahlberg, T., Ziegler, T., Ikonen, N., Puhakka, T., Heikkinen, T. Early oseltamivir treatment of influenza in children 1-3 years of age: a randomized controlled trial. Clin Infect Dis 2010;51:887–894

IV Heinonen, S., Silvennoinen, H., Lehtinen, P., Vainionpää, R., Ziegler, T., Heikkinen, T. Effectiveness of inactivated influenza vaccine in children aged 9 months to 3 years: an observational cohort study. Lancet Infect Dis 2011:11:23–29

1 INTRODUCTION

As media attention around influenza peaked during the recent 2009 influenza H1N1 pandemic, influenza may well be the best-known viral infection by the general public at the moment. The global importance of influenza is largely due to the continuous antigenic evolution of the virus and its exceptional ability to cause pandemics. The mortality rates are usually highest during the pandemics, and the overall mortality due to the devastating 1918 pandemic is estimated to have ranged between 50 and 100 million (Johnson and Mueller, 2002). Although influenza pandemics certainly pose a serious public health threat, pandemics occurred only 4 times during the last century, whereas interpandemic seasonal influenza is circulating in the community virtually every winter season. Consequently, a large part of the overall burden of influenza illness is due to seasonal epidemics, and the World Health Organization (WHO) estimates that annually 250,000 – 500,000 deaths are caused by seasonal influenza (WHO, 2009a).

A substantial part of the overall burden of influenza is carried by children. Although the mortality figures are low in children, the attack rates of influenza are consistently highest in the pediatric population (Monto and Sullivan, 1993), and children frequently develop complications requiring antibiotic treatment (Heikkinen et al., 2004). Furthermore, young children are hospitalized due to influenza at rates similar to adults with underlying conditions (Neuzil et al., 2000). The importance of children's influenza is further emphasized by their salient role in the spread of the disease in the community (Loeb et al., 2010).

This study was undertaken to assess different aspects of the control of influenza in children. We sought for signs and symptoms that could help clinicians in diagnosing influenza. We further assessed the performance of different diagnostic methods during the early stage of the illness and determined the efficacy of early oseltamivir treatment. In addition, we estimated the effectiveness of the inactivated influenza vaccine among young children.

2 REVIEW OF THE LITERATURE

2.1 Influenza viruses

Influenza viruses are negative-stranded, segmented, enveloped RNA viruses that belong to the orthomyxoviridae family. Three types of influenza viruses have been identified; influenza A, B and C, of which influenza A viruses are of the greatest importance. Influenza A viruses are not only responsible for the majority of seasonal epidemics, but are also capable of causing pandemics. All the previous pandemics with virological documentation have been caused by influenza A viruses. Influenza B viruses cause seasonal epidemics, but they are considered unable to cause pandemic outbreaks. Influenza C has been studied less and is considered to be of little importance to humans causing only local epidemics with mild symptoms, although in children clinical presentation comparable to influenza A and B has been described (Matsuzaki et al., 2006). This review will, however, focus on influenza A and B viruses.

2.1.1 Viral structure

The genome of influenza A and B viruses consists of 8 negative-stranded RNA segments. Each RNA segment codes for one or two proteins, and the whole genome contains codes for a total of 10 and 11 different proteins in influenza A and B viruses, respectively. These proteins have different roles in viral replication or they act as structural proteins. The gene segments are situated in the viral core and are associated with nucleoproteins (NP) to form ribonucleoproteins (RNPs). RNA polymerase components B1, B2 and A (PB1, PB2 and PA) are bound to the RNPs and resultant RNP complexes act as viral transcriptase units (Noda et al., 2006). These RNP complexes are surrounded by a layer of matrix 1 (M1) proteins that are the most abundant structural proteins of the virus. The external layer of the virus consists of a lipid membrane and glycoprotein envelope formed by two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). These glycoproteins are also major antigenic determinants that define the subtype and the strain of the virus.

HA has an important role in the binding of the virus in the cellular sialic acid residues and subsequent entry of the virus in to the cell. In addition, HA is the primary viral antigen to which the host's antibody response is directed. Consequently, it is also the main target molecule of vaccination against influenza. NA has an important role in the release of newly formed viruses from the surface of the infected cell. With its enzymatic activity, NA cleaves the sialic acid residues where the progeny viruses are attached, thus releasing them from the host cell. NA is the target molecule of the antiviral drugs oseltamivir and zanamivir, which are sialic acid analogues that inhibit the activity of NA. The influenza A virus envelope contains also matrix 2 (M2) protein which acts as a channel protein controlling the uncoating of the viral particle after endocytosis (Schnell and Chou, 2008). M2 is the target protein of the antiviral drugs amantadine and rimantadine.

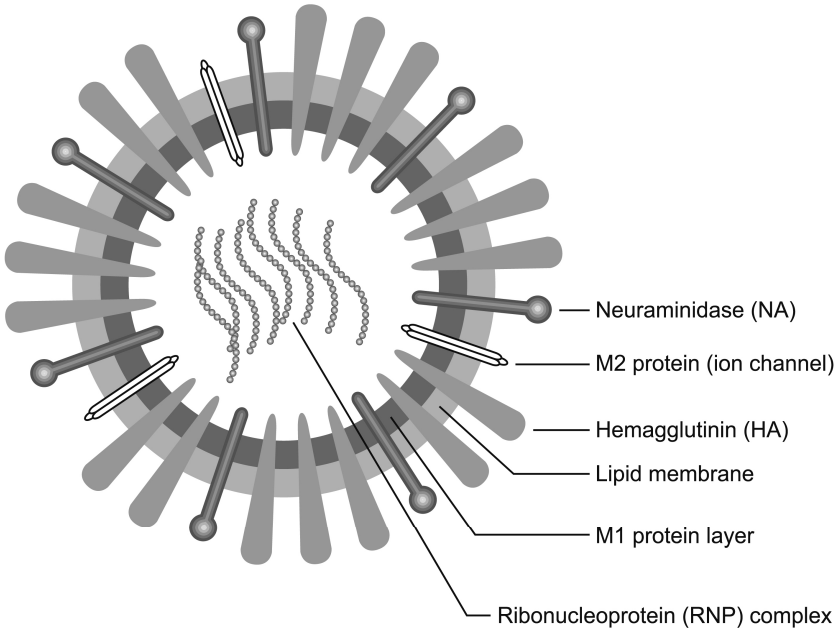


Figure 1. Structure of influenza A virus.

Influenza A viruses are divided into subtypes according to their surface glycoproteins. Sixteen different subtypes of hemagglutinin (H1-H16) and 9 subtypes of neuraminidase (N1-N9) have been identified (Fouchier et al., 2005) (Table 1). All of these subtypes have been isolated in aquatic birds that are considered the primary reservoir for influenza (Webster et al., 1992). Only four subtypes have been described to cause epidemics in humans (H1N1, H1N2, H2N2, H3N2). Small outbreaks of other subtypes in humans have been reported without confirmed sustained human-to-human transmission (H5N1, H7N7 and H9N2) (Claas et al., 1998, Fouchier et al., 2004, Peiris et al., 1999). Few subtypes cause epidemics also in swine and horses. In addition, influenza A viruses have been isolated from several other mammals, including dogs, cats, seals, whales and minks (Webster et al., 1992). Influenza B viruses are not divided into subtypes. However, the antigenic evolution of influenza B viruses has lead to the development of two antigenically distinct lineages, referred to as B/Victoria-like and B/Yamagata-like lineages. Influenza B primarily infects humans, although it has also been isolated from seals (Osterhaus et al., 2000).

Table 1. Prevalence of different hemagglutinin (H) and neuraminidase (N) subtypes in influenza A viruses, isolated in aquatic birds, horses, swine and humans. Subtypes in parentheses have been isolated only sporadically in humans without confirmed sustained human-to-human transmission. (Adapted from Ziegler and Heikkinen, 2010)

	Aquatic Birds	Horses	Swine	Humans
H1	+		+	+
H2	+			+
H3	+	+	+	+
H4	+			
H5	+			(+)
H6	+			
H7	+	+		(+)
H8	+			
H9	+			(+)
H10	+			
H11	+			
H12	+			
H13	+			
H14	+			
H15	+			
H16	+			
N1	+		+	+
N2	+		+	+
N3	+			
N4	+			
N5	+			
N6	+			
N7	+	+		(+)
N8	+	+		(+[1])
N9	+			

[1]N8 has not been isolated in humans but serological evidence of an earlier epidemic exists.

2.1.2 Replication

In humans, influenza viruses target primarily the epithelial cells of the respiratory tract. The viral HA binds to the sialic acid residues on the epithelial cell surface. This binding triggers receptor-mediated endocytosis, a process where the virus enters the cell in an endosome. Once in the cell, the pH of the endosome starts to decrease, which leads to conformational changes in the HA and the uncoating of the endosome that allows the release of the viral RNA into the cell cytosol (Matlin et al., 1981, Schnell and Chou, 2008).

In the cell, the viral RNA is transported to the nucleus where transcription takes place. Negative-sense RNA is transcribed to positive-sense messenger RNA (mRNA) and complementary RNA (cRNA) by a polymerase complex that consists of three viral proteins: PB1, PB2 and PA. Synthesized mRNA is transported to the cytoplasm where it is translated into viral proteins, while cRNA is used in the nucleus to synthesize viral RNA copies for the new viruses being produced. Newly synthesized viral RNA forms

again RNP complexes that move towards the cell surface where the new virus particles are assembled. In the process of budding, virus particles receive the lipid membrane with HA and NA and move outside of the host cell. The virus particle still remains attached to the outer surface of the cell and NA activity is needed to cleave the connection between sialic acid residues and HA to finally release the newly formed progeny virus.

2.1.3 Antigenic evolution

An important feature of the influenza A virus is that is has two distinct ways to escape pre-existing immunity directed against it. Antigenic drift refers to continuous antigenic evolution and the small changes that occur in the HA and NA antigens of the circulating seasonal influenza viruses, whereas an antigenic shift denotes the emergence of a new influenza virus subtype with pandemic potential. Antigenic drift occurs also in influenza B viruses, but antigenic shift is not possible, as there are no subtypes in influenza B viruses.

2.1.3.1 Antigenic drift

The replication of influenza viruses is an error-prone process where mutations occur frequently. One reason for this is the lack of proofreading mechanisms associated with the viral RNA polymerase. The majority of the hosts' antibody responses and neutralizing antibodies are directed against HA, which is the major antigenic determinant of the influenza virus. Therefore, the accumulation of point mutations in the gene coding for antigenic domains of HA may give rise to new virus variants that are antigenically different from the parent virus, and consequently are not effectively neutralized by the pre-existing antibodies. This continuous and gradual process of antigenic changing is referred to as antigenic drift.

As a consequence of antigenic drift, an earlier influenza vaccination or even prior infection with the same subtype virus may not provide protection against forthcoming viruses, as the strains may be antigenically distinct. Thus, antigenic drift lays the basis for repeated seasonal epidemics and causes the need for annual vaccinations with a regularly updated vaccine composition (Carrat and Flahault, 2007).

2.1.3.2 Antigenic shift

Antigenic shift denotes an introduction of a new influenza virus subtype into the human population. Due to a lack of pre-existing immunity, this newly emerged strain has the potential to spread efficiently in the population and cause a pandemic. An antigenic shift may occur by three different mechanisms.

First, a new subtype may be directly transmitted to humans from another host species. An example of this kind of direct transmission is the highly pathogenic avian influenza virus H5N1. Previously, H5N1 was considered to be solely an avian influenza virus, and the virus had not been isolated from other species than birds. However, in 1997, the H5N1 virus was isolated from a three-year-old boy who died from severe influenza pneumonia in Hong Kong (Claas et al., 1998), suggesting that the virus had adapted to the human host. By August 9, 2011, 564 confirmed H5N1 cases in humans were reported to the World Health Organization (WHO), with 330 deaths (WHO, 2011a).

Fortunately, the virus has not established a sustainable human-to-human transmission, although sporadic clusters have been reported (Ungchusak et al., 2005, Zaman et al., 2011).

A second mechanism of antigenic shift is the reassortment of viral genes (genetic reassortment). This process may occur if one cell is simultaneously infected with two influenza A viruses of a different subtype. During the assembly of the new virus, the RNA segments of two different strains may get mixed. This results in a new combination of viral genes and the emergence of a novel influenza strain unknown to our immune defenses. Pigs are considered optimal hosts and mixing vessels where the genetic reassortment could occur, as they can be easily infected by swine, avian and human influenza viruses (Scholtissek et al., 1985, Ito et al., 1998). The novel, swine originated H1N1 strain that caused the recent 2009 pandemic was a reassortment of two different strains that had been isolated earlier in pigs (Dawood et al., 2009). However, genetic reassortment has been described to take place also in humans infected with two different influenza A virus subtypes; a H1N2 virus was isolated from a patient infected simultaneously with the H1N1 and H3N2 subtypes of viruses (Nishikawa and Sugiyama, 1983).

The third potential mechanism of antigenic shift is a re-emergence of an old influenza virus strain. It is possible that a virus, which has circulated earlier, could remain in a hidden state for years or decades when no antigenic changes would occur. During this time, the immunity in the population wanes and if such a virus is reintroduced into the human population, the majority of the people would not have a pre-existing immunity directed against it. Although no new virus has been created in this process, it is often considered as a form of antigenic shift due to lack of pre-existing immunity in the population. This kind of re-emergence could occur, for example, if a frozen strain of H2N2 would be released from a laboratory into the population. When the H1N1 subtype re-emerged in 1977, two decades after it was previously detected, it was closely related to the strain isolated in 1950 (Nakajima et al., 1978), and was suspected to have accidentally escaped from a laboratory source (Webster et al., 1992). The re-introduction of H1N1 in 1977 caused the Russian flu, a mild pandemic affecting primarily children and teenagers.

2.2 Epidemiology

2.2.1 Seasonal influenza

Seasonal influenza causes significant morbidity and mortality everywhere in the world. WHO estimates that seasonal influenza epidemics result in three to five millions cases of severe illness and 250,000 – 500,000 deaths annually (WHO, 2009a). Although a great majority of the deaths are among the elderly, the impact of influenza is substantial also in children. The attack rates are highest among children who also frequently suffer from bacterial complications, such as acute otitis media (AOM). In addition, young children are hospitalized at similar rates as adults with high-risk conditions.

2.2.1.1 Seasonality

In the temperate zones, influenza epidemics occur annually during the winter months. In Finland, as well as in other Northern hemisphere countries, the epidemic is usually timed between December and May (Peltola et al., 2003), while in the Southern hemisphere, the peak is normally between July and October (Lambert et al., 2005). Typically, the epidemic lasts approximately 60 days (Neuzil et al., 2000, Peltola et al., 2003), although, at least during some outbreaks, influenza viruses can circulate in the community throughout the winter season (Heikkinen et al., 2003). However, in the tropics, similar seasonal distribution is not usually present (Moura, 2010). In Vietnam, for example, influenza viruses are detected year round (Nguyen et al., 2009), and in Hong Kong, two separate peaks can be detected annually (Chiu et al., 2002). Several explanations for the seasonality of the epidemics have been proposed, including varying transmission of influenza viruses in different temperature and humidity levels (Lowen and Palese, 2009), seasonal variations in social behavior, and various host associated factors. However, the definitive reason still remains unknown and it is plausible that the seasonality of influenza is a complex, multi-factorial phenomenon (Lofgren et al., 2007).

Previously it was thought that seasonal epidemics move from one continent to another following a seasonal tendency. However, recent studies on antigenic evolution suggest that seasonal influenza has its epicenter in East and South-East Asia in a region of continuous viral circulation where the majority of the antigenic changes take place. According to this hypothesis, the epicenter acts as a reservoir for influenza, from where the new seasonal strains are seeded to temperate regions every year in the beginning of the cold season (Russell et al., 2008, Rambaut et al., 2008).

2.2.1.2 Attack rates

Several factors affect the severity of influenza epidemics. The attack rate estimates vary according to circulating strain and existing immunity in the population. Study specific factors, such as the measured outcome and the characteristics of population studied, also have an impact on the estimates. However, children have been consistently reported to suffer from the highest attack rates of all the age groups (Glezen and Couch, 1978, Monto and Sullivan, 1993). In the most severe seasons, rates of virologically confirmed influenza infections have exceeded 30% in pre-school aged children (Glezen and Couch, 1978). In a prospective cohort study conducted in Turku, Finland, during two consecutive influenza seasons in 2000-2002, the average annual attack rates of culture-confirmed influenza were 18% in children aged ≤6 years and 14% in 7-13-year-old children (Heikkinen et al., 2004). In the first season when the A(H1N1) strain dominated, the attack rates where similar in all age groups. By contrast, during the second season, which was dominated by the A(H3N2) strain, the attack rates were highest among children <3 years, and only sporadic cases were seen in school aged children. A potential explanation for the age dependent variations seen is the lack of pre-existing immunity in the youngest children. In a 25-year prospective cohort study, the attack rates of culture-confirmed influenza in children ranged from 1% to 19%, according to the severity of the season, and averaged to 9.5% (Neuzil et al., 2002).

2.2.1.3 Outpatient visits

Although the majority of influenza episodes do not require medical consultation, influenza still places a substantial burden on health care systems. Periods when influenza is circulating in the community are associated with increases in the rates of outpatient visits and hospitalizations. In North-American studies based on surveillance data and diagnoses obtained from administrative and insurance databases, the annual estimates of influenza-associated physician visits have ranged from 6 to 28 per 100 children (Neuzil et al., 2000, Menec et al., 2003). In a study among children aged <5 years using laboratory- confirmed endpoints, influenza resulted annually in 5.0 to 9.5 outpatient visits per 100 children and 0.6-2.7 emergency department visits per 100 children (Poehling et al., 2006a). In the same study, influenza accounted for 10 to 19% of the visits for fever or respiratory infections during influenza seasons.

2.2.1.4 Hospitalizations

In a large cohort study based on United States (US) insurance data over 19 years, the estimated annual rates of excess hospitalizations associated with influenza ranged from 4 to 104 per 10,000 children per year (Neuzil et al., 2000). The highest rates were seen in the youngest children, <6 months of age, and the rates decreased with increasing age. In another similar study, 23.1 hospitalizations per 10,000 children were estimated in children <2 years of age annually (Izurieta et al., 2000). These studies have relied on epidemiological data, and slightly lower estimates have been observed in studies assessing laboratory- confirmed influenza hospitalizations. In children aged <5 years, the estimates have ranged from 9 to 12 hospitalizations per 10,000 children per year (Mullooly and Barker, 1982, Poehling et al., 2006a), and in children <18 years, 1 to 3 laboratory-confirmed hospitalizations per 10,000 children per year were observed during a 5- year surveillance (Dawood et al., 2010). In a recent retrospective study over 16 years, the average annual rate of laboratory-confirmed influenza hospitalization in Finland was 3.6 per 10,000 children aged ≤16 years (Silvennoinen et al., 2011a). The hospitalization rates where the highest in the youngest children, and in children aged <1 year the annual incidence was 22.5 per 10,000 children. Influenza A accounted for 82% of all hospitalizations and influenza B for 18%. The rates of hospitalizations have been consistently highest in the youngest children, and children aged <1 year have been estimated to get hospitalized due to influenza- related illnesses at rates similar to those in adults with high-risk conditions (Neuzil et al., 2000). Hospitalizations further increase the risk for nosocomial influenza infections. In a retrospective Australian study, nosocomial infections accounted for 26% of the critical influenza infections in children (Kappagoda et al., 2000).

2.2.1.5 Mortality

Although a great majority of mortality associated with seasonal influenza occurs in the elderly population, pediatric deaths are not exceptional. In a large US study based on nationwide surveillance data from 1976-1999, the annual death rates for children aged <1 years and 1-4 years were 0.3 and 0.2 deaths per 100,000 children, respectively (Thompson et al., 2003). During the 2003-2004 influenza season, 153 laboratory-confirmed deaths were reported in children in the US (Bhat et al., 2005). The death rate was highest, 0.88 per 100,000 children, in infants <6 months of age. Surprisingly, a

majority of these children had been previously healthy, with underlying medical conditions reported only in 33% of the children. Thereafter, the number of laboratory-confirmed pediatric deaths in the US has decreased, ranging from 46 to 88 deaths annually. Simultaneously, the proportion of influenza-associated pediatric deaths with evidence of bacterial co-infections, especially infections caused by *Staphylococcus aureus*, has increased from 6% to 38% (Finelli et al., 2008, Peebles et al., 2011).

2.2.1.6 Economic impact

The economic burden of influenza is comprised of direct and indirect costs. Direct costs include pharmaceutical costs and costs of medical care, whereas indirect costs result from productivity losses due to work absenteeism and premature deaths. In the US, seasonal influenza has been estimated to result annually in 10.4 billion USD of direct, and 16.3 billion USD of indirect costs (Molinari et al., 2007). In Norway, a country with a population and social security system comparable to Finland, the overall direct costs of seasonal influenza were estimated at 22 million USD, and indirect costs of 231 million USD, totaling at 253 million USD annually (Xue et al., 2010).

In Finland, the direct costs of influenza in children aged 6 – 35 months were estimated at 3.7 million € (Salo et al., 2006). When also indirect costs were included, the overall costs of influenza in this age group totaled 7.1 million €. With respect to children's influenza, indirect costs are mainly due to parental absenteeism when employees are taking care of their sick children. In a Finnish prospective cohort study, 57% of the parents of influenza-infected children aged ≤6 years were absent for at least one day, and the mean duration of absenteeism (among parents absent for ≥1 day) was 3.6 days (Heikkinen et al., 2004).

2.2.2 Pandemic influenza

The emergence of a novel type of influenza virus that the population has no direct immunity against poses a threat of a severe, globally-spreading epidemic referred to as a pandemic. Historical documents since 1510 are suggestive of several previous influenza pandemics, but due to the lack of scientific evidence the focus of this review is on the five virologically documented pandemics that have occurred since the 20th century.

2.2.2.1 1918 H1N1 "Spanish flu"

The 1918 pandemic caused by the A(H1N1) strain is by far the most devastating of documented pandemics. The first outbreaks of unusually severe respiratory illness were reported in March 1918, simultaneously in three distinct locations in the US. However, the true geographic origin of the pandemic virus still remains unsolved. The 1918 pandemic was characterized by three separate waves. The first wave rapidly spread from the US to Europe during the spring of 1918, with the US military troops on their way to fight in World War I. In Europe, the first outbreak was reported in Madrid, Spain, in May 1918, hence the name "Spanish flu". From Europe, the epidemic continued its spread to Asia. During summer months in the northern hemisphere, the pandemic ceased, but was followed by a second, more severe wave in

the autumn of 1918. The third wave occurred in most parts of the world in the spring of 1919 (Taubenberger and Morens, 2006).

The global death toll is estimated at 50 million, but may well go up to 100 million due to presumable underreporting (Johnson and Mueller, 2002). The overall case fatality is estimated at >2.5%, compared to significantly lower estimates of <0.1% in other pandemics. Mortality was highest in children aged <5 years and in the elderly, but different from other pandemics, high rates of mortality were observed also among healthy adults aged 20-40, creating a "W"-shaped age specific mortality curve characteristic for the 1918 pandemic (Taubenberger and Morens, 2006). The majority of deaths in all age-groups were likely caused by secondary bacterial pneumonia (Morens et al., 2008).

The 1918 pandemic was caused by an influenza A(H1N1) strain that later continued to circulate in the population as a seasonal virus (Glezen, 1996). Actually, all the isolated pandemic and seasonal influenza A viruses that have caused epidemics in humans after 1918 have been descendants of the 1918 virus, including drifted H1N1 viruses and reassorted H3N2 and H2N2 viruses containing key genes from the 1918 virus (Taubenberger and Morens, 2006). Despite great scientific efforts, the origin and the basis of the devastating pathogenicity of the 1918 virus remains unclear. In 2005, the 1918 virus was reconstructed using plasmid-based reverse genetics, and subsequent studies have confirmed its extraordinary virulence in animal models (Tumpey et al., 2005). The genetic analyses indicate that the 1918 virus was an avian-like virus. However, it is genetically highly distinct from the contemporary avian strains, suggesting that there might be another, yet unidentified, source for the 1918 virus (Taubenberger and Morens, 2006).

2.2.2.2 1957 H2N2 "Asian flu"

After the 1918 pandemic, the A(H1N1) strain continued to circulate and caused milder, seasonal epidemics from the 1920s to 1950s. In March 1957, a new pandemic virus emerged in Yunan Province of China and rapidly spread to Hong Kong. The virus was isolated already in May 1957, and was soon found to be antigenically different from the previous influenza strains. Later the strain was designated A(H2N2), a genetic reassortant virus that had received its HA, NA and PB1 genes from an avian virus and the rest of the genes from the circulating A(H1N1) seasonal virus (Hilleman, 2002). From Asia, the virus spread first to Australia and Europe, reaching the US during the summer. Within six months, the pandemic spanned the globe, causing a second wave occurring in the autumn of 1957. The H2N2 soon replaced A(H1N1) as the main seasonal virus and continued to cause epidemics until 1968 (Glezen, 1996).

During the first wave of the 1957 pandemic ~70,000 excess deaths were estimated in the US, totaling 115,700 deaths for the entire pandemic period of 1957-1960 (Glezen, 1996). Less accurate data are available for the global mortality. Young children, elderly, and pregnant women were identified to be at increased risk. However, this time there was no similar peak seen in the mortality of the working age adults as seen during the 1918 pandemic.

2.2.2.3 1968 H3N2 "Hong Kong flu"

As with the 1957 pandemic, the 1968 pandemic virus also emerged in China in May 1968. From there, the epidemic rapidly spread to Hong Kong and caught the attention of Western media. The epidemic first spread in Asia during the summer of 1968 and was not detected in the US until September. In the US, the epidemic peaked in December 1968, and was estimated to result in ~100,000 excess deaths during the 4-year pandemic period from 1968 to 1971, making it significantly milder than the two previous documented pandemics (Glezen, 1996).

This time the pandemic was caused by a newly emerged A(H3N2) strain. This virus contained avian origin HA and PB1 genes, while the other six genes, including the NA gene, were from circulating seasonal A(H2N2). This antigenic similarity between the H3N2 and H2N2 and the existing immunity in the population against H2N2 viruses was considered to account for the milder pandemic caused by H3N2 (Viboud et al., 2005). Again, the new pandemic H3N2 strain rapidly superseded the earlier seasonal H2N2 strain and has continued to circulate as a seasonal virus since then.

2.2.2.4 1977 H1N1 "Russian flu"

The first outbreaks of the "Russian flu" were reported in northern China in May 1977. The epidemic spread in Russia during the summer of 1977, and throughout the rest of the world in 1978. The 1977 epidemic was not caused by a newly emerged virus, but an old A(H1N1) strain that had not been detected for nearly two decades (Gregg et al., 1978). The re-emerged A(H1N1) strain closely resembled the A(H1N1) strain isolated in 1950 (Nakajima et al., 1978), and it was suspected that the new strain had been accidentally released from a laboratory source (Webster et al., 1992). Because similar A(H1N1) viruses had been circulating before 1957, the majority of the adult and elderly population had some degree of immunity directed against this strain. Therefore, the pandemic was confined almost entirely to children and teenagers who had high attack rates exceeding 50% (Fox et al., 1982). Fortunately, the excess mortality was low and remained at similar levels as during seasonal epidemics.

After the pandemic, A(H1N1) continued to circulate as a seasonal virus, but unlike in earlier pandemics, it failed to replace the previous seasonal strain A(H3N2). Consequently, two influenza A strains currently circulate, causing seasonal epidemics. As "Russian flu" was milder and distinct from previous pandemics in several ways, it is not always listed as a pandemic and may be considered instead as a severe seasonal epidemic.

2.2.2.5 2009 H1N1 "Swine flu"

The 2009 pandemic virus emerged in Mexico with first clinical cases reported by CDC on April 17, 2009. The virus was soon identified as a swine origin novel reassortment A(H1N1) strain, containing genes from two different viruses circulating in pigs. Six genes were similar to those in a triple-reassortant virus isolated earlier in pigs in Northern America, and two genes were most closely related to viruses circulating in pigs in Eurasia (Dawood et al., 2009). From Northern and Central America, the epidemic rapidly spread worldwide, and by the end of May 15,510 virologically

confirmed cases in 53 countries were reported to WHO (WHO, 2009a). On June 11, 2009, WHO declared that the first pandemic of the 21st century had begun.

The pandemic period lasted officially until August 2010. In the Southern hemisphere countries, the outbreaks occurred during the winter months, typical for the seasonal epidemics, while in some Northern hemisphere countries, two peaks were observed: first, a milder peak in the spring / summer of 2009, and a more severe peak in the autumn / winter of 2009-2010. On the basis of seroprevalence studies, the overall attack rate has been estimated at ~20%, and children were the age group most frequently affected with attack rates exceeding 40% (Zimmer et al., 2010, Miller et al., 2010). Similarly, hospitalization rates were highest among children, and in the US, 117 hospitalizations per 100,000 children aged 0-17 years per year were estimated (Shrestha et al., 2011). This is 4-11 times greater than the previously published rates for seasonal influenza in the period of 2003-2008 (Dawood et al., 2010). At the same time, unexpectedly low hospitalization rates were seen in the elderly population. Less than 10% of all hospitalizations occurred in adults aged ≥65 years (Jain et al., 2009, Skarbinski et al., 2011), and in this age group, 70 hospitalizations per 100,000 population per year was estimated, which is 75% lower than the estimations during previous seasonal epidemics (Shrestha et al., 2011).

The early reports from Mexico suggested a case fatality ratio of 0.4% (Fraser et al., 2009). However, this estimation was probably biased due to the concentration of laboratory diagnostics in the more severe cases, and recent reports suggest significantly lower mortality (Girard et al., 2010). A UK based study estimated an overall case fatality ratio of 0.026% (Donaldson et al., 2009). In a recent population-based analysis in the US, the mortality was estimated at 0.12 deaths per 100,000 population (Fowlkes et al., 2011). A great majority (>90%) of the deaths occurred in the age group <65 years. This is in stark contrast to seasonal epidemics when the mortality is consistently highest in people aged ≥65. Increased mortality occurred also in children. In the US, 317 pediatric deaths were reported (Cox et al., 2011). The mean age of the children was higher than during the seasonal epidemics (median 6.2 years), and a majority of the children had high risk underlying conditions. The identified risk factors for severe disease included various underlying medical conditions, pregnancy and obesity (Girard et al., 2010).

The milder illness and the lower mortality rates in the elderly might be explained by the higher frequency of cross-protective antibodies against pandemic 2009 H1N1 in the older age groups. In a large serologic study, cross-reactive antibodies against the 2009 A(H1N1) strain were detected only in 4% of subjects born after 1980, whereas in samples from subjects born between 1910 and 1929, protective antibodies were detected in 100% of the samples tested (Hancock et al., 2009). The subjects born in the beginning of the 20th century are likely to have been exposed to the 1918-like H1N1 virus, which may have induced immunity that protected them also against the pandemic 2009 H1N1.

2.3 Pathogenesis

2.3.1 Transmission

Influenza virus transmission from an infected subject to a susceptible host is considered to occur via three different mechanisms: droplet, airborne, and contact transmission. Contact transmission can be further divided into direct and indirect contact transmission. Experimental and observational studies in humans and animals have indicated that all three mechanisms are possible. However, the relative importance of each of the mechanisms in a natural setting remains unclear. Furthermore, air temperature and humidity seem to have an effect on the different transmission mechanisms.

2.3.1.1 Contact transmission

In direct contact transmission, the pathogen is transmitted in a direct physical contact between the infected subject and the susceptible host (e.g. hand shaking), whereas in an indirect contact transmission, the pathogen is first transferred from the infected subject to an intermediate object and later from this contaminated object to the susceptible host. Influenza viruses have been shown to remain viable in various non-porous surfaces, such as plastic and steel, as well as in fomites like cloths, papers and tissues, for up to 8-48 hours. From these contaminated surfaces and fomites, the virus could be further transferred to the hands, suggesting that indirect contact transmission is possible. (Bean et al., 1982).

2.3.1.2 Droplet transmission

Droplet transmission refers to transferring pathogens via respiratory droplets produced by the infected person, for example when sneezing, coughing or speaking. These droplets are propelled into the nasal, oral or conjunctival mucosa of the susceptible host. The term droplet (usually) refers to large particles with a diameter of >5-10μm. These particles spread only a short distance (usually <1 m) and do not stay suspended in the air. Therefore, it is considered that this mode of transmission is not affected by ventilation or air-handling procedures.

Several studies in mice and ferrets have provided evidence that contact between the animals is not required for the transmission of influenza. Schulman and Kilbourne demonstrated that influenza was efficiently transmitted between mice physically separated with a double mesh-wire screen to maintain a 2 cm separation (Schulman and Kilbourne, 1963). In humans, several observational outbreak studies describe a more effective spread of infection to contacts within a short distance of index patients compared to contacts within a longer distance (Brankston et al., 2007, Han et al., 2009). This pattern of spread is suggestive for contact or droplet spread.

2.3.1.3 Airborne transmission

Airborne transmission occurs via the dissemination of pathogens through aerosols and droplet nuclei. Aerosols are produced in the same manner as large droplets, whereas droplet nuclei result when large droplets evaporate. These particles are small (diameter <5-10 μm) and light enough to remain suspended in the air for longer periods and may

be carried over long distances by the air currents. Therefore, they provide a potential mechanism for long-range infections. Controlling airborne transmission requires special air-handling and ventilation measures. In addition, airborne transmission is considered unpreventable by using surgical masks, as the inhaled particles are small enough to pass through the mask. Instead, more effective respirators would be needed to adequately protect from aerosols. (Tellier, 2006).

Many experimental and observational studies lend support for the occurrence of airborne transmission (Tellier, 2006). In ferrets, influenza has been described to transmit over longer distances that would not be expected with large droplets only (Andrewes and Glover, 1941). In humans, an observational study of an outbreak among airplane staff and passengers reported high attack rates and a pattern of spread suggestive of airborne transmission (Moser et al., 1979). However, due to limited experimental data on humans, there is still controversy over the role and occurrence of different transmission mechanisms. Some experts emphasize the importance of contact and droplet transmission, whereas others consider airborne transmission more significant (Tellier, 2007, Lemieux et al., 2007).

Ambient temperature and relative humidity play an important role in all of the transmission mechanisms, as these factors affect the stability and viability of influenza viruses. Guinea pig models have shown that a dry and cold environment favors airborne transmission and that this type of transmission is blocked in a warm and humid environment (Lowen et al., 2007). In contrast, the contact transmission was not affected by an increase in temperature or relative humidity (Lowen et al., 2008). These findings have lead to an interesting hypothesis regarding the varying seasonality of influenza epidemics. Investigators propose that in temperate zones where influenza epidemics occur seasonally during the winter months, aerosol transmission dominates, and in tropical areas, with more stable year-round circulation, transmission would occur mostly through direct or indirect contact (Lowen and Palese, 2009).

2.3.2 Pathophysiology

After the virus is transmitted to the respiratory tract mucosa of a susceptible host, it binds to the epithelial cells lining the upper and probably also lower airways, infects the cells and starts to replicate (Shinya et al., 2006). The replication process leads to lysis and necrosis of the epithelial cells and ultimately desquamation of the respiratory lining. In uncomplicated influenza, the pathophysiological changes are usually that of a tracheo-bronchitis (Kuiken and Taubenberger, 2008). Mucosal inflammation causes local oedema and the hypersecretion of mucus. In most cases, influenza is a self-limiting illness confined to the respiratory tract and the respiratory symptoms result from the local inflammatory process, whereas the systemic symptoms are caused by the production of inflammatory cytokines, including TNF-α, IL-10 and particularly IL-6, whose levels correlate positively with the severity of the symptoms (Kaiser et al., 2001, Eccles, 2005). However, in rare, severe cases of influenza, viremia may occur, and the virus has been detected also in the CNS and heart (Naficy, 1963, Kuiken and Taubenberger, 2008).

2.3.3 Viral load and shedding

The inoculation period in influenza ranges from 1 to 3 days. Viral shedding peaks at the onset of the symptoms or during the following 1-3 days, and then starts to gradually decline (Lau et al., 2010). In adults, viral shedding lasts on average for 5 days (Carrat et al., 2008, Lau et al., 2010). In children and in the immunocompromised, higher viral loads have been described, and shedding may last for longer periods (Hall et al., 1979, Frank et al., 1981, Cheng et al., 2009, Anton et al., 2010). Not all infected subjects, however, develop symptomatic infections. Estimates on the proportion of asymptomatic infections during the 2009 pandemic have ranged from 10% to 50% (Aho et al., 2010, Papenburg et al., 2010). Viral shedding has been described to occur also during asymptomatic infections and during the inoculation period prior to the occurrence of symptoms (Lau et al., 2010).

2.4 Clinical presentation

2.4.1 Signs and symptoms

Non-complicated influenza is typically characterized by respiratory symptoms, such as cough, rhinitis and pharyngitis, as well as systemic symptoms, including fever, weakness, malaise, headache and myalgia (Cox and Subbarao, 1999). In a prospective cohort study including 353 outpatient-treated children of ≤13 years with laboratory-confirmed influenza, fever was the most common symptom (Silvennoinen et al., 2009). Fever at ≥38°C was reported in 90% of the children, and 20% of the children aged <3 years had a high fever of ≥40°C. Rhinitis and cough was reported in 78% and 77% of the children, respectively. Other symptoms were less frequent; sore throat was reported in 36% of the children aged ≥3 years, headache was present in 26% of the children, and 7% had myalgia. Nine percent of the children had gastrointestinal symptoms. In this study, there were no differences in the clinical presentation between children infected with influenza A or B. In another study including children aged <17 years referred to the emergency department of a tertiary hospital, fever was again a major symptom, and a temperature of ≥38°C was reported in 94% and 89% of children with influenza A and B, respectively (Peltola et al., 2003). In children with influenza A, the incidences of other symptoms were as follows: cough 67%; rhinitis 66%; headache 26%; vomiting 19%; abdominal pain 10%; and myalgia 6%. The clinical features were comparable in children with influenza A and B infections, except for rhinitis that was less common (56%), and myalgia that was more common (15%) in children with influenza B. These results emphasize the salience of fever in the symptomatology of influenza in children. The degree of fever has also shown to have a positive correlation with viral load and nasal and plasma IL-6 levels (Kaiser et al., 2001). Other symptoms, such as cough, headache and myalgia that are often considered essential in the symptomatology of influenza and have been reported in >90% of adults with influenza (Monto et al., 2000), were considerably less frequent in children.

In young children and infants, influenza may present as a sepsis-like illness (Dagan and Hall, 1984). A recent study from Finland showed that the most common reason for influenza-associated hospitalization in children <6 months of age was a sepsis-like

illness, which accounted for 52% of the admissions in this age group (Silvennoinen et al., 2011b). In all children aged ≤16 years, respiratory symptoms accounted for 38% of the admissions, and 15% of the children were hospitalized due to acute neurologic conditions, mainly due to febrile convulsions.

The most detailed data on the duration of the illness can be obtained from placebo-controlled influenza antiviral trials. In adults, the median duration of illness has been 4-5 days and fever has lasted 2-3 days (Nicholson et al., 2000, Treanor et al., 2000). Similar durations were reported also in children. In a study among children aged <12 years, the median duration of the illness was 5.7 days, with a longer duration (6.7 days) seen in younger children ≤2 years (Whitley et al., 2001). In the same study, the median duration of fever was 2.8 days.

2.4.2 Complications

Influenza predisposes to bacterial complications. Several mechanisms for this have been proposed. Influenza causes epithelial damage that can facilitate the adherence and invasion of the bacteria. Furthermore, the cytokine response induced by the influenza infection may favor the adherence of bacteria by up-regulating receptors for bacterial binding (McCullers, 2006). In children, the most common complication is acute otitis media (AOM). In different studies, AOM has been diagnosed in ~40% of children aged <3 years, and in ~20% of children aged 3-6 years, with influenza (Heikkinen et al., 2004, Winther et al., 2010). Influenza viruses can be detected in the middle-ear fluid of children with influenza- associated AOM. However, in most cases, influenza viruses are isolated alongside with bacteria, particularly *Streptococcus pneumoniae* (Heikkinen et al., 1999), suggesting the presence of viral-bacterial co-infection.

Another frequent complication of influenza is pneumonia. Influenza-associated pneumonia may be primary viral pneumonia or a secondary bacterial co-infection (Ruuskanen et al., 2011, Lahti et al., 2006). However, it is often difficult to distinguish between bacterial and viral etiologies. In outpatient-treated children, pneumonia is rare, and in a prospective cohort study only 2% of the children <13 years with laboratory-confirmed influenza were diagnosed with pneumonia (Heikkinen et al., 2004). In a hospital setting, pneumonia is more common. In a retrospective Finnish study, radiologically confirmed pneumonia was diagnosed in 14% of children <16 years, who were evaluated in a tertiary hospital with laboratory-confirmed influenza (Lahti et al., 2006). 81% of these children had radiological and laboratory parameters suggestive of primary viral etiology. In children, the course of viral pneumonia is usually benign (Lahti et al., 2006, Ruuskanen et al., 2011).

Severe, progressive viral pneumonia is a rare but feared complication of influenza. Risk factors for this condition are both host and pathogen specific, with a higher risk observed in the elderly population, during pregnancy and in those with underlying illnesses. Higher rates of severe viral pneumonia have been observed in the context of the pandemics (Hers et al., 1958, Skarbinski et al., 2011), as well as in those infected with avian influenza viruses, probably due to a lack of pre-existing immunity and different preferences in the site of viral binding (Shinya et al., 2006, Kuiken and Taubenberger, 2008). Viral pneumonia may lead to diffuse alveolar damage and

severely impair the gas exchange function of the lungs. The most severe cases may lead to the development of acute respiratory distress syndrome (ARDS) (Kuiken and Taubenberger, 2008). Severe, secondary bacterial pneumonias complicating influenza have been reported both in adults (Oliveira et al., 2001) and in children (Finelli et al., 2008), with the most commonly isolated pathogens being *Streptococcus pneumoniae* and *Staphylococcus aureus.*

Laryngitis may be considered as a complication of influenza, although it is rather a presentation of the primary viral infection of the upper respiratory tract. The course of laryngitis caused by influenza viruses is more severe than that caused by parainfluenza viruses (Peltola et al., 2002) Complications outside the respiratory tract are rare and include central nervous system (CNS) manifestations, myocarditis and myositis (Kuiken and Taubenberger, 2008). In a large retrospective study among 683 hospitalized children aged <17 years with influenza, one child was diagnosed with myocarditis and three children with myositis, febrile convulsion occurred in 21 children, encephalitis was diagnosed in 5 children and status epilepticus in 4 children (Peltola et al., 2003).

Influenza and its complications are commonly treated with antibiotics. In the population level, influenza is estimated to account for 3 to 9 courses of antibiotics per 100 children annually (Neuzil et al., 2000). In clinical studies, the proportion of children diagnosed with influenza requiring antibiotics has ranged from 9% to 42% in different age groups and averaged at 28% (Whitley et al., 2001, Heikkinen et al., 2004).

2.5 Diagnosis

Compared to other viral respiratory infections, specific antiviral drugs are available for treating influenza. This emphasizes the importance of differentiating between influenza and the common cold. In addition, diagnosing influenza may help clinicians to avoid unnecessary courses of antibiotics and to arrange for required isolation measures. Furthermore, continuous surveillance of the epidemiological situation and detection of the antigenic changes in influenza viruses are mainly dependent on the specimens obtained for clinical purposes.

2.5.1 Clinical diagnosis

Symptoms of influenza in children are generally considered similar to those in other viral respiratory infections (Peltola et al., 2003, Silvennoinen et al., 2009). In adults, a combination of cough and fever has been associated with the increased likelihood for influenza during an epidemic (Monto et al., 2000). However, in children the situation is more complex. Other respiratory viruses frequently co-circulate during influenza epidemics, and in children influenza causes only a minority of febrile respiratory infections, even during the peak weeks of the epidemics (Zambon et al., 2001, Heikkinen et al., 2003, Poehling et al., 2006a). Moreover, children may not be able to describe their subjective symptoms reliably, which may further hinder the diagnosis. In a Finnish study, the overall sensitivity of the clinical diagnosis of influenza in children

<13 years was only 38%, and the positive predictive value was 32% (Peltola et al., 2005).

Few studies have assessed symptoms predicting influenza in children. Friedman and Attia identified the triad of cough, headache and pharyngitis as an accurate predictive model with a sensitivity of 80% (95% CI: 69-91%) and specificity of 79% (95% CI: 67-89%) (Friedman and Attia, 2004). This study was conducted in a highly-selected population of children < 17 years, treated at a tertiary hospital emergency room. Sočan et al. identified fever ≥38°C, headache, cough and the absence of abnormal breathing sounds as predictors for influenza in children <15 years. This study included both hospitalized and outpatient-treated children (Socan et al., 2010). Ohmit and Monto explored symptomatic predictors for influenza in 1-12-year-old children recruited in two clinical antiviral trials (Ohmit and Monto, 2006). In the first trial, in children aged 5-12 years, a combination of cough and fever predicted influenza with a positive predictive value (PPV) of 83% (95% CI: 79-88%), whereas in the other trial, cough and headache independently predicted influenza. Instead, in children 1-4 years of age, no clinically useful predictors were identified, with myalgia being the sole symptom reported more often in children with influenza. However, these antiviral trials aimed at recruiting the highest possible number of influenza-positive children, which may at least partly explain the remarkably high rate of influenza-positive children in the study population (66-74%). Furthermore, positive respiratory syncytial virus (RSV) rapid tests results were used as exclusion criteria in half of the patients. Consequently, these results may not be directly generalized to normal, outpatient-treated children.

2.5.2 Laboratory diagnosis

Viral isolation by culture has long been considered as the golden standard for influenza diagnostics. However, in recent years the reverse transcriptase polymerase chain reaction (RT-PCR) assay has started to supersede viral isolation as a reference method, due to its greater sensitivity and increasing availability (Weinberg et al., 2004). Antigen detection with different laboratory-based techniques, or as rapid point-of-care (POC) tests, are widely available. Although rapid to perform, they usually have a lower sensitivity compared to viral culture and RT-PCR. Serological assays are nowadays used mainly to assess the response to vaccination, or in seroprevalence studies, and are rarely used for diagnostic purposes. The performance of all diagnostic tests is highly dependent on the quality of the sample, which in turn may be influenced by several factors, including the site, method and timing of the sample collection, as well as the age of the patient.

2.5.2.1 Viral culture

Viral culture was the first virological method for diagnosing influenza. In this procedure, the cell culture, commonly Madin-Darby canine kidney cells, is inoculated with the specimen. The detection of the influenza virus is based on the cytopathic effect that it causes in the cell cultures, and subsequent specific identification tests (Playford and Dwyer, 2002). The conventional viral culture takes 4-5 days, but the process may be precipitated to 2-3 days with different immunological staining methods (Waris et al., 1990, Playford and Dwyer, 2002). Viral culture is still often considered

the golden standard of diagnosing influenza, although, when compared to RT-PCR, the yield of influenza is lower. In a single pediatric study, the sensitivity of conventional viral culture was 59% when compared to RT-PCR (Weinberg et al., 2004).

2.5.2.2 Antigen detection

Different immunological techniques have been developed for detecting influenza virus antigen. These include indirect and direct immunofluorescence (IF), enzyme-linked immunosorbent assay (ELISA) and time-resolved fluoroimmunoassay (TR-FIA). All these techniques are based on the usage of influenza virus-specific antibodies. In the IF and TR-FIA techniques, antibodies with fluorescent stains are used to detect influenza viruses in the specimen. In ELISA, antibodies are linked with an enzyme, and when a substrate is added the enzyme converts it into a detectable signal. These tests are usually rapid to perform and the results may be available within 1-2 days. Some of these tests are able to differentiate between influenza A and B, but not to determine the subtype. The performance of these tests differs, but in general, the sensitivity is 60-90% and the specificity 90-100% when compared to viral culture (Playford and Dwyer, 2002, Uyeki, 2003).

Several rapid antigen detection tests that can be performed at the point of care (POC) are available. These tests are usually lateral flow immunochromatographic assays that are targeted against influenza virus nucleoprotein (NP). POC tests are easy to perform, do not require laboratory expertise or equipment and can provide results within 10-30 minutes. The majority of the tests detect both influenza A and B viruses, and some tests are able to distinguish between them. However, the performance of these tests is highly variable. POC tests are considered highly specific, but reported sensitivities range from 20 to 85%, with lower sensitivities reported for influenza B (Cazacu et al., 2003, Cazacu et al., 2004, Agoritsas et al., 2006, Poehling et al., 2006b, Grijalva et al., 2007, Rouleau et al., 2009, Cheng et al., 2009, Ghebremedhin et al., 2009).

2.5.2.3 Reverse transcriptase polymerase chain reaction

Diagnosing influenza with reverse transcriptase polymerase chain reaction (RT-PCR) is based on the amplification and detection of viral RNA. First, the extracted viral RNA is transcribed to complementary deoxyribonucleic acid (cDNA) by a reverse transcriptase enzyme. This process is followed by a polymerase chain reaction (PCR), where the cDNA is exponentially amplified by the polymerase enzyme in cyclically changing temperatures. Different primers targeted against various influenza gene sequences including HA and NP can be used. Finally, the amplified DNA is detected either real-time or in the final PCR products using electrophoresis, nucleic acid staining, or fluorescence detection methods.

RT-PCR is currently the most sensitive method for diagnosing influenza (Weinberg et al., 2004). It may have advantages over viral isolation, especially if the specimen is obtained at a later stage of the illness and the viral load is already decreasing, or if the sample quality is low for some other reason. Furthermore, RT-PCR can be used to type and subtype the detected virus. Previously, RT-PCR was considered a slow and expensive method, but due to development of more automated techniques the results

may be available already within a few hours, and RT-PCR is being increasingly used also for clinical purposes.

2.5.2.4 Serology

Serological diagnosis of influenza is based on demonstrating a rise in the titres of specific antibodies in paired serum samples. Antibody titres can be measured using different methods, including hemagglutination inhibition (HAI), microneutralization, enzyme immunoassay (EIA) and complement fixation tests. Usually, a fourfold increase in the antibody titres between the samples is considered diagnostic. The need for two samples, a baseline sample obtained during acute illness, and a convalescent sample obtained 10-14 days later, substantially limits the usefulness of serology in clinical practice (Cox and Subbarao, 1999). Today the use of serology is mainly restricted to seroprevalence and vaccine immunogenicity studies.

2.5.2.5 Factors affecting performance of diagnostic tests

Various factors may affect the performance of diagnostics tests based on viral culture, RT-PCR and antigen detection. The sensitivities of the tests vary between different age groups, with the highest sensitivities reported in children (Steininger et al., 2002, Ruest et al., 2003, Steininger et al., 2009, Cheng et al., 2009). This is apparently associated with the higher viral loads and longer duration of viral shedding observed in children (Hall et al., 1979, Frank et al., 1981, Cheng et al., 2009). Higher sensitivities have been reported for a specimen obtained in the early course of an illness when compared to samples obtained at a later stage (Gordon et al., 2009, Stripeli et al., 2010). This reflects the patterns of viral shedding, which peaks within 0-3 days after the onset of the symptoms and then starts to gradually decrease (Hall et al., 1979, Frank et al., 1981, Carrat et al., 2008, Lau et al., 2010).

Presumably, the most important factor affecting the yield of the influenza virus is the quality of the sample, which, in turn, is dependent on the site and method of collection. A nasopharyngeal aspirate is often considered as the specimen of choice for everyday clinical purposes in children. However, the sensitivity of a nasal swab is ~90% when compared with a nasopharyngeal aspirate for the detection of influenza viruses in children, and the method is more pleasant for the patient and easy to perform in any setting, as it does not require any special equipment (Heikkinen et al., 2001, Heikkinen et al., 2002). The sensitivity of nasal swabs may be further enhanced by using flocked swabs (Abu-Diab et al., 2008, Chan et al., 2008) or polyurethane foam swabs (Scansen et al., 2010), or by collecting the swab from the nasopharynx (Agoritsas et al., 2006).

2.6 Treatment

2.6.1 Adamantanes

Adamantanes (M2 inhibitors) are the older generation of influenza antiviral drugs. This group consists of two drugs, amantadine and rimantadine. The mechanism of action for these drugs relies on their ability to interfere with the function of the M2 viral protein. M2 acts as a channel protein and by blocking this channel after the endocytosis of the viral particle, adamantanes prevent the uncoating of the virus and consequent release of

viral RNA in the host cell cytoplasm (Schnell and Chou, 2008). Adamantanes are basically active against all influenza A subtypes, but not against influenza B viruses that lack M2 protein. However, the widespread resistance of the circulating influenza A strains against adamantanes considerably limits the usage of these drugs in the current situation (Bright et al., 2006, CDC, 2010b).

2.6.1.1 Amantadine

Amantadine was first approved by FDA in 1966 for chemoprophylaxis against influenza A(H2N2), and later in 1976 for the treatment and prophylaxis against all influenza A infections. Currently, it is registered in the US for the treatment and prophylaxis of influenza A in children ≥1 year of age. In Finland, amantadine is currently available for treatment and prophylaxis against influenza A in subjects aged 15-65 years. The efficacy of amantadine in the treatment of influenza A in children has been evaluated in three RCTs. In two Japanese studies that included both adults and children, amantadine reduced the mean duration of fever in children statistically significantly, but no exact time of reduction was provided (Kitamoto, 1968, Kitamoto, 1971). When data from these studies where combined in the recent Cochrane review (including a total of 313 children ≤16 years of age), no statistically significant difference between amantadine and the placebo groups were observed in the occurrence of fever on day three of the treatment (RR 0.37; 95% CI: 0.08 to 1.75) (Galvao et al., 2008). In a UK based RCT that included both children and adults, a statistically significant reduction of 28 hours in the duration of the illness was observed in children treated with amantadine (Galbraith et al., 1971). Amantadine could be considered as an option in treating influenza A encephalitis, if the strain is assumed susceptible. Although prospective trials are lacking, amantadine has been described to have a high degree of CSF penetration with potentially therapeutic concentrations (Geskey and Thomas, 2004). Adverse events associated with amantadine include CNS symptoms (such as anxiety, dizziness and insomnia) and gastrointestinal symptoms (such as nausea and anorexia). Although these symptoms were infrequently reported in pediatric studies (Galvao et al., 2008), chemoprophylaxis studies in adults have reported that at least one CNS symptom was present in 13% of the patients (Dolin et al., 1982).

2.6.1.2 Rimantadine

The other adamantane derivative, rimantadine, was approved by the FDA in 1993 for chemoprophylaxis against influenza A in adults and children ≥1 year and for the treatment of influenza A in adults. Rimantadine is not currently available in Finland. Two RCTs on the efficacy of rimantadine in the treatment of influenza A in children have been published. In both studies, rimantadine was compared to acetaminophen (paracetamol). Hall et al. found significantly greater reduction in fever and improvement of symptom scores in children treated with rimantadine (Hall et al., 1987). In another similar study by Thompson et al., no such clinical benefits were observed (Thompson et al., 1987). In both studies, the viral shedding was decreased in the rimantadine group at the beginning of the treatment, but it then subsequently increased to a similar or higher level than in acetaminophen group at the end of the treatment. The greatest benefit of rimantadine over amantadine is the lower frequency of CNS related adverse events (Dolin et al., 1982). In pediatric studies, rimantadine

was not associated with an increased risk for adverse events (Hall et al., 1987, Thompson et al., 1987, Galvao et al., 2008).

2.6.2 Neuraminidase inhibitors

The neuramidase inhibitors, zanamivir and oseltamivir, are the second generation of influenza antivirals. Neuraminidase is a surface glycoprotein that has an important role in the release of new progeny viruses from the infected host cell after viral replication. With its enzymatic activity, neuraminidase cleaves the sialic acid residues where the newly formed viruses are attached (Figure 2). Neuraminidase inhibitors were designed on the basis of the three-dimensional, x-ray crystallography structure of influenza neuraminidase specifically to fit the catalytic site of the molecule (Colman et al., 1983, Von Itzstein et al., 1993). By binding to the active site pocket of the neuraminidase and inhibiting its activity, these drugs prevent the release of new viruses and consequently limit the infection (Moscona, 2005) (Figure 2).

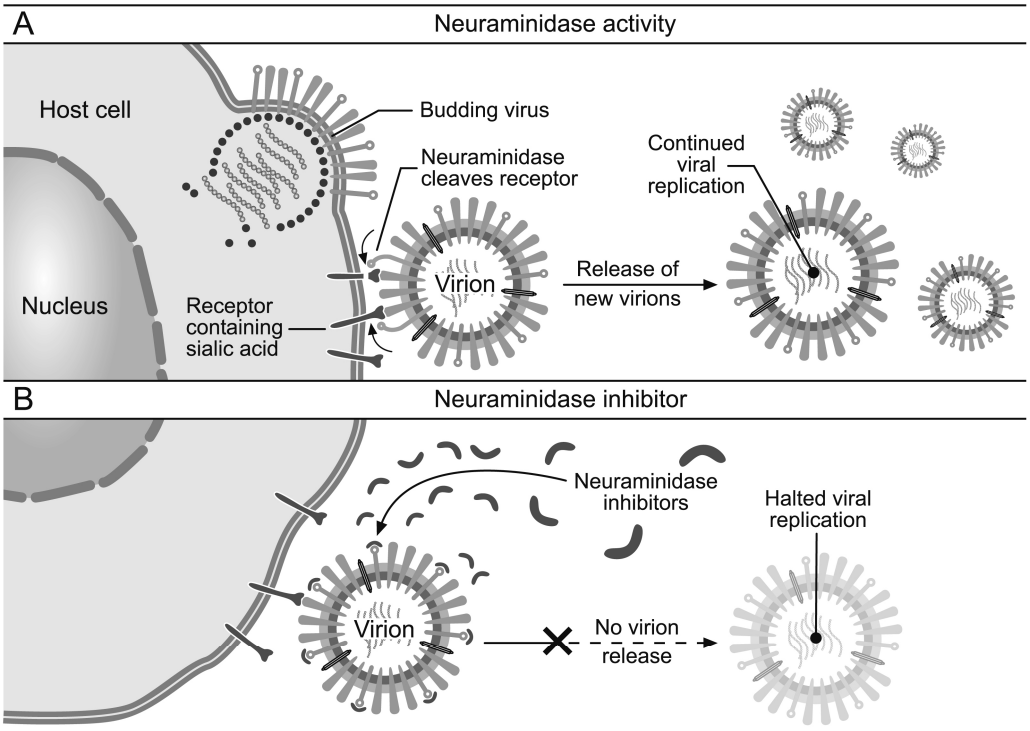


Figure 2. Mechanism of action of neuraminidase inhibitors. A) Normally neuraminidase cleaves the sialic acid receptors where the newly formed virions (i.e. viral particles) are attached. **B)** Neuraminidase inhibitor blocks the enzymatic activity of neuraminidase, prevents the release of newly formed virions and halts the viral replication. (Adapted from Moscona, 2005)

2.6.2.1 Zanamivir

Zanamivir was the first neuraminidase inhibitor-class drug on the market and was approved by the FDA and the European regulatory authorities in 1999. Currently, its

indications in Finland include the treatment and chemoprophylaxis of influenza A and B in adults and children ≥5 years of age. Zanamivir is not bioavailable orally, and currently the only approved form of dosage is the dry powder for inhalation. This form of direct delivery into the respiratory tract results in high concentrations of the drug in the respiratory tract epithelial cells - the site where the majority of the viral replication takes place. In adults, zanamivir treatment started within 36-48 hours from the onset of symptoms reduced the duration of the illness by 1.0-2.5 days (Hayden et al., 1997, Silagy et al., 1998, Monto et al., 1999, Mäkelä et al., 2000). Similar benefits have been described in children. In the only published RCT on children, zanamivir treatment started within 36 h from the onset of symptoms reduced the median time to symptom alleviation by 1.25 (95% CI: 0.5 to 2.0) days in children aged 5-12 years (Hedrick et al., 2000). In another unpublished phase III study (trial identifier: NAI30028) in the same age group, the median time to the alleviation of symptoms was reduced only by 0.5 days (GSK, 2010). Zanamivir is well tolerated in children and in adults. Due to the post marketing reports of zanamivir-associated bronchospasm in patients with asthma or COPD, zanamivir is not recommended for these patient groups (Fiore et al., 2011). Zanamivir was, however, also safely administered in two studies involving patients with underlying airway diseases (Murphy et al., 2000, Cass et al., 2000).

Intravenous zanamivir is not officially approved by the authorities, but it has been approved for compassionate use in critically ill patients in Europe by the EMA (EMA, 2011a), and it is also available in the U.S. under an Emergency Investigational New Drug (EIND) application to the U.S. Food and Drug Administration (FDA) (FDA, 2011). Several case reports have been published suggesting that intravenous zanamivir would be safe and considerable option, especially if an oseltamivir-resistant strain is suspected (Kidd et al., 2009, Gaur et al., 2010, Dulek et al., 2010). In two influenza A-infected children with immunosupression due to hematologic malignancies, oseltamivir treatment was replaced or combined with intravenous zanamivir after clinical deterioration during the oseltamivir treatment. In one child, this led to a gradual improvement of the clinical condition (Gaur et al., 2010), whereas the other child experienced a progressive decrease in respiratory status and died 22 days after the initiation of intravenous zanamivir (Dulek et al., 2010). In both children, the oseltamivir-resistant influenza A strain carrying the H274Y neuraminidase mutation was isolated.

2.6.2.2 Oseltamivir

Oseltamivir was approved by the FDA for adults in 1999 and for children in 2000. In Europe, oseltamivir was approved by the EMA in 2002. Currently, it is approved for treatment and prophylaxis against influenza A and B in adults and children ≥1 year of age. Oseltamivir is administered as oseltamivir phosphate, an inactive prodrug which is metabolized in the liver to its active compound oseltamivir carboxylate. The forms of dosage include capsules and powder for oral suspension. Similarly to zanamivir, an intravenous formulation of oseltamivir has been approved by the EMA for compassionate use to treat severely ill patients (EMA, 2010b)

2.6.2.2.1 Efficacy

In adults, oseltamivir started within 36-48 hours from the onset of symptoms has been described to shorten the duration of the illness by 1.0-1.5 days (Treanor et al., 2000, Nicholson et al., 2000). Two RCTs on the efficacy of oseltamivir in children have been published. Whitley et al. studied the efficacy in 695 children aged 1-12 years. In this study, oseltamivir treatment started within 48 hours from the onset of symptoms shortened the duration of the illness in children with confirmed influenza infection (n=452) by 1.5 days. In addition, oseltamivir treatment reduced the incidence of acute otitis media by 44% (Whitley et al., 2001). The other RCT included 335 children aged 6-12 years diagnosed with asthma severe enough to require a regular medical follow-up. In this study, the time to the alleviation of symptoms was 1.0 day shorter in the oseltamivir group among influenza-infected children who completed the study per protocol (n=162), but the difference was not statistically significant (Johnston et al., 2005). In this study, greater improvements of pulmonary function were observed in the oseltamivir group, measured by FEV1. The effect of oseltamivir on influenza-related complications in children with underlying medical conditions was evaluated in an epidemiological, health insurance database-derived study (Piedra et al., 2009). In this study, oseltamivir treatment was associated with significant reductions in the diagnoses of respiratory illnesses other that pneumonia (including mainly asthma attacks), episodes of acute otitis media and all-cause hospitalizations during the 14 day follow-up period after the influenza diagnosis.

Oseltamivir treatment started within 48 hours from the onset of symptoms has been associated with a reduced viral load and shorter duration of viral shedding (Hayden et al., 1999b, Whitley et al., 2001, Lee et al., 2009, Li et al., 2010), both important factors in reducing the infectivity and limiting the spread of the disease.

Influenza A and B viruses both carry the neuraminidase molecule, and oseltamivir is principally active against both types of influenza (Hayden et al., 1999b, Hayden et al., 2000b). However, there are reports of lower clinical efficacy of oseltamivir against influenza B compared to influenza A (Kawai et al., 2006, Sugaya et al., 2007). Whether this has been associated with the emergence of oseltamivir-resistant influenza B strains (Hatakeyama et al., 2007), or the baseline lower mean inhibitory concentration of oseltamivir for influenza B (Sheu et al., 2008), is unclear.

Because of the pathogenesis of influenza and the mechanism of action of neuraminidase inhibitors, to be effective these drugs need to be initiated in the early course of the illness. Both oseltamivir and zanamivir are recommended to commence as early as possible, and no later than 48 hours from the onset of symptoms (Fiore et al., 2011). The importance of the early initiation of the treatment was demonstrated by Aoki et al. in a study including 1,426 healthy adults (Aoki et al., 2003). Earlier initiation of oseltamivir treatment was strongly associated with shorter illness duration, and oseltamivir treatment initiated within 0-12 h after the onset of symptoms shortened the duration of the illness by 3.1 days, compared to treatment started at 48 h.

On the other hand, in severely ill patients requiring hospitalization, antiviral treatment may be beneficial also when initiated at a later stage. McGeer et al. described the outcomes of 512 patients hospitalized for confirmed influenza in Canada (McGeer et

al., 2007). In these patients, oseltamivir treatment significantly reduced the risk of death (OR, 0.21; 95% CI, 0.06–0.80), although in 71% of the patients the treatment was started later than 48 h from the onset of symptoms. Lee et al. described that in hospitalized adults, oseltamivir treatment started within 4 days after the onset of symptoms improved survival (aHR 0.27; 95% CI: 0.13 to 0.55) and reduced the viral load and duration of viral shedding (Lee et al., 2010a).

2.6.2.2.2 Safety and tolerability

Oseltamivir is generally well-tolerated. In pediatric RCTs, the only adverse events associated with oseltamivir treatment were gastrointestinal, with ~6% excess vomiting and ~2% excess stomachaches in the oseltamivir group (Whitley et al., 2001, Johnston et al., 2005). There have been post marketing reports of neuropsychiatric adverse events in Japanese children taking oseltamivir. However, an extensive safety review indicated that these incidents were more likely to be associated with the influenza illness itself than the drug (Toovey et al., 2008). This conclusion is further supported by the low CNS penetration levels of oseltamivir (Jhee et al., 2008).

Oseltamivir is not officially approved to be used in children under 1 year of age. However, there are several retrospective reports that oseltamivir has been used with safety and without excess adverse events also in infants <1 year (Tamura et al., 2005, Okamoto et al., 2005, Kimberlin et al., 2010b, Siedler and Skopnik, 2010). During the 2009 pandemic, the FDA and EMA expanded the indication of oseltamivir under the Emergency Use Authorization (EUA) to temporarily include also children younger than 1 year.

2.6.3 Other antiviral drugs

Peramivir is a long-acting neuraminidase inhibitor being developed for the treatment of influenza. It is currently in phase III studies. In clinical trials, a single dose (300 or 600mg) of intravenous peramivir has been described to shorten the duration of the illness by one day when compared to placebo (trial identifier: 0722T0621), and perform similarly when compared with orally administered oseltamivir (trial identifier: 0815T0631) in outpatients with uncomplicated influenza. In a smaller study among hospitalized patients with severe influenza, intravenous peramivir 200 or 400 mg daily for 5 days was compared to oral oseltamivir, and no differences were reported between the three treatment groups (trial identifier: BCX1812-201). Peramivir was generally well tolerated. The most common adverse events reported were diarrhea, nausea, vomiting and neutropenia. The safety and efficacy of peramivir have not been studied in pediatric patients (FDA, 2009). Due to the acceptable safety profile, lack of other influenza antivirals with intravenous dosage, and the potential benefits peramivir could offer, the FDA approved the usage of intravenous peramivir during the 2009 pandemic in hospitalized adults and children under the EUA application (Birnkrant and Cox, 2009). According to a case report, intravenous peramivir was successfully used under the EUA application to treat a patient with severe influenza illness and immunosuppression due to hematopoietic stem-cell transplantation (Campbell et al., 2010).

Laninamivir is a long-acting multimeric compound of zanamivir. It is currently in phase III trials. Two different doses (40 mg and 20 mg) of single laninamivir inhalation were compared to a 5-day course of oral oseltamivir in 186 children aged 3-9 years (Sugaya and Ohashi, 2010). A single laninamivir inhalation or the first dose of oseltamivir was administered within 36 hours from the onset of symptoms. No difference was observed between the groups in children infected with A(H3N2) and influenza B viruses in either dosage of laninamivir. By contrast, a statistically significant reduction of 61-66 h in the duration of the illness was observed in children infected with A(H1N1) viruses. It should be, however, noted that this study was conducted during the H1N1 season when virtually all viruses were resistant to oseltamivir carrying the H274Y mutation, and therefore no efficacy could have been expected in A(H1N1) infected children receiving oseltamivir. In a similar study among adults, no differences between the treatment groups were seen (Watanabe et al., 2010).

Ribavirin is an older antiviral drug that is active against both DNA and RNA viruses and approved for the treatment of hepatitis C and respiratory syncytial virus (RSV) infections. It has also been described to possess antiviral activity against influenza viruses *in vitro* by inhibiting the RNA polymerase (Graci and Cameron, 2006). However, only limited data are available from clinical trials in humans (Chan-Tack et al., 2009). Intravenous ribavirin is available in the U.S. under an EIND application to the FDA. Favipiravir is another antiviral agent inhibiting RNA polymerase. It does not inhibit DNA polymerase and is considered less cytotoxic than ribavirin (Furuta et al., 2009). Favipiravir has shown protective effectiveness against influenza in mice (Furuta et al., 2002) and clinical phase I/II trials are underway. Other influenza antivirals under development include cyanovirin-N, a carbohydrate-binding protein which interferes with the entry of viruses into the cell by binding to surface glycoproteins of the viral envelope, and DAS181, a conjugated sialidase that removes the sialic acid residues acting as binding sites for influenza viruses on the surface of the respiratory epithelial cells (Boltz et al., 2010).

2.6.4 Antiviral resistance

2.6.4.1 Adamantanes

Adamantane-resistant influenza A strains were previously isolated frequently in the clinical treatment and prophylaxis trials (Hall et al., 1987, Hayden et al., 1989), or after other exposure to these drugs (Shiraishi et al., 2003). However, only low levels (<1%) of adamantane-resistant strains were observed in global surveillance studies until the beginning of the 21st century (Ziegler et al., 1999). After the year 2000, the number of adamantane-resistant A(H3N2) strains started to increase rapidly (Bright et al., 2005) leading to widespread resistance observed in the 2005 season, with 92% of the A(H3N2) strains isolated resistant to adamantanes (Bright et al., 2006). Adamantane resistance first appeared to emerge less frequently among A(H1N1) strains. The oseltamivir-resistant seasonal A(H1N1) strain with H274Y neuraminidase mutation that emerged in 2008 remained susceptible to adamantanes. This strain, however, was rapidly superseded by the adamantane-resistant pandemic 2009 A(H1N1) strain, and during the 2009-2010 influenza season, virtually all A(H1N1) and A(H3N2) strains tested were resistant to adamantanes (CDC, 2010a). Consequently, adamantanes are

not currently recommended, either for treatment or prophylaxis against influenza. The molecular basis of adamantane resistance has been characterized, and it is associated with an aminoacid substitution at position 26, 27, 30, 31, or 34 in the transmembrane region of the M2 protein. Mutations in these positions have been described to result in complete cross-resistance between amantadine and rimantadine (Belshe et al., 1988).

2.6.4.2 Neuraminidase inhibitors

No resistant strains against zanamivir or oseltamivir were detected in the large-scale global surveillance study conducted in 1996-1999, before the introduction of neuraminidase inhibitors into clinical practice (McKimm-Breschkin et al., 2003). This suggested that there was no naturally occurring resistance against these drugs. In the clinical zanamivir trials, no resistant strains were recovered after zanamivir treatment (Barnett et al., 2000, Hedrick et al., 2000) or during prophylaxis (LaForce et al., 2007). There is a single report on the development of a zanamivir-resistant influenza B strain in an immunocompromised child during prolonged zanamivir treatment (Gubareva et al., 1998), and sporadic reports of both influenza A and B isolates with reduced susceptibility detected in surveillance studies (Monto et al., 2006, Sheu et al., 2008, Thorlund et al., 2011, Yates et al., 2010, NISN, 2010). In influenza B viruses, zanamivir resistance has been associated with mutations D198N, R152K and R371K at the active site of neuraminidase (Gubareva et al., 1998, Monto et al., 2006, Sheu et al., 2008). In influenza A(H1N1) and A(H3N2) neuraminidases, mutation H126N and several substitutions in the D151 residue, respectively, have been associated with reduced susceptibility to zanamivir.

In the manufacturer-sponsored clinical oseltamivir trials, resistant strains were isolated in 0.3% of adults and 4.1% of children treated with oseltamivir (Aoki et al., 2007). No resistant isolates were detected in the prophylaxis trial including children and adults (Hayden et al., 2004). In a descriptive study from Japan, where oseltamivir use is substantially higher than in any other country, oseltamivir resistant viruses were isolated in 18% of the children treated with oseltamivir (Kiso et al., 2004). Oseltamivir resistance emerges at higher rates in the A(H1N1) subtype; In a UK study conducted in 2005-2007, oseltamivir-resistant viruses were recovered from 27% of the children infected with A(H1N1), from 3% of the children infected with A(H3N2) and from 0% of the children infected with influenza B, all of whom were treated with oseltamivir (Stephenson et al., 2009). Detected mutations associated with oseltamivir resistance include H275Y, Y155H and H126N in A(H1N1)viruses, and E119V, R292K and D151V in A(H3N2) viruses (McKimm-Breschkin et al., 2003, Monto et al., 2006, Sheu et al., 2008, Lackenby et al., 2008b). Surveillance studies indicated that until the 2007-2008 season, oseltamivir-resistant strains were circulating at very low levels, both globally (<1%) (Monto et al., 2006, Sheu et al., 2008), as well as in Japan (≤3%) (Tashiro et al., 2009). In addition, the oseltamivir-resistant strains isolated were less fit (Ives et al., 2002) and showed impaired transmissibility in ferrets (Herlocher et al., 2004).

The situation changed rapidly during the 2007-2008 northern hemisphere season. Oseltamivir-resistant influenza A(H1N1) strains with an H274Y mutation in the neuraminidase were isolated in several European countries. The highest rate was detected in Norway, where 68% of A(H1N1) viruses isolated were resistant to

oseltamivir (Lackenby et al., 2008a). Overall, approximately 20% of the A(H1N1) viruses tested were resistant to oseltamivir in Europe during the 2007-2008 season (Meijer et al., 2009). This rapid rise in the oseltamivir-resistant viruses seems to have emerged in the absence of drug selection pressure (Kramarz et al., 2009). In the subsequent 2008-2009 northern hemisphere season, A(H3N2) was the dominant strain in Europe, and this subtype remained susceptible to oseltamivir, but in the USA influenza A(H1N1) dominated, and virtually all seasonal A(H1N1) viruses tested carried the H274Y mutation and were resistant to oseltamivir (CDC, 2009a). The swine origin pandemic A(H1N1) strain emerged in Mexico in April 2009, and rapidly spread around the world. By the following 2009-2010 northern hemisphere season, pandemic A(H1N1) had became the dominant strain and had replaced the former seasonal A(H1N1) strain entirely. Pandemic A(H1N1) that seems to continue circulating as a new seasonal A(H1N1) strain has remained to a large extent susceptible to oseltamivir. There are sporadic reports of oseltamivir-resistant pandemic A(H1N1) viruses, detected usually in association with oseltamivir treatment and immunosuppression (Wang et al., 2010, Tramontana et al., 2010, Dulek et al., 2010), and globally the proportion of oseltamivir-resistant pandemic A(H1N1) strains has remained <2% (WHO, 2011b). However, recently a cluster of oseltamivir-resistant pandemic A(H1N1) viruses were detected in Australia among 16 subjects not exposed to oseltamivir treatment. This suggests that the resistant viruses may be already readily transmissible among humans (ECDC, 2011).

2.7 Prevention

2.7.1 Vaccination

Vaccination is the most important method of controlling seasonal and pandemic influenza. The key to effective vaccination is a continuous global surveillance, which enables the detection of antigenic changes in the circulating seasonal influenza viruses, as well as rapid identification of emerging novel strains with pandemic potential. Influenza vaccines are generally safe and well tolerated. The vaccines are effective in preventing influenza illness if a good match between the circulating strains and the vaccine strains is achieved. However, limited data exists on the efficacy of influenza vaccines in young children.

2.7.1.1 Evolution of influenza vaccines

2.7.1.1.1 Inactivated vaccine

The discovery of the influenza A virus in 1933 (Smith et al., 1933) started the era of influenza vaccine development. The first influenza vaccines were inactivated (killed) whole virus vaccines that were introduced in the 1940s. Influenza vaccine development relied then – and still does to a large extent – on the cultivation of influenza viruses in the allantoic sac of embryonated hens' eggs. The cultivated viruses were then purified, concentrated and subsequently inactivated (killed) with formaldehydin (Francis, 1953).

The inactivated whole virus vaccine, administered intramuscularly or subcutaneously, proved to be highly immunogenic and effective in preventing influenza illness

(Francis, 1953). In the 1960s, a split vaccine was developed with the aim to reduce the reactogenicity associated with the whole cell vaccine. The split vaccine was formed by disrupting the viral particles with detergents. Later, a subunit vaccine was derived from the split vaccine by enriching the vaccine with the glycoproteins hemagglutinin (HA) and neuraminidase (NA) after the disruption of the viral particle. Both the split and subunit vaccines were found to be immunogenic and effective with a reduced reactogenicity, and these vaccines are today still the most commonly used influenza vaccine types in the world. However, due to lower immunogenicity compared to the whole virus vaccine, two doses of split and subunit vaccines might be needed in immunologically naïve subjects, or in the context of the emergence of a novel type of virus (antigenic shift) (Hilleman, 2002, Ellebedy and Webby, 2009).

2.7.1.1.2 Adjuvanted vaccine

Since the development of influenza vaccines, different adjuvants have been studied and used as a way to enhance the immunogenicity and efficacy of the vaccines as well as to spare vaccine antigen. Aluminium salts were among the first adjuvants used concurrently with influenza vaccines. At present, two adjuvanted influenza vaccines are licensed for clinical use. MF59 is a squalene-based oil-in-water emulsion that was approved as a seasonal vaccine adjuvant for the elderly in 1997, and it was also used in the monovalent vaccine prepared during the 2009 H1N1 pandemic. Another squalene-based oil-in-water adjuvant, tocopherol-containing AS03 was used in combination with the 2009 pandemic vaccine.

2.7.1.1.3 Live attenuated vaccine

Live influenza vaccine was studied already in the 1930s after the isolation of the influenza virus (Francis, 1953). The live vaccine was widely used in the former Soviet Union (Kendal, 1997), but it was not until 2003 that the live attenuated influenza vaccine (LAIV) was approved in the US by the FDA. The approved intranasally administered LAIV is a reassortant virus that consists of a cold-adapted influenza virus backbone, where the concurrently circulating strain HA and NA are inserted (Maassab and Bryant, 1999). The cold adaptation has been achieved by a serial passage in cold temperatures. The cold-adapted virus is able to replicate in the lower temperature (33-34°C) of the upper airways, but the virus loses its ability to replicate in the higher temperature (37°C) of the lower airways (Wareing and Tannock, 2001). The mucosal immune response achieved by the intranasal administration mimics the natural infection and causes a broad activation of the immune system, including both local neutralizing antibody and cell-mediated responses. Theoretically, this could lead to broader protection against different influenza virus strains (Ellebedy and Webby, 2009). LAIV is approved in the US for persons aged 2-49 years (Fiore et al., 2010), and in Europe for children aged 2-<18 years (EMA, 2010a).

2.7.1.2 Vaccine composition

Both live and inactivated seasonal influenza vaccines are trivalent and consist of the HA of three different influenza strains: A(H1N1), A(H3N2) and B. Due to the continuous antigenic evolution of influenza viruses (referred to as antigenic drift), the strains included in the vaccine need to be regularly updated. Based on the global surveillance data, WHO evaluates twice a year which strains are most likely to

circulate during the forthcoming influenza season and gives recommendations on the vaccine composition to vaccine manufacturers accordingly. A monovalent influenza vaccine, consisting of only one strain, is not commonly used anymore in the context of a seasonal influenza. However, a monovalent vaccine is an option when a novel influenza strain emerges (antigenic shift), and it was the type of vaccine used during the 2009 pandemic. Currently, several vaccine manufacturers are developing a quadrivalent influenza vaccine that includes two influenza A strains (H1N1 and H3N2) and two influenza B strains (one from the Yamagata-like and one from the Victoria-like lineage).

2.7.1.3 Safety

2.7.1.3.1 Inactivated vaccine

In general, influenza vaccines are considered safe, and serious adverse events are rare in all age groups (Zangwill and Belshe, 2004, Jefferson et al., 2010). Local reactions such as redness, induration, swelling and pain at the injection site have been reported in 3-71% of the children vaccinated with non-adjuvanted TIV. Mild systemic reactions, such as fever, irritability, malaise and respiratory symptoms were reported in 4-16% of the children (Zangwill and Belshe, 2004, Neuzil et al., 2001, Englund et al., 2005, Neuzil et al., 2006). The frequency of post vaccination fever varies by age, with higher rates (12%) observed in younger children aged 1-5 years, and lower rates (5%) seen in older children aged 6-15 years (Neuzil et al., 2001). Furthermore, large scale population-based studies indicated no increase in clinically important medically attended events during a two week period after a TIV vaccination in children aged 6-23 months (Hambidge et al., 2006), or in children aged <18 years (France et al., 2004). The majority of the currently marketed TIVs are approved for children ≥6 months of age. TIV was, however, administered with safety and without excess adverse events also in a study conducted among infants aged 6-12 weeks (Englund et al., 2010).

Guillain-Barré syndrome (GBS) is a neurological disorder, commonly presenting as an acute inflammatory demyelinating polyradiculoneuropathy. It is considered to have an autoimmune origin and is commonly triggered by an infectious agent. Several studies have indicated association with influenza or ILI and GBS (Lehmann et al., 2010). During the 1976 national influenza vaccine campaign in the US against swine origin A(H1N1)/NJ/76 influenza, an increase in the incidence of GBS was observed and the vaccine campaign was suspended prematurely. Subsequent analyses of the surveillance data confirmed association between the vaccine and GBS (Schonberger et al., 1979). Several studies have thereafter explored the possible association between GBS and the seasonal influenza vaccine, but most of these studies were unable to establish a link between GBS and the vaccine (Lehmann et al., 2010). In two studies based on vaccine safety surveillance data, a small increase of approximately one additional case per million vaccinated people was observed after the seasonal influenza vaccination (Lasky et al., 1998, Haber et al., 2004). However, when considering the overall risk-benefit assessment, these data must be interpreted carefully. Despite a slight increase in the GBS observed after the vaccination, there is a more clear association between GBS and the influenza illness (Lehmann et al., 2010). Therefore, preventing influenza with vaccination may simultaneously protect from influenza-associated GBS.

2.7.1.3.2 Adjuvanted vaccine

Vaccine adjuvants are designed to boost immune responses and consequently adjuvanted vaccines may be more reactogenic. In studies conducted among children, adjuvants MF59 and AS03 have been associated with slightly increased numbers of local reactions when compared with non-adjuvanted vaccines (Vesikari et al., 2009, Waddington et al., 2010, Vesikari et al., 2011). AS03 was also associated with an increase in systemic reactions with 22% of the children <5 years having fever ≥38°C after the second dose (Waddington et al., 2010).

In Finland, an increase in the incidence of narcolepsy in children was observed after the pandemic influenza vaccine campaign in 2010. This raised concerns over the potential association between narcolepsy and the vaccine, especially the AS03 adjuvant included in the pandemic vaccine used in Finland. By 25 August, 2011, 98 new cases of narcolepsy following vaccination had been reported to the Finnish National Institute for Health and Welfare (THL) (THL, 2011). In their carefully conducted epidemiological assessment, THL confirmed that there was a link between the vaccine and narcolepsy. In the age group of 4-19 years, children that had received the AS03-adjuvanted vaccine had 12.7 (95 % CI 6.1-30.8) fold risk of developing narcolepsy when compared to unvaccinated children. A similar increase in the risk was not observed in adults. The AS03-adjuvanted vaccine was used in several European countries, as well as in Canada, and more than 30 million doses where administered during the pandemic (EMA, 2011b). However, an increase in the narcolepsy diagnoses in children was observed only in Finland and in Sweden (Läkemedelsverket, 2011), with sporadic events reported also in France and Norway.

Narcolepsy is a complex neurological disorder considered to have an autoimmune origin, and predisposing HLA types have been identified. Triggering factors previously associated with narcolepsy include antecedent respiratory and streptococcal infections (Picchioni et al., 2007, Aran et al., 2009). Recently, a significant seasonal distribution in the onset of narcolepsy was reported in a 13-year retrospective study from China (Han et al., 2011). The incidence of narcolepsy peaked in April and was at its lowest in November, with a 6.7 fold increase from trough to peak. In this study, an increase in the occurrence of narcolepsy was observed after the 2009 pandemic but this increase is unlikely to be associated with influenza vaccination as only 5.7% of the subjects had been vaccinated against pandemic influenza.

Although the epidemiological studies in Finland and Sweden confirmed an association between the vaccine and narcolepsy, the mechanisms for this are unclear (THL, 2011, Läkemedelsverket, 2011). The restricted geographical localization of the increase in narcolepsy cases in Nordic countries suggests that there might be a genetic or environmental factor that in combination with the vaccine increases the risk for narcolepsy. In Finnish children diagnosed with narcolepsy, all 41 children tested had the HLA DQB1*0602 allele associated with increased narcolepsy risk. In addition, one fourth of the children were found to have antibodies reacting with the AS03 adjuvant included in the vaccine. Whether the development of these antibodies contributes to the increased risk for narcolepsy remains to be solved. In Finland, the use of the AS03-adjuvanted vaccine in children was suspended in August 2010, and in July 2011 the

EMA also restricted the use of the vaccine in subjects <20 years only to situations when other influenza vaccines are not available (EMA, 2011b).

2.7.1.3.3 Live attenuated vaccine

Intranasally administered LAIV certainly lacks the injection site reactions associated with the intramuscular administration of TIV. On the other hand, LAIV is designated to replicate in the upper airways of the recipient, which may account for the mild systemic reactions and respiratory tract symptoms associated with LAIV. In a large pre-licensure, placebo-controlled trial, adverse events reported more frequently among children in the vaccine group included runny nose and nasal congestion (58% vs. 47%), fever (15% vs. 11%) and decreased activity (16% vs. 12%) (Belshe et al., 1998). Fever is reported more commonly after the first dose of LAIV, compared to TIV (5.4% vs. 2.0%) (Belshe et al., 2007). Post-licensure safety assessment in subjects aged 5-49 years did not indicate a significant increase in rare and severe adverse events associated with LAIV (Izurieta et al., 2005). In a large placebo-controlled safety assessment, a statistically significant increase in asthma diagnoses was observed in children 18-35 months of age (Bergen et al., 2004). Similarly, an increase in wheezing episodes in children <24 months receiving LAIV was observed in a large study comparing the efficacy of LAIV and TIV (Belshe et al., 2007). In the same study, a statistically significant increase in hospitalizations for any cause was detected in children aged <12 months. These findings have raised uncertainty about the safety of LAIV among young children and in subjects with asthma. Therefore, LAIV is currently approved in the U.S. and in the EU only for children ≥2 years without a history of wheezing.

2.7.1.4 Immunogenicity

The standard method of assessing the immunogenicity of influenza vaccines is to measure antibody responses with the hemagglutination inhibition (HAI) test. This assay indirectly measures the amount of virus-specific antibody in the sera by comparing the ability of serum from a vaccinated subject to compete with animal-derived erythrocytes in binding to influenza viruses. HAI titer ≥1:40, or four-fold increase in the postvaccination titer, are universally used limits for seroconversion in clinical studies. The other widely used test to measure antibody responses and the immunogenicity of the vaccine is the microneutralization assay, in which the serum is mixed with influenza viruses and the ability of this mixture to infect cells in cell cultures is evaluated (Rowe et al., 1999). Recently, more modern methods have been introduced to evaluate B and T-cell responses, as well as changes in gene expression, following an influenza vaccination (He et al., 2006, Sasaki et al., 2007, Zhu et al., 2010). Although immunogenicity studies are important and give acceptable projections of the potential of the vaccine, immunogenicity does not directly compare to clinical efficacy. Therefore, the actual efficacy of the vaccine needs to be determined in clinical studies.

2.7.1.4.1 Inactivated vaccine

Generally, children have weaker antibody responses compared to adults, and therefore two doses of vaccine are recommended to previously unvaccinated children aged ≤9 years (Fiore et al., 2010). A study conducted in children aged 5-8 years indicated that

two doses of TIV were needed to achieve protective levels of antibodies (HAI ≥1:40), especially in children with low levels (HAI ≤1:10) at the baseline (Neuzil et al., 2006). Of these children seronegative at the baseline, only 11-35% reached HAI levels of ≥1:40 after the first dose of the vaccine. After the second dose, the rates of seroconversion increased to 48-85%, with higher responsiveness observed against influenza A than B types. Of the children seropositive at the baseline (HAI ≥1:10), 85-100% had seroconverted already after the first dose of the vaccine. Similar findings were also observed in another similar study conducted in children aged 6-23 months (Englund et al., 2005). These findings indicate the need for two doses in previously naïve children.

In a study evaluating the immunogenicity of non-adjuvanted inactivated monovalent vaccine against the pandemic 2009 A(H1N1) strain in children aged 6 months to 9 years, 93% of the children responded to a single 15 μg dose of the vaccine (Nolan et al., 2010). The 15 μg dose used in this study was higher than the standard 7.5 μg dose of each of the three strains used in seasonal vaccines in children aged 6-35 months in the US. The association between the robust response, noted especially in the youngest children, after the first dose and the higher 15 μg dose used needs to be further studied. Other potential explanations include differences in the type of the vaccine, viral strains, or the HAI assay used. In contrast to the US, in Finland the recommended dose for all children aged 6-35 months includes 15 μg of each of the three vaccine strains.

Recently, some researchers have suggested that by preventing natural influenza infections with annual influenza vaccinations with TIV, we simultaneously might prevent the development of cell-mediated heterosubtypic immunity against the strains not included in the vaccine, and consequently children receiving an annual seasonal vaccination could be more vulnerable in the occurrence of a novel pandemic virus (Bodewes et al., 2009). Bodewes et al. reported that animals vaccinated with seasonal TIV are more vulnerable to a subsequent H5N1 infection (Bodewes et al., 2011b). However, in humans the data are very limited. In children, antecedent H3N2 infection was associated with a lower risk to contract a pandemic H1N1 infection (Cowling et al., 2010b). Recently, children who had received TIV annually were reported to have lower age-dependent levels of virus specific CD8+ T cells, compared to never-vaccinated controls (Bodewes et al., 2011a). However, in this study, the groups were not fully comparable, as cases consisted of children with cystic fibrosis, and controls did not have any underlying conditions. On the other hand, previous seasonal influenza vaccination did not have any effect on the risk of contracting a pandemic A(H1N1) infection in a recent case-control study in children (Nelson et al., 2011). Nevertheless, the role of cell mediated responses and heterosubtypic immunity in the clinical protection against influenza infection is poorly understood, and more research in this area is warranted.

2.7.1.4.2 Adjuvanted vaccine

In two studies conducted in children 6-35 months and 6-71 months of age, MF59-adjuvanted seasonal TIV was compared to non-adjuvanted TIV (Vesikari et al., 2009, Vesikari et al., 2011). The MF59-adjuvanted vaccine was more immunogenic, with significantly higher HAI geometric mean titers (GMT) against all three vaccine strains. The differences in seroconversion rates were most evident in the youngest children and

against influenza B. Furthermore, MF59 induced higher cross-reactivity against mismatched influenza A strains. Another adjuvant, AS03, has been studied in children 6 months to 12 years of age together with the monovalent inactivated split vaccine against the pandemic 2009 A(H1N1) strain. The AS03-adjuvanted vaccine was found to be more immunogenic than its comparator, the non-adjuvanted whole virion vaccine, with a 99% seroconversion rate after two doses of the vaccine (Waddington et al., 2010).

2.7.1.4.3 Live attenuated vaccine

One dose of LAIV has been described to induce antibody responses against influenza A(H3N2) and B strains, but two doses are needed to obtain immunity against influenza A(H1N1) in a majority of previously unvaccinated children (Gruber et al., 1996, Belshe et al., 1998). In a study conducted among children aged 15-71 months, seroconversion after the first vaccine dose against influenza A(H1N1), A(H3N2) and B was detected in 16%, 92% and 88% of the children, respectively. After the second dose, 61% of the children had seroconverted against A(H1N1) and 96% against both, A(H3N2) and B (Belshe et al., 1998). Similarly to TIV, two doses of LAIV are recommended to previously unvaccinated children <9 years of age (Fiore et al., 2010).

When compared to TIV, LAIV has been demonstrated to induce weaker systemic antibody responses, especially in previously seronegative subjects (Sasaki et al., 2007). However, LAIV is a stronger inductor of mucosal IgA response measured from nasal wash specimens. The strong mucosal response, likely mediated by the nasal administration route, is considered as an important factor in the protective efficacy of LAIV (Beyer et al., 2002). While both LAIV and TIV have been described to elicit significant effector B cell responses, memory B cells were detected more frequently following TIV administration (Sasaki et al., 2007). On the contrary, stronger T cell responses were observed in children receiving LAIV compared to TIV recipients (He et al., 2006).

2.7.1.5 Efficacy

The efficacy estimates of influenza vaccines in children range from no efficacy to ~85%. The great variations may partly be explained by the heterogenicity of the studies conducted. The age distribution and other demographic data have varied between the studies. A more important factor, however, is the endpoint measured. Studies using non-specific endpoints, such as influenza-like-illness (ILI), may result in an underestimation of the vaccine effectiveness as ILI is commonly caused by other viral infections, even during confirmed influenza activity (Zambon et al., 2001, Heikkinen et al., 2003). Therefore, laboratory-confirmed endpoints should nowadays be standard for all influenza vaccine efficacy studies. It has been suggested that the term “efficacy” should be used only in the context of RCTs using laboratory-confirmed endpoints, whereas the term “effectiveness” could be used more widely when reporting results from studies with different study designs and endpoints. The focus of this review is on the studies using laboratory-confirmed endpoints.

Another factor that contributes to the variability in the results of efficacy studies is that the efficacy of the influenza vaccine varies from season to season. The antigenic match between the vaccine and the circulating strain is probably the most important

determinant of the efficacy of an influenza vaccine (Skowronski et al., 2007, Belongia et al., 2009). Another significant factor is the incidence of influenza during the study period. If influenza is absent or circulating at very low levels, this may prevent detecting any significant efficacy, even if the vaccine would be highly efficacious (Hoberman et al., 2003, Beran et al., 2009). For these reasons, studies conducted over several seasons with varying vaccine match and incidence of illness are required.

2.7.1.5.1 Inactivated vaccine

Several studies have evaluated the efficacy of TIV against seasonal influenza in children (Table 2). Heikkinen et al. reported an 83% reduction in the incidence of laboratory-confirmed influenza A in children 1-3 years of age vaccinated with TIV (Heikkinen et al., 1991). In the same study, also the incidence of influenza-associated AOM was reduced by 83% among TIV recipients. In an RCT conducted in children aged 1-16 years over five consecutive influenza seasons, Neuzil et al. reported that TIV was 91% effective against laboratory-confirmed A(H1N1) and 77% effective against A(H3N2) (Neuzil et al., 2001). In an RCT in children 6-24 months of age by Hoberman et al, the vaccine efficacy (VE) was 66% during the first season when influenza was circulating at moderate levels but no efficacy was observed during the second season when influenza was circulating at exceptionally low levels (Hoberman et al., 2003). Recently, Vesikari et al. reported VE of 43% for non-adjuvanted TIV in an RCT in children 6-71 months of age. This study was conducted during two consecutive influenza seasons and the majority of the cases were caused by vaccine-matched influenza A(H3N2) viruses (Vesikari et al., 2011).

Several case-control studies have been conducted in children aged 6-59 months with VE estimates ranging from no significant efficacy to 86% (Shuler et al., 2007, Szilagyi et al., 2008, Eisenberg et al., 2008, Joshi et al., 2009, Cochran et al., 2010, Kelly et al., 2011, Katayose et al., 2011). In studies extending over several influenza seasons, variations in the VE estimates were detected between the seasons (Table 2). Despite cumulative data from virologically confirmed case-control studies, only a limited number of RCTs in young children have been published. The latest Cochrane review concluded that in children <2 years, the efficacy of TIV is similar to placebo (RR 0.55, 95% CI: 0.18 to 1.69) (Jefferson et al., 2008). This conclusion was, however, largely based on the paucity of evidence rather than evidence of low efficacy.

Table 2. Studies assessing effectiveness of TIV against laboratory-confirmed influenza in children <5 years.

Study	Age of the children (months)	Study design	Diagnostic methods	Study conducted	N (cases/controls) (vaccinees/controls)	Vaccine effectiveness (95% CI)
Heikkinen et al. 1991	7-50	Controlled open label	Antigen detection	1988-1989	187/187	**83 %** (59–93)
Hoberman et al. 2003	6-24	Randomized controlled trial	Viral culture	1999-2000 2000-2001	273/138 252/123	**66%** (34–82) -**7%** (-247 to 67)
Shuler et al. 2007	6-59	Case-control	Antigen detection (POC test)	2003-2004	290/580	**49 %** (30–60)
Szilagyi et al. 2008	6-59	Case-control	RT-PCR or viral culture	2003-2004 2004-2005	74/622 95/647	**52%** (-100 to 90) **7%** (-80 to 50)
Eisenberg et al. 2008	6-59	Case-control	RT-PCR or viral culture	2003-2004 2004-2005	288/744 197/1305	**44%** (-42 to 78) **57%** (28–74)
Joshi et al. 2009	6-59	Case-control	RT-PCR or viral culture	1999-2007	103/103	**86 %** (29–97)
Cochran et al. 2010	6-23	Case-control	RT-PCR or Viral culture or antigen detection	2003-2004 2004-2005 2005-2006	213/951 29/124 58/273	**5%** (-80 to 50) -**50%** (-350 to 50) **59%** (1–80)
Katayose et al. 2011	6-71	Observational cohort	Antigen detection (POC test)	2002-2008	6933/6968	A: **52%** (47–56) B: **59%** (52–64)
Kelly et al. 2011	6-59	Case-control	RT-PCR and Viral culture	2008	48/24	A: **82%** (21–96) B: **43%** (-39 to 77)
Vesikari et al. 2011	6-71	Randomized controlled trial	RT-PCR	2007-2009	1772/993	**43%** (15–61)

Children are major disseminators of influenza and play an important role in the spread of the illness in communities. The indirect benefits of vaccinating children have been described to extend to non-vaccinated members of the community. Already during the 1968 A(H2N2) pandemic, Monto et al. reported that vaccinating schoolchildren prevented respiratory infections effectively in the entire community (Monto et al., 1969). In Japan, mandatory influenza vaccination among schoolchildren from 1962 to 1987 was associated with significant reductions in the excess influenza-associated mortality among older people (Reichert et al., 2001). In a recent cluster-randomized trial conducted among Hutterite colonies by Loeb et al., children aged 3-15 years were vaccinated either with TIV or placebo. In those colonies where children received TIV, non-vaccinated adults experienced 61% less laboratory- confirmed influenza infections compared to colonies were children received placebo (Loeb et al., 2010).

2.7.1.5.2 Adjuvanted vaccine

Limited data exist on the efficacy of adjuvanted vaccines in children. Recently, Vesikari et al. compared the efficacy of MF59-adjuvanted TIV with non-adjuvanted TIVs and control vaccines in children aged 6-71 months (Vesikari et al., 2011). The absolute VE of the adjuvanted TIV (compared with placebo) was 86%, and the relative VE of the adjuvanted vs. non-adjuvanted TIV was 75%. In a Canadian case-control study where 39% of the participants were children, the VE of an AS03-adjuvanted monovalent pandemic vaccine was 93% (Skowronski et al., 2011).

2.7.1.5.3 Live attenuated vaccine

LAIV has been extensively studied in children, with several large RCTs conducted in different parts of the world. In a meta-analysis including all manufacturer-sponsored trials, the overall VE against any type of laboratory-confirmed influenza, regardless of antigenic match, was 72% for all children aged 6 months to 17 years and 69% for younger children aged 6-35 months. VE against strains similar to the vaccine strain was 85% for A(H1N1); 76% for A(H3N2) and 73% for influenza B (Rhorer et al., 2009). Importantly, LAIV has been described to induce cross-protection also against antigenically mismatched strains (Belshe et al., 2000, Halloran et al., 2007). Belshe et al reported 86% VE against the variant A(H3N2) strain that was antigenically different from the strain included in the vaccine (Belshe et al., 2000). Despite the good efficacy observed also in the youngest children, LAIV is not licensed to be used in children <2 years of age due to increased wheezing in this age group (Bergen et al., 2004, Belshe et al., 2007).

In children, LAIV seems to be more effective than TIV. In a large RCT comparing LAIV and TIV in children 6-59 months of age, 55% less cases of laboratory-confirmed influenza were observed among children vaccinated with LAIV (Belshe et al., 2007). The relative efficacy of LAIV compared to TIV was 58% against mismatched strains and 45% against matched strains. In contrast, in a similar study conducted in adults, LAIV was less effective than TIV, with 50% less laboratory-confirmed influenza cases among TIV recipients (Monto et al., 2009). These studies did not include placebo groups, and consequently it was not possible to estimate the absolute efficacy of the vaccines against influenza. Although two doses of LAIV are required for optimal protection in previously unvaccinated children, significant levels of protection (60-87%) were observed in children 2-6 years of age already after the first vaccine dose

(Block et al., 2009). Similar to TIV, vaccinating schoolchildren with LAIV has been described to induce indirect herd immunity and reduce respiratory infections in the entire community (Glezen et al., 2010).

2.7.1.6 Influenza vaccine recommendations

In the US, healthy children 6-24 months of age were first recommended a routine influenza vaccination in 2003. The recommendation was expanded in 2006 to cover all children aged 6-59 months, and in 2008 further expanded to cover all children aged 6 months to 18 years. Currently, the US Advisory Committee on Immunization Practices (ACIP) recommends universal influenza vaccination for all persons aged ≥6 months (Fiore et al., 2010). Also the Canadian National Advisory Committee on Immunization recommends routine influenza vaccination for children aged 6 to 24 months (Orr, 2004). In Europe, routine influenza vaccination is recommended for healthy children only in a few countries, including Austria, Finland, Estonia, Latvia, Slovakia and Slovenia (Mereckiene et al., 2010). Of the European countries, Finland was the first and only country to implement the recommendations when the annual influenza vaccination of children aged 6-35 months was included in the national immunization program in 2007. The decision to include young children in the vaccination program was largely based on detailed data on the burden of illness (Heikkinen et al., 2004), together with an extensive cost-effectiveness evaluation (Salo et al., 2006). In Finland, as well as in most other developed countries, the influenza vaccine is recommended also for persons aged ≥65 years, pregnant women and those with underlying medical conditions.

2.7.1.7 The future of influenza vaccines

Despite the great progress made in the field of vaccine development, the efficacy and the production capacity of influenza vaccines still remain suboptimal. Several approaches have been taken to enhance the immunogenicity and the efficacy, especially in subgroups considered less responsive, such as children and the elderly. Different adjuvants (Vesikari et al., 2009), higher doses (Couch et al., 2007) and the subcutaneous administration of the vaccine (Holland et al., 2008) are studied and used as a way to improve the efficacy of current vaccines, especially in the elderly. New approaches are adopted to expedite vaccine production and increase the production capacity. A shift from egg-culture based vaccines to vaccines produced in cell cultures may facilitate vaccine production also in the context of the seasonal epidemics (Frey et al., 2010, Barrett et al., 2011), but the greatest benefits of this technique might be observed only in the threat of a new pandemic when the time constraints could be stricter. Furthermore, if the emerging pandemic would be of avian origin, this might significantly restrict the availability of hens' eggs and subsequently hinder the egg based vaccine production.

Recombinant DNA techniques represent a completely novel approach to vaccine development. In the recombinant technique, the HA gene of the influenza virus is cloned into a viral vector (e.g. baculovirus). Cell cultures infected with the vector start to express the HA, which is subsequently harvested, purified and used as a vaccine. The recombinant HA vaccine has shown promising results in preliminary clinical trials (Treanor et al., 2007) and is currently in phase 3 clinical trials. When several

recombinant vectors expressing different influenza viral proteins (e.g. HA, NA and M1) are used simultaneously to infect the cell culture, the expressed gene products self-assemble to form virus-like particles, which have shown promise as vaccine candidates (Kang et al., 2009). Other examples of the recombinant technique include using viral vectors (e.g. adenovirus) to directly deliver influenza proteins to the immune system without causing the illness itself (Van Kampen et al., 2005). DNA plasmids, where HA and NA genes of influenza have been introduced, have also been studied as vaccine candidates (Jones et al., 2009). All the above mentioned vaccine candidates are already at least in phase 1 clinical trials. (Lambert and Fauci, 2010).

Although facilitating and enabling more rapid vaccine production without restrictions placed by the availability of hens' eggs, all the previously mentioned approaches are dependent on the antigenic match between the vaccine and the circulating strain. To overcome this problem, a lot of effort is made to discover a universal influenza vaccine that would induce cross-protection against all influenza types. Such a vaccine would not be affected by the constant antigenic drift of the viruses and could also offer protection against antigenically shifted strains with pandemic potential. The major targets studied include the conserved epitopes of M1 protein, nucleoprotein (NP), HA and the conserved external domain of M2 protein (Du et al., 2010).

2.7.2 Antiviral prophylaxis

Antiviral chemoprophylaxis can be divided into two entities. Post-exposure prophylaxis (PEP) refers to the initiation of an antiviral regimen to the close contacts of an index patient with confirmed or suspected influenza. Usually PEP is applied within the household setting. Sometimes it is employed at a larger scale at the institutional level. Seasonal prophylaxis denotes the long-term use of the antiviral agent throughout the influenza season. It could be considered in patients with immunosuppression or other underlying conditions that increase the risk of influenza complications.

2.7.2.1 Post-exposure prophylaxis

The efficacy of rimantadine in PEP within a household setting was evaluated in a single RCT. Rimantadine (200 mg per day), or placebo, was administered for the index person and all family members for 10 days. In this study, no differences were observed in the occurrence of secondary infections between the groups (Hayden et al., 1989).

Both neuraminidase inhibitors, zanamivir and oseltamivir, have been shown to be effective in preventing influenza transmission and secondary cases within households. Zanamivir was investigated as PEP for adults and children aged ≥5 years in two RCTs. When initiated to household contacts of an index person with suspected or confirmed influenza, zanamivir was associated with an ~80% reduction in the confirmed secondary influenza cases (Hayden et al., 2000a, Monto et al., 2002).

Oseltamivir PEP was 89% effective in subjects ≥12 years of age in preventing confirmed influenza within the household contacts of an index patient who was not treated with oseltamivir (Welliver et al., 2001). In another study that was conducted in subjects aged ≥1 year and where the index patient received oseltamivir treatment at a normal dosage, PEP with oseltamivir was still effective and reduced the rate of

secondary infections by 84% (Hayden et al., 2004). In the sub-analysis of pediatric patients 1-12 years of age, the protective efficacy was 80%.

A larger scale PEP in a closed setting (such as hospitals, schools and military units) that aims to geographical containment of the epidemic, is referred to as "ring chemoprophylaxis". A recent report from Singapore describes that outbreaks in military units in Singapore during the 2009 pandemic were efficiently contained by administering oseltamivir PEP to all contacts of an identified influenza-positive index subject in addition to standard outbreak control measures (Lee et al., 2010b). Before the initiation of oseltamivir prophylaxis, 6.4% of the personnel were infected. The attack rate was reduced to 0.6% after oseltamivir intervention. There are further reports that oseltamivir was successfully used to control outbreaks also in a boys' summer camp among children aged 8-14 years (Kimberlin et al., 2010a), as well as in a residential facility for patients undergoing hematopoietic stem cell transplantation (Vu et al., 2007).

2.7.2.2 Seasonal prophylaxis

Adamantanes have been shown to be effective in seasonal prophylaxis against influenza A infections. In adults, amantadine and rimantadine have shown similar efficacy in preventing 65-90% of influenza cases (Dolin et al., 1982, Jefferson et al., 2006). In children, the reported efficacies of seasonal prophylaxis with amantadine and rimantadine have been 89% and 100% against symptomatic influenza A infections, respectively (Finklea et al., 1967, Clover et al., 1986, Crawford et al., 1988, Galvao et al., 2008). The benefits of children's rimantadine treatment were also extended to the family members of treated children who experienced a significantly lower incidence of influenza. The use of adamantanes, however, is not currently recommended, due to the development of widespread resistance.

Zanamivir was evaluated for seasonal prophylaxis in two RCTs. In healthy adults, a 4-week course of inhaled zanamivir, 10 mg once daily, prevented 67% of symptomatic, laboratory-confirmed influenza cases (Monto et al., 1999). In a similar study among patients with high-risk conditions, the protective efficacy was 83% (LaForce et al., 2007).

The efficacy of oseltamivir in seasonal prophylaxis was explored in an RCT in low-risk adults. A 6-week course of oseltamivir, 75 mg once a day, was 76% efficacious in preventing laboratory-confirmed influenza when compared to placebo (Hayden et al., 1999a). Another similar study was conducted among elderly patients living in nursing homes. Eighty percent of the subjects were vaccinated against influenza. In this study, a 6-week course of oseltamivir reduced the incidence of laboratory-confirmed influenza by 92% compared to placebo (Peters et al., 2001).

2.7.3 Non-pharmaceutical prophylaxis

Non-pharmaceutical prophylaxis includes measures that aim to reducing influenza transmission both on the individual and community level. These measures are applied to some extent also during the seasonal epidemics. However, the importance of non-

pharmaceutical measures is highlighted in the context of a pandemic when vaccines and antivirals may not be readily available in sufficient numbers.

Commonly used measures at a personal level include hand hygiene, face masks and cough etiquette. However, limited data exists on the efficacy of these interventions. Hand washing and use of alcohol-based hand rubs have been demonstrated to clear effectively influenza viruses from human hands (Grayson et al., 2009), emphasizing the importance of these basic procedures. A randomized, controlled cluster study from Hong Kong assessed the efficacy of improved hand hygiene, with or without face masks, compared to placebo in preventing household transmission of influenza (Cowling et al., 2009). The adherence to interventions was low in this study and no statistically significant difference was observed in the secondary attack rate between the groups. However, in the subgroup analysis of those households where the measures were introduced within 36 hours from the onset of the index person's illness, a statistically significant reduction was observed in the attack rates among participants who employed improved hand hygiene and used face masks, suggesting that to be effective, these measures should be employed early in the course of the illness. There is some evidence to support the use of face masks during an illness to protect others, while fewer data exist to support the use of masks to prevent becoming infected (Cowling et al., 2010c). No difference was observed between surgical masks and N95 respirators in protective efficacy against influenza among health care workers during a seasonal influenza epidemic (Loeb et al., 2009).

During a pandemic, more strict measures at the community level may be considered. Pandemic preparedness plans have consisted of different mitigation strategies, including travel restrictions, border screening, school closures, case isolation and quarantine. In mathematical modeling, border closures have been estimated to have only little effect (Ferguson et al., 2006), and similar to border entry screening, may delay the spread of a pandemic by 1-2 weeks (Cowling et al., 2010a). School closures during the peak of a pandemic could reduce the peak attack rate by 40%, but the impact on the overall attack rate may be little (Ferguson et al., 2006, Cauchemez et al., 2008). Case isolation and household quarantine could be effective in reducing the attack rate, but only if a high compliance is achieved (Ferguson et al., 2006).

3 AIMS OF THE STUDY

The specific objectives of this study were:

I To identify signs and symptoms that could predict influenza in children and could help clinicians to differentiate between influenza and other respiratory infections *(Study I)*.

II To assess the feasibility of diagnosing influenza in young children during the early stage of illness using POC rapid tests and laboratory-based methods *(Study II)*.

III To determine the efficacy of early oseltamivir treatment started within 24 hours from the onset of illness in children aged 1-3 years *(Study III)*.

IV To estimate the effectiveness of the inactivated influenza vaccine against laboratory-confirmed influenza in children 9 months to 3 years of age *(Study IV)*.

4 MATERIALS AND METHODS

Details of the materials and methods are presented in the original publications.

4.1 Participants and study design

Study I (*Signs and symptoms predicting influenza*) was a matched case-control study, for which data were derived from a prospective cohort study conducted during two consecutive influenza seasons in 2000-2001 and 2001-2002 (Heikkinen et al., 2004). In the autumn of each study year, children aged < 13 years were recruited from local day care centers, family day care and schools. No exclusion criteria were employed. The parents of the participating children were asked to bring their child to a specific study clinic every time the child developed fever or symptoms of respiratory infection. The study office was open from 9 October, 2000 through 20 May, 2001, and from 1 October, 2001 through 19 May, 2002. During each visit, the child was examined by a study physician and the signs and symptoms were recorded on a structured form. During each new illness episode, a nasal swab was obtained for virological analyses.

Of a total of 372 episodes of laboratory-confirmed influenza, all 353 influenza-positive children from whom complete data on the clinical presentation were available were included in this analysis as cases (two children were excluded because of a confirmed double viral infection and 17 children due to incomplete data on the degree of fever). For each influenza-positive case, we selected an influenza-negative control child who provided the closest match to the case with respect to gender, age and timing of the visit.

For **studies II-IV**, data were derived from a prospective cohort study that was carried out during the influenza seasons of 2007-2008 and 2008-2009 in Turku, Finland. Prior to the beginning of each influenza season, we recruited children aged 1-3 years through mailed announcements and local advertisements. There were no exclusion criteria for enrolment. A total of 1,185 children were enrolled in the study; the numbers of children in the cohorts during the first and second season were 631 and 554, respectively. When active surveillance indicated the circulation of influenza viruses in the community, a study clinic was opened. The clinic was open throughout the influenza season, from January 14 through April 9, 2008, and from January 7 through March 26, 2009, every day, including weekends and holidays. The parents of the enrolled children were asked to bring their child to the study clinic whenever the child developed fever or signs of respiratory infection. In the study clinic, a clinical examination was performed by a study physician and three nasal swabs were obtained for virological analyses at least once during each respiratory episode.

In the first part of **study II** (*Influenza diagnostics during early stage of illness*), we included all 61 children from the placebo arm of the oseltamivir trial (study III) who had laboratory-confirmed influenza (viral culture, TR-FIA, or RT-PCR) diagnosed at any time within 8 days from the onset of symptoms. In the second part of this study, we included 158 children to whom influenza rapid tests were performed within 24

hours from the onset of symptoms. Rapid tests were performed routinely for all febrile children (≥38°C) without respiratory symptoms, but also to any other children at the discretion of the study physician.

Study III (*Efficacy of early oseltamivir treatment*) was a randomized, placebo-controlled, double-blind trial. To be eligible for the treatment trial, the child had to have a fever of ≥38°C for <24 hours. In addition, the child had to have at least one symptom of respiratory infection (cough, rhinitis or sore throat) or a positive influenza rapid test result. Exclusion criteria were as follows: suspicion of invasive bacterial infection; virologically confirmed infection other than influenza; known immunosuppression; uncontrolled underlying medical condition; oseltamivir treatment within 4 weeks; allergy to oseltamivir; and participation in another trial with an investigational drug. A total of 409 children were randomized to the study treatment.

Study IV (*Effectiveness of influenza vaccination*) was an observational cohort study that was conducted during the influenza season of 2007-2008. This was the first season after the inclusion of influenza vaccination of all children aged 6-35 months into the national childhood vaccination program in Finland. The study cohort consisted of all 631 children enrolled for the season 2007-2008.

4.2 Intervention and data collection

In **Studies I-II** there were no interventions. The data on the signs and symptoms (Study I) were obtained from standardized case report forms filled out by the study physician during each visit.

Children included in **Study III** were randomized at a ratio of 1:1 to receive orally administered oseltamivir suspension or a matching placebo. The first dose of the study drug was administered within 24 hours from the onset of fever and the treatment was continued twice a day for 5 days. Standard weight- based dosage was used for oseltamivir; 30 mg twice daily for children weighing ≤15.0 kg, and 45 mg twice daily for children weighing 15.1–23.0 kg.

A follow-up visit was scheduled on study days 5-8. In addition, the parents were encouraged to bring their child to the study clinic for re-evaluation whenever they deemed it necessary. During each visit, the child was examined by the study physician and three nasal swabs were obtained if the child was symptomatic. Pneumatic otoscopy, tympanometry and acoustic reflectometry were used in diagnosing acute otitis media (AOM). The diagnosis of AOM was based on the detection of middle ear fluid, signs of inflammation of the tympanic membrane and at least one symptom of acute infection.

Symptoms diaries were filled out by the parents twice daily on study days 1-7, and once a day on study days 8-21. At each time-point, the parents recorded the temperature of the child; presence and severity (on a 4-point scale: 0=absent, 1=mild, 2=moderate, 3=severe) of symptoms; the child's return to normal activities; the child's absenteeism from the day care; parental absenteeism from work due to the child's illness; the use of relief medication and antibiotics, as well as compliance to the study treatment.

In Study IV, the vaccination was offered and the children were vaccinated as part of the national vaccination program in their local health care centers. The children received a 0.5 ml dose of the trivalent inactivated subunit vaccine. Previously unvaccinated children received two doses of the vaccine with a 4-week interval. The vaccination campaign took place in October-November 2007, and all vaccinations were given before the beginning of the influenza season. For the purposes of this study, the age of the children was calculated as of November 1, 2007.

The vaccination status of the children was derived from a questionnaire filled out by the parents before the beginning of the clinical phase of our study. The season 2007-2008 was the first one when the children's influenza vaccination was included in the national vaccination program. Before that season, influenza vaccination of children was very rare (Heikkinen et al., 2004, Blank et al., 2008). We therefore classified children who had received two vaccinations in 2007 as fully vaccinated, and children who had received only one dose as partly vaccinated.

The strains included in the vaccine for season 2007-2008 were A/Solomon Islands/3/2006 (H1N1), A/Wisconsin/67/2005 (H3N2) and B/Malaysia/2506/2004. Influenza A viruses detected in Finland during the 2007-2008 season were almost entirely of the subtype A(H1N1), with only sporadic detections of A(H3N2). Both of these subtypes matched well with the H1 and H3 strains included in the vaccine. In contrast, all influenza B viruses detected in Finland during the 2007-2008 season were Yamagata-like viruses that were lineage-level mismatched with the Victoria-like viruses included in the vaccine.

4.3 Viral sampling

All specimens for viral detection were collected with nasal swabs. In **Study I,** we used cotton swabs, whereas in **Studies II-IV,** flocked swabs (Copan, Italy) were employed, with the exception of the samples for influenza rapid tests in Study II that were obtained with the polyester swab provided with the kit.

4.4 Virological analyses

In **Study I,** the detection of influenza viruses was based on viral culture in Madin-Darby canine kidney cells, followed by immunoperoxidase staining with monoclonal antibodies (Waris et al., 1990). In **Studies II-IV,** all samples were tested with the above-described method of viral culture and antigen detection (TR-FIA) (Nikkari et al., 1989). In addition, samples from febrile children (≥38°C) that remained negative with the viral culture and antigen detection were further tested with RT-PCR for influenza A and B viruses. The sample was considered positive if an influenza virus was detected with any of these methods. In **Study II,** influenza rapid tests (Actim Influenza A & B, Medix Biochemica, Finland) were performed immediately at the point of care according to the manufacturer's instructions.

4.5 Outcomes, data analysis, and statistical methods

In all the studies, where applicable, the Mann-Whitney *U* test was used for comparing the differences in medians; an unpaired *t* test was used to compare the differences in means; the χ^2-test or the Fisher exact test was used for comparing the differences in proportions; and one way ANOVA was used to compare the differences in continuous variables between the groups. The Wilcoxon (survival) test was used for comparing the survival curves in the time-to-event analyses. All statistical analyses were performed with SAS, version 9.2 or StatsDirect, version 2.7.7. Two-sided P values < 0.05 were considered statistically significant.

In **Study I,** we performed initially a univariate conditional logistic regression analysis to compare the signs and symptoms among matched pairs of influenza-positive cases and influenza-negative controls. To further identify signs and symptoms independently predicting influenza, we performed a multivariate conditional logistic regression analysis with all the variables reaching a P value <0.1 in the univariate analysis included in the model. The results were reported as odds ratios (OR) with 95% confidence intervals (CI). The likelihood ratio (LR) of a positive test was calculated as sensitivity/(1-specificity), and the likelihood ratio of a negative test was calculated as (1-sensitivity)/specificity. Subjective symptoms (headache, sore throat, and myalgia) were analyzed only among children ≥3 years of age.

In **Study II,** we estimated the sensitivity of diagnosing influenza during the early stage of the illness with conventional laboratory methods (viral culture, TR-FIA, RT-PCR), by determining the proportion of children (among all influenza-positive children) in whom the influenza virus was detectable already from the samples collected within 24 hours from the onset of symptoms. In the second part of the study, we assessed the performance of the influenza rapid test by comparing the results of the rapid tests to the composite reference of the viral culture, TR-FIA, and RT-PCR. The reference test was considered positive if any of these tests yielded a positive result.

The primary outcome of **Study III** was the development of AOM in children with laboratory-confirmed influenza. Secondary outcomes included the time to resolution of illness; time to resolution of all symptoms; time to resolution of fever (≤37.5°C); parental absenteeism from work due to the child's illness; and use of relief medication. All efficacy outcomes were analyzed in influenza-positive children in whom the treatment was started within 24 hours from the onset of symptoms. The primary outcome was analyzed also among a predefined subgroup of children in whom the treatment was initiated already within 12 hours from the onset of symptoms.

The time to resolution of illness was defined as the interval from the administration of the first dose of the study medication to the first time when the following conditions were met simultaneously and lasted so for at least 24 hours: temperature ≤37.5°C; rhinitis and cough either absent or mild; and the child appeared healthy and had returned to normal activities. If fever reappeared, or if cough or rhinitis worsened to moderate or severe levels during the 5-day study medication period, the duration of these symptoms was calculated until the first time that the above listed conditions were

again met after the worsening of these symptoms. *The time to resolution of all symptoms* required a total absence of all symptoms.

The sample size calculations were based on the hypotheses that 50% of the children fulfilling the inclusion criteria will have influenza; 30% of influenza-infected children receiving placebo will develop AOM; and oseltamivir treatment will prevent 60% of the AOM cases. With a 5% level of significance and 80% power, the number of influenza-infected children needed in each group was 77.

All efficacy analyses were performed among children with laboratory-confirmed influenza who had received at least one dose of study drug, and who fulfilled all inclusion criteria. Safety and tolerability were analyzed among safety population that consisted of all children, regardless of their influenza status, who had received ≥1 dose of the study drug and for whom any follow-up information was available. Children who had received ≥8 doses or 80% of the designated amount of the study drug were considered compliant to the treatment.

The main outcome in **Study IV** was the incidence of symptomatic laboratory-confirmed influenza. The primary effectiveness of the vaccine was evaluated by comparing the incidence of influenza between the fully vaccinated and unvaccinated children in the follow-up cohort. The vaccine effectiveness (VE) was calculated as (1-relative risk) x 100. VE was analyzed separately for any influenza, influenza A, and influenza B, and subgroup analyses were performed for children <2 years and ≥2 years of age.

To address the possibility that some children in the cohort might not have been brought to the study clinic during an influenza illness, we performed a case-control analysis, where we included only children who made at least one visit to the study clinic. Children who tested positive for influenza at least once during the follow-up period were cases, and children whose all samples remained negative for influenza were controls. The cases and controls were not matched. In this analysis, partly vaccinated children were classified as unvaccinated, and VE was calculated as (1-odds ratio) x 100.

4.6 Ethics

Written informed consent was obtained from the parents of the participating children, and the study protocols were approved by the Ethics Committee of the Hospital District of Southwest Finland.

5 RESULTS

5.1 Signs and symptoms predicting influenza (I)

5.1.1 Univariate analysis

In this study, we included 353 influenza-positive cases and 353 matched controls. In the univariate analysis, fever was the strongest predictor of influenza. The higher the fever, the stronger it was associated with influenza. The highest OR (58.46, 95% CI 19.36-176.53) was observed for fever ≥40.0°C. Other symptoms significantly associated with influenza in the univariate analysis included an impaired general condition (OR 3.78, CI 1.81-7.88); abdominal symptoms (OR 2.23, CI 1.16-4.29); pharyngitis (OR 1.48, CI 1.03-2.12); headache (OR 3.35, CI 1.95-5.76); and myalgia (OR 3.00, CI 1.09-8.25).

5.1.2 Multivariate analysis

We performed multivariate analyses including all symptoms with P<0.1 in the univariate analysis in the multivariate models. Because subjective symptoms (headache, myalgia and sore throat) were analyzed only in children ≥3 years of age, we performed two separate multivariate analyses; one restricted to children aged ≥3 years, where all symptoms (including subjective symptoms) were included; and the other analysis in all children where subjective symptoms were excluded. In both analyses, fever was the only symptom significantly associated with influenza. In children aged ≥3 years, the OR for temperature ranges of 38-38.9°C; 39-39.9°C and ≥40.0°C were 19.11 (95% CI 7.44-49.09); 31.90 (CI 11.28-90.23); and 59.18 (CI 14.25-245.73), respectively. In all children, the OR for fever ranged from 13.55 (CI 6.90-26.63) to 50.10 (CI 16.25-154.45) for temperatures 38-38.9°C and ≥40.0°C, respectively.

5.1.3 Likelihood ratios

The positive likelihood ratios (LR) also increased with gradual elevations in the temperature (Table 3). The highest positive LR (6.00) was observed for fever ≥38.0°C. On the other hand, a lack of fever was a strong negative predictor of influenza; negative LR for fever ≥38.0°C was 0.16. Other signs and symptoms that significantly increased the likelihood of influenza were an impaired general condition (LR 3.50); gastrointestinal symptoms (LR 2.07); and pharyngitis (LR 1.36). In children ≥3 years of age, the likelihood of influenza was increased by the presence of headache (LR 2.60) and myalgia (LR 2.43).

Table 3. Frequencies, sensitivities, specificities, positive and negative LRs for different signs and symptoms among influenza-positive cases (n=353) and matched influenza-negative controls (n=353).

Sign or symptom	Frequency in cases N (%)	Frequency in controls N (%)	Sensitivity, % (95% CI)	Specificity, % (95% CI)	LR of a positive test (95% CI)	LR of a negative test (95% CI)
Fever ≥38.0°C	317 (89.8)	126 (35.7)	90 (86-93)	64 (59-69)	2.52 (2.19-2.92)	0.16 (0.11-0.22)
Fever ≥39.0°C	177 (50.1)	49 (13.9)	50 (45-55)	86 (82-90)	3.61 (2.74-4.79)	0.58 (0.52-0.65)
Fever ≥40.0°C	42 (11.9)	7 (2.0)	12 (9-16)	98 (96-99)	6.00 (2.80-12.96)	0.90 (0.86-0.93)
Rhinitis	274 (77.6)	275 (77.9)	78 (73-82)	22 (18-27)	1.00 (0.92-1.08)	1.01 (0.77-1.33)
Cough	272 (77.1)	251 (71.1)	77 (72-81)	29 (24-34)	1.08 (0.99-1.18)	0.79 (0.62-1.02)
Pharyngitis	91 (25.8)	67 (19.0)	26 (21-31)	81 (77-85)	1.36 (1.03-1.80)	0.92 (0.84-0.99)
Impaired general condition	35 (9.9)	10 (2.8)	10 (7-14)	97 (95-99)	3.50 (1.79-6.89)	0.93 (0.89-0.96)
Gastrointestinal symptoms	31 (8.8)	15 (4.3)	9 (6-12)	96 (93-98)	2.07 (1.15-3.73)	0.95 (0.91-0.99)
Conjunctivitis	30 (8.5)	22 (6.2)	9 (6-12)	94 (91-96)	1.36 (0.81-2.30)	0.98 (0.93-1.02)
Laryngitis	24 (6.8)	14 (4.0)	7 (4-10)	96 (93-98)	1.71 (0.91-3.23)	0.97 (0.93-1.01)
Wheezing	9 (2.6)	14 (4.0)	3 (1-5)	96 (93-98)	0.64 (0.29-1.43)	1.01 (0.99-1.05)
Headache	65 (25.7)	25 (9.9)	26 (20-32)	90 (86-94)	2.60 (1.71-3.99)	0.82 (0.76-0.89)
Sore throat	90 (35.6)	80 (31.6)	36 (30-42)	68 (62-74)	1.13 (0.88-1.44)	0.94 (0.83-1.07)
Myalgia	17 (6.7)	7 (2.8)	7 (4-11)	97 (94-99)	2.43 (1.05-5.63)	0.96 (0.92-1.00)

5.2 Influenza diagnostics during the early stage of illness (II)

5.2.1 Detection of influenza within 24 hours from the symptom onset

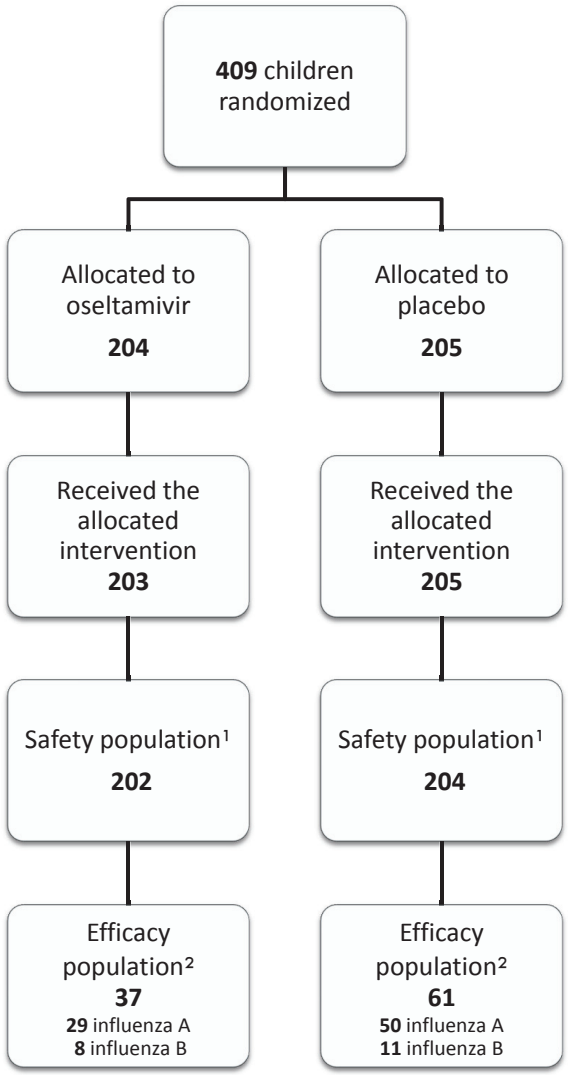
This analysis included all 61 children from the placebo arm of the oseltamivir treatment trial (Study III) in whom influenza was eventually confirmed with conventional laboratory methods (viral culture, TR-FIA or RT-PCR). The influenza virus was already detectable from the samples obtained within 24 hours from the onset of the illness in 56 of 61 children, yielding a sensitivity of 92% (95% CI 82–97%). The sensitivity was 94% (CI 83-99%) for detecting the influenza A virus and 82% (CI 48-98 %) for the influenza B virus (P=0.2). No significant differences were observed between the samples obtained within 12 h and 12-24 h from the onset of symptoms (P>0.9), between vaccinated and unvaccinated children (P=0.5), and between children <2 years and 2-3 years of age (P>0.9).

5.2.2 Sensitivity of the influenza rapid test

A total of 158 children with rapid tests performed within 24 hours from the onset of fever were included in this analysis. Influenza was confirmed with reference methods in 39 (25%) children. The overall sensitivity of the rapid test was 77% (95% CI 61-89%) and its specificity was 99% (CI 95-100%). The sensitivity was 90% (CI 74-98%) for influenza A and 25% (CI 3-61%) for influenza B (P<0.001). For any influenza, the positive predictive value (PPV) was 97% (CI 83-100%) and the negative predictive value (NPV) was 93% (CI 87-97%).

5.3 Efficacy of early oseltamivir treatment (III)

A total of 409 children were randomized to the study treatment, and 408 received the study drug. A simplified flow chart of the study is shown in Figure 3. A total of 98 children were diagnosed with laboratory-confirmed influenza and included in the efficacy analyses. Influenza A was diagnosed in 79 (80.6%) children and influenza B in 19 (19.4%) children. The mean age of the 98 children with influenza was 2.4 years (SD 0.8). Of these children, 37 (37.8%) were aged 1 to <2 years; 32 (32.7%) were 2 to <3 years; and 29 (29.6%) were 3 to <4 years of age. No statistically significant differences were observed between the groups with respect to age and sex distribution; day care attendance, prematurity; diagnosis of asthma; or uptake of the influenza vaccination for the season. Before randomization, the mean highest measured fever was 38.9° in both groups. AOM was diagnosed before randomization in 5 (13.5%) children in the oseltamivir group and in 6 (9.8%) children in the placebo group (P=0.74).



[1]The safety and tolerability was assessed in a population that consisted of all randomized children who had received at least one dose of the study drug and from whom any follow-up information was available.
[2]All efficacy analyses were performed among children with laboratory-confirmed influenza who had received at least one dose of study drug, and who fulfilled all inclusion criteria.

Figure 3. Simplified flow chart of the randomized controlled trial of early oseltamivir treatment.

5.3.1 Incidence of acute otitis media

There was no statistically significant reduction in the incidence of AOM in children in whom oseltamivir treatment was started within 24 hours from the onset of symptoms. However, in children in whom the treatment was initiated within 12 hours from the onset of symptoms, oseltamivir decreased the incidence of AOM by 85% (95% CI 25-97%; P=0.02) (Figure 4).

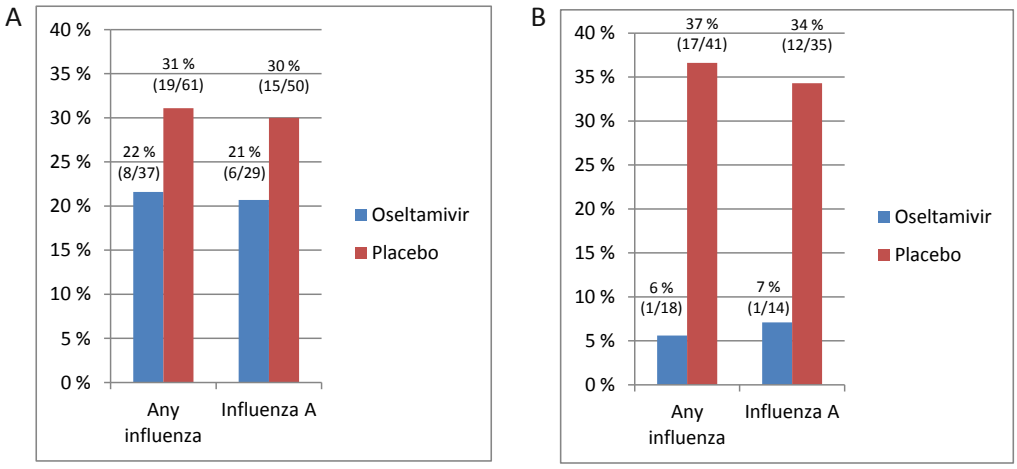


Figure 4. Incidence of acute otitis media in children with laboratory-confirmed influenza in whom the treatment was initiated within 24 hours from the onset of symptom (Panel A) and within 12 hours from the onset of symptoms (Panel B)

5.3.2 Duration of illness

The median time to resolution of illness was shortened in the oseltamivir group by 1.4 days (4.3 vs. 5.7 days; P=0.004) in children with any influenza and by 3.5 days (3.0 vs. 6.5 days; P=0.002) in children with influenza A (Figure 5). In unvaccinated children with any influenza, the time to resolution of illness was shortened by 2.9 days (4.3 vs. 7.3 days; P=0.009). In unvaccinated children with influenza A, the time to resolution of illness was shortened by 4.0 days (3.4 vs. 7.3; P=0.006). No efficacy was observed in children with influenza B (4.4 vs. 4.7 days; P=0.93).

The median time to resolution of all symptoms was shortened in the oseltamivir group by 2.8 days (10.4 vs. 13.3 days; P<0.001) in all children with any influenza, and by 4.6 days (9.4 vs. 14.0 days; P=0.002) in children with influenza A.

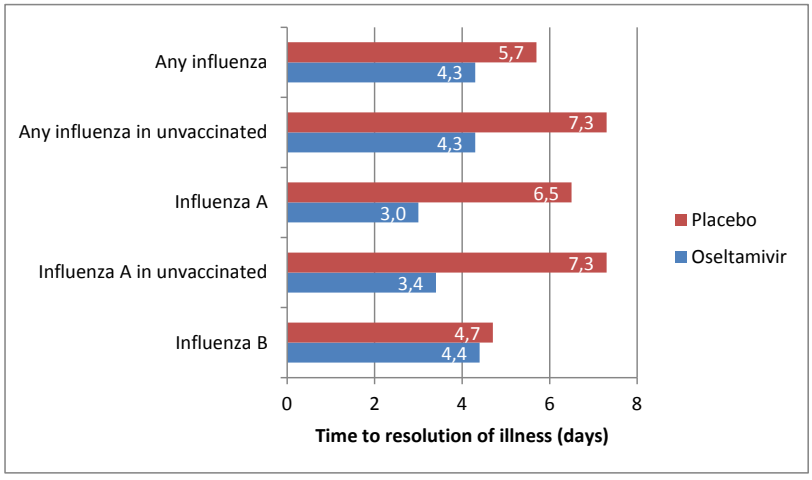


Figure 5. The time to resolution of illness (days) according to the type of influenza and the vaccination status of the children.

5.3.3 Other outcomes

Among the parents of children with any influenza, oseltamivir shortened the median duration of work absenteeism by 2.0 days (P=0.01); the median absenteeism was 0.0 days (interquartile range [IQR]: 0.0-2.0) in the oseltamivir group and 2.0 days (IQR: 0.0-4.0) in the placebo group. Among the parents of children with influenza A infection, oseltamivir reduced the absenteeism by 3.0 days (P=0.07), with the median absenteeism being 0.0 days (IQR: 0.0-2.0) in the oseltamivir group and 3.0 days (IQR: 0.0-4.0) in the placebo group. The mean number of doses of antipyretics and/or analgesics was reduced in the oseltamivir group by 1.5 (4.4 vs. 5.9; P=0.03) in children with any influenza and by 1.8 (4.3 vs. 6.1; P=0.01) in those with influenza A. With respect to influenza B, no impact on any of these outcomes was observed.

5.3.4 Safety and tolerability

Safety and tolerability were assessed in a safety population that consisted of all 406 children who had received at least one dose of the study drug and from whom any follow-up information was available. Vomiting was the only adverse event that was reported more commonly in the oseltamivir group (29.2% vs. 18.6%; P=0.01). No significant differences between the groups were observed with respect to diarrhea, abdominal pain, irritability, decreased appetite, fatigue or headache. The proportion of children who discontinued the treatment prematurely was 5.4% in the oseltamivir group and 2.5% in the placebo group (P=0.12). The compliance to the study medication was 92.1% in the oseltamivir group and 96.6% in children who received placebo (P=0.05).

5.4 Effectiveness of influenza vaccination (IV)

5.4.1 Incidence analysis

Of a total of 631 children in the follow-up cohort, 154 (24.4%) were fully vaccinated, 21 (3.3%) were partially vaccinated, and 456 (72.3%) were unvaccinated. Among all children aged 9 months to 3 years (mean 2.13 years; SD 0.78) the incidence of any influenza was 13.4% in the unvaccinated children and 4.5% in the fully vaccinated children (Figure 7), which translates to a vaccine effectiveness (VE) of 66% (95% CI 29-84%; P=0.003) against any influenza. The VE against the well-matched influenza A was 84% (CI 40-96%; P=0.003). Against the mismatched influenza B viruses, no significant VE was observed (VE 45%,CI-34 to 78%; P=0.20).

In the subgroup analysis of children aged 9 months to <2 years (N=278), the VE was 66% (95% CI 9-88%; P=0.03) against any influenza and 79% (CI 21-95%; P=0.02) against influenza A. In the older children aged 2-3 years (N=353), the VE against any influenza was 63% (CI -5 to 88%; P=0.06), and while none of the fully vaccinated children in this age group contracted influenza A, the VE against influenza A was 100% (CI 6-100%; P=0.05). No significant effectiveness against influenza B was observed in either of the subgroup analyses.

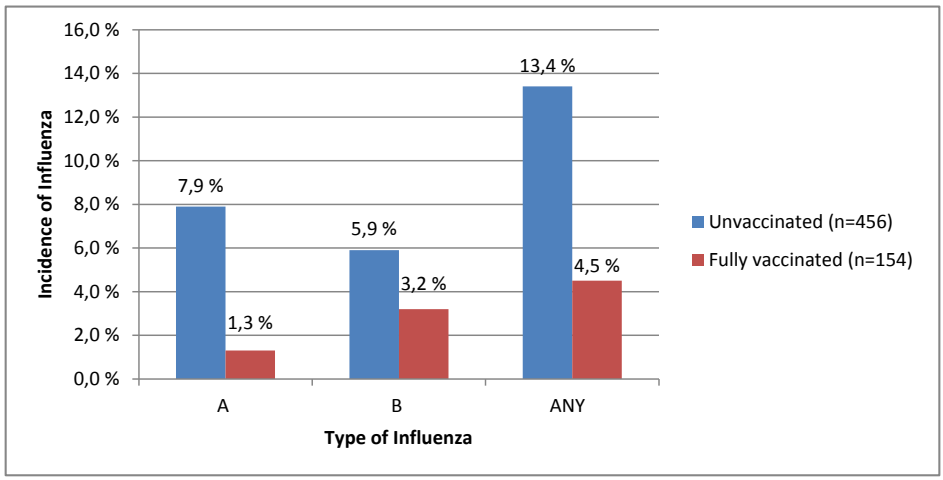


Figure 6. Incidence of influenza in unvaccinated (n=456) and fully vaccinated children (n=154).

5.4.2 Case-control analysis

In the case-control analysis conducted among 340 children who visited the study clinic at least once during the study period, the vaccine effectiveness estimates were largely similar to the incidence analysis. VE estimates were 72% (95% CI 35-88%) against any influenza; 85% (CI 37-96%) against influenza A; and 48% (CI -38 to 81%) against influenza B.

6 DISCUSSION

6.1 Clinical diagnosis

Before the development of effective antiviral treatments, the specific diagnosis of viral respiratory infections was considered less important. The use of viral diagnostics was mainly research-oriented or limited for surveillance purposes. Recently, the development of novel diagnostic and treatment options has increased the interest in the specific viral diagnosis of respiratory infections. Better recognition of viral infections has also the potential to decrease the unnecessary use of antibiotics (Woo et al., 1997, Bonner et al., 2003, Benito-Fernandez et al., 2006, Jennings et al., 2009).

Influenza still remains the only viral respiratory infection that can be effectively treated with specific antivirals. However, laboratory diagnostics are not available in many settings, and distinguishing influenza from other respiratory infections on clinical grounds alone poses a challenge to clinicians. This is the case especially with children, in whom respiratory infections caused by other viruses are very common and account for the majority of respiratory infections, even during the influenza epidemics (Zambon et al., 2001, Heikkinen et al., 2003, Sakkou et al., 2011). In the youngest children, the diagnosis is further hindered by their inability to describe their subjective symptoms.

In our study, we found out that fever was the only symptom that independently predicts influenza in unselected children <13 years of age, with the odds ratios increasing with incremental elevations of fever. The presence of an impaired general condition, gastrointestinal symptoms, headache, myalgia and pharyngitis increased the likelihood of influenza, but these symptoms did not reach statistical significance in the multivariate model. To our knowledge, this study is the first one assessing the predictive symptoms of influenza in unselected outpatient children. Our results are largely accordant with previous studies conducted in children, in which various pre-selection criteria were employed. In studies including hospitalized children with a high suspicion of influenza, Sočan et al found fever ≥38°C, headache, cough, and a lack of abnormal breathing sounds to be associated with influenza (Socan et al., 2010), whereas Friedman and Attia identified a triad of cough, headache, and pharyngitis to predict influenza with a sensitivity of 80% and specificity of 78% (Friedman and Attia, 2004). Ohmit and Monto assessed the symptoms predicting influenza in children as part of two clinical trials of the antiviral drugs oseltamivir and zanamivir (Ohmit and Monto, 2006). Varied inclusion criteria where employed in these two studies, including the presence of fever ≥37.8°C and a negative result in the rapid antigen detection test for RSV. In children aged 5-12 years, fever ≥38.2°C, cough, and headache were found to be independent predictors of influenza, with PPVs for a combination of fever and cough ranging from 71% to 83%, whereas in children 1-4 years of age, the only symptom that increased the likelihood of influenza was myalgia, with a PPV of 73%. When interpreting the relatively high PPV values reported in that study, one must take into account that the overall incidence of influenza was remarkably high and ranged from 66% to 74%. As the PPV of a test depends on the incidence of illness, these results may not be directly generalizable to the normal child population, where the

attack rates are approximately 15-25% during an average influenza season (Heikkinen et al., 2004).

On the contrary to other studies, we found cough to have no value in predicting influenza in children. Although cough was a common symptom of influenza and was present in 77% of the influenza-positive children, it was also reported in 71% of children without influenza. Cough is often considered an essential feature of influenza, and in a large study in adults, Monto et al. reported the combination of fever (≥37.8°C) and cough to have a PPV of 79% in predicting influenza (Monto et al., 2000). In that study, cough was present in 93% of the subjects with influenza, compared to 80% of those without influenza.

Our results suggest that during influenza epidemics, fever increases the probability of influenza, and the higher the fever is, the more likely the child is to have influenza. However, other viruses causing febrile respiratory infections commonly co-circulate with influenza, and even during confirmed epidemics, only a minority of children's respiratory infections are actually caused by influenza viruses (Zambon et al., 2001, Heikkinen et al., 2003, Sakkou et al., 2011). This decreases the predictive value of the signs and symptoms and hinders the clinical diagnosis. Furthermore, the clinical presentation of influenza in children seems to differ from that described in adults, and symptoms such as cough, headache and myalgia that are often associated with influenza in adults, seem to have no predictive value in children. Therefore, virological confirmation is usually required for a reliable diagnosis and the optimal use of antiviral drugs in children.

6.2 Virological diagnostics

Previous studies have shown that in children with influenza, the viral shedding peaks within 3 days from the onset of the illness, and during this period the sensitivity of diagnostic tests is expected to be the greatest. However, limited data have been available on the accuracy of different diagnostic methods during the very early stage of the illness when influenza antivirals could be of greatest benefit. In our study, the influenza virus was detectable from the specimens obtained within 24 hours from the onset of symptoms with at least one of the conventionally used laboratory methods (viral culture, TR-FIA, RT-PCR) in 92% of the children in whom the influenza virus infection was eventually confirmed. Furthermore, the sensitivity of an influenza rapid test during the first 24 hours of the illness was 90% for detecting the influenza A virus. These results suggest that, at least with respect to influenza A, both the conventionally used laboratory methods and point of care (POC) rapid tests are reliable in children already during the first day of the illness. The low sensitivity (25%) of the rapid test for detecting influenza B viruses may limit the use of rapid tests in the context of influenza B epidemics, although we only evaluated the performance of a single test, and the number of influenza B-positive children in our study was too small for firm conclusions to be made.

In previous studies assessing the accuracy of influenza rapid tests, the specificity of the test has been excellent, but the sensitivity has varied greatly. Sensitivities from 20 to 85% have been reported in different studies (Cazacu et al., 2003, Cazacu et al., 2004,

Agoritsas et al., 2006, Poehling et al., 2006b, Grijalva et al., 2007, Rouleau et al., 2009, Cheng et al., 2009, Ghebremedhin et al., 2009). Several potential factors may affect the sensitivity of a test and contribute to the discrepancy between the previous reports. Children are known to shed viruses in larger amounts (Hall et al., 1979, Frank et al., 1981, Cheng et al., 2009) and for longer periods, and consequently the diagnostic tests are reported to be more sensitive in younger age groups (Steininger et al., 2002, Ruest et al., 2003, Steininger et al., 2009, Cheng et al., 2009, Hawkes et al., 2010, Stripeli et al., 2010). The delay from the onset of symptoms to the collection of the specimen also affects the sensitivity. In two studies where the sensitivities were reported separately for samples taken within two days and after two days from the onset of symptoms, the sensitivity of the samples obtained earlier ranged from 70 to 86%, whereas the sensitivities of the samples taken at a later stage ranged from 49 to 65% (Hawkes et al., 2010, Stripeli et al., 2010). In the only study reporting the sensitivity of rapid tests performed within 24 hours from the onset of symptoms, the sensitivity was 77% (Watanabe et al., 2009), exactly the same as the overall sensitivity in our study. In another study conducted in Nicaraguan children, the sensitivity of the samples collected during 2-3 days after the onset was higher (74-75%) compared to samples obtained either 1 or 4 days after the onset of symptoms (52-58%) (Gordon et al., 2009).

The site and method of collection are important factors affecting the yield of the virus, and consequently they have a significant impact on the sensitivity of a test. A nasopharyngeal aspirate is a widely used method, and it is considered to provide good-quality samples. In our study, we used nasal swabs that are easy to obtain in any setting and are more pleasant for the patient. The sensitivity of these two methods is comparable; when compared with nasopharyngeal aspirate, the sensitivity of nasal swabs was found to be 91-92% for detecting influenza viruses in children (Heikkinen et al., 2001, Heikkinen et al., 2002). The sensitivity of the nasal swab samples may be further increased by using flocked swabs or by obtaining the sample from the nasopharynx (Abu-Diab et al., 2008, Chan et al., 2008).

We found that the rapid test used was significantly more sensitive for detecting influenza A than influenza B viruses (90% vs. 25%). With conventional laboratory methods, there was also a non-significant trend towards better sensitivity for the detection of influenza A during the first 24 hours of the illness (94% vs. 82%). In previous studies, the results have been variable. A few studies have reported lower sensitivity for rapid tests in detecting influenza B viruses (Cazacu et al., 2003, Hurt et al., 2007, Cruz et al., 2008), whereas no significant differences were observed in other studies reporting the sensitivities separately for influenza A and B (Rouleau et al., 2009, Ghebremedhin et al., 2009). There are several potential explanations for the observed differences in detecting influenza A and B viruses. Immunological differences may explain why different antigens are detected with varying sensitivity, and dissimilar assay methods may be used for the detection of different types of viruses. Another potential explanation was provided by Lau et al. when they demonstrated that in a naturally acquired influenza A infection, the viral shedding peaks at the time of the onset of symptoms, whereas in an influenza B infection, the peak is reached only 3 days after the onset of the illness (Lau et al., 2010). Consequently, lower replication rates at the early stage of the illness could explain the observed lower sensitivity for detecting influenza B viruses.

Our study was conducted during seasonal epidemics dominated by influenza A(H1N1) and A(H3N2) strains. The emergence of the pandemic 2009 A(H1N1) strain raised concerns about the performance of rapid influenza tests for the detection of the novel strain, and rather low sensitivities were reported in the early phase of the pandemic (CDC, 2009b). Most commercially available rapid tests are, however, based on detecting the highly conserved influenza virus nucleoprotein (NP), and similar analytical and clinical sensitivities have been reported for both pandemic and seasonal strains especially in children (Chan et al., 2009, Hawkes et al., 2010). Nevertheless, the performance of rapid tests may be affected by the genetic and antigenic changes that occur in the viruses, and the performance of these tests should be re-evaluated annually (Landry, 2011).

In addition to enabling the rational use of antivirals, influenza rapid tests may provide also other benefits. In several pediatric studies, the use of influenza rapid tests has been associated with significant reductions in auxiliary laboratory testing and chest radiographs, as well as the length of stay in the emergency department (Bonner et al., 2003, Benito-Fernandez et al., 2006, Sharma et al., 2002, Poehling et al., 2006b, Doan et al., 2009). Rapid tests have also been shown to reduce the use of antibiotics and hospitalizations (Woo et al., 1997, Bonner et al., 2003, Benito-Fernandez et al., 2006, Jennings et al., 2009). In an era of increasing antibiotic resistance, all possibilities to cut down the unnecessary courses of antibiotics are highly valuable.

6.3 Treatment

We found that early oseltamivir treatment started within 24 hours from the onset of symptoms was highly effective against influenza A and shortened the duration of the illness by 3.5 days in all influenza A-infected children and by 4.0 days among unvaccinated children infected with influenza A. The duration of the illness in children infected with any influenza (influenza A or B) was shortened by 1.4 days. This is mainly explained by the absence of any efficacy against influenza B that accounted for 19% of all confirmed influenza infections in our study. In addition, we found that oseltamivir treatment significantly reduced the days of absenteeism from work among the parents of influenza-infected children.

In the previous study by Whitley et al., oseltamivir treatment started within 48 hours shortened the duration of the illness by 1.5 days and reduced the incidence of AOM by 44% (Whitley et al., 2001). The children in that study were older (mean age, 5 years) than the children in our study; in the subgroup of children ≤2 years in the Whitley study, oseltamivir shortened the duration of the illness only by 0.9 days. Although our study was not designed to compare the treatments started within 48 and 24 hours, some cautious comparisons can be made with the Whitley study on the basis that the median durations of the illness in the placebo groups were analogous (5.7 days), and definitions for the time to resolution of the illness were similar in both studies. Aoki et al. demonstrated in adults that the earlier the oseltamivir treatment was started, the shorter was the duration of the illness (Aoki et al., 2003). In that study, oseltamivir treatment started within 12 hours from the onset of symptoms shortened the duration of

the illness by 3.1 days when compared to those in whom the treatment was initiated 48 h after the illness onset.

Our primary endpoint was the development of AOM as a complication of influenza. In children in whom oseltamivir treatment was initiated within 24 hours from the onset of the illness, we recorded 31% less episodes of AOM, but the difference was not statistically significant. In the predefined subgroup analysis among children in whom the treatment was initiated within 12 hours, we recorded a statistically significant 85% reduction in the incidence of AOM. These findings suggest that to be able to prevent AOM as a complication of influenza, oseltamivir treatment should be initiated very rapidly after the onset of symptoms. The reasons for this are unclear, but one potential explanation is that the influenza virus-associated inflammatory response in the nasopharyngeal mucosa and Eustachian tube dysfunction may proceed so rapidly that in otitis-prone children the point of no return is reached early in the course of the illness. In the natural course of an influenza A infection, the viral load peaks at the time of the onset of symptoms, or soon after this, and then starts to gradually decline (Lau et al., 2010). This may at least partly explain the short time window for an effective treatment.

In our study, we found oseltamivir ineffective against influenza B infection. Several large-scale in vitro surveillance studies have shown that influenza B viruses are less susceptible to neuraminidase inhibitors than influenza A viruses (Sheu et al., 2008, McKimm-Breschkin et al., 2003, Monto et al., 2006). This observation is based on higher mean 50% inhibitory concentration values at the baseline, and actual resistant mutations occur only rarely (Hatakeyama et al., 2007). The clinical importance of these findings has remained unclear. Whitley et al. did not report detailed efficacy data separately for influenza A and B, but described that oseltamivir shortened the duration of fever, cough and coryza in children with influenza B by 1.1 days (Whitley et al., 2001). However, in several Japanese observational studies oseltamivir has been less effective against influenza B compared to influenza A. This difference was emphasized in young children in whom influenza B viruses were also shed for longer times (Sugaya et al., 2007, Kawai et al., 2006, Kawai et al., 2007).

As oseltamivir cannot be expected to be effective against any other viruses than influenza viruses, the efficacy analyses in our study were limited to influenza-positive children. This differs from the traditional intention-to-treat (ITT) approach routinely employed in the context of RCTs (Hollis and Campbell, 1999). However, because we aimed to recruit the highest possible number of influenza-positive children, the treatment was given to all febrile children fulfilling the inclusion criteria. In normal clinical practice, this would lead to large-scale unnecessary use of antivirals, as only 25% of the randomized children in our study actually had influenza. An alternative approach could have been to require a positive rapid test result for inclusion in the study. In that situation, the efficacy could have been analyzed among all randomized children using the conventional ITT approach. However, at the time of designing the study, there was uncertainty about the performance of the influenza rapid tests during the early phase of the illness. Furthermore, although our results afterwards indicated that the rapid test used was sensitive in detecting influenza A viruses already during the early phase of the illness, its sensitivity was low for influenza B viruses, and

consequently we would have missed the majority of influenza B-positive children in our study.

We were able to start the antiviral treatment within 24 hours from the onset of fever. This means that in most cases, the children were evaluated at the study clinic during the same day when the symptoms begun, or the next day at the latest. The children had easy access to the study clinic, which was open also during evenings and weekends. However, in real life there are several obstacles to the practical implementation of early antiviral treatment of influenza. In the absence of the signs of severe illness, the patients often seek healthcare only after having been ill for a few days when the delay is already too long for influenza antivirals to be beneficial. Diagnostic difficulties may further complicate the situation, especially for children in whom the clinical diagnosis is inaccurate (Peltola et al., 2005). Influenza rapid tests may not be available and large scale testing may not be feasible. However, the great benefits of early oseltamivir treatment seen especially in children with influenza A indicate the need to seek ways to deal with these problems. During the recent 2009 pandemic, different ways to manage influenza patients and deliver antivirals were explored. In the UK, antivirals were made available through telephone and online self-care services, and specific influenza clinics were established in many countries. When considering the costs and labor associated with the use of an influenza rapid test, one should keep in mind the other benefits they could potentially provide in decreasing auxiliary testing, the use of antibiotics and hospitalizations (Woo et al., 1997, Bonner et al., 2003, Benito-Fernandez et al., 2006, Jennings et al., 2009). Furthermore, the interest in specific viral diagnostics may increase in the future if the efforts in developing specific drugs against other respiratory viruses are successful (Moscona et al., 2010, Lanier et al., 2011, Olszewska et al., 2011, Rollinger and Schmidtke, 2011). In the end, things often depend on money, and whether we should aim to large-scale early diagnostics, and the treatment of outpatient children with influenza is mainly a question of costs and benefits. Therefore, a proper cost-effectiveness analysis might be helpful in guiding treatment recommendations for influenza antivirals in children. However, in the current situation, if we are able to diagnose a child with influenza A during the early stage of the illness, our results strongly support the initiation of oseltamivir treatment.

6.4 Vaccination

Despite the availability of antiviral drugs, vaccination still remains the most important method of controlling influenza, both in the community and at individual levels. In young children, the efficacy of influenza vaccination has still been a controversial issue. Based on limited data, the recent Cochrane review concluded that the efficacy of the inactivated influenza vaccine in children <2 years of age is similar to placebo (Jefferson et al., 2008). In our study, however, we found that in young children, the efficacy of the inactivated vaccine was 84% against the well-matched influenza A viruses, whereas no efficacy was observed against the mismatched influenza B strain. Importantly, the vaccine was effective against influenza A also in the subgroup of children <2 years. The burden of illness is particularly high among the youngest children (Neuzil et al., 2000, Izurieta et al., 2000, Heikkinen et al., 2004) and consequently, an effective vaccine would provide great benefits in this age group. Our

results strongly suggest that the inactivated vaccine is effective also in young children if the vaccine strain matches well with the circulating strain, and that the efficacy is more dependent on the similarity of the strains than the age of the vaccine recipient (Heikkinen and Heinonen, 2011).

Several potential explanations exist on the observed poor efficacy of the vaccine against influenza B in our study. The most important reason is presumably the lineage-level mismatch between the vaccine and the circulating influenza B strains. Currently, two genetically distinct lineages of influenza B viruses co-circulate, and previous studies in adults have shown that the vaccine gives only little or no protection at all against lineage-level mismatched influenza B strains (Skowronski et al., 2009, Beran et al., 2009, Belshe, 2010). As the current trivalent influenza vaccines consist of two influenza A strains and of only one influenza B strain, the selection of the correct influenza B strain is a great challenge. The difficulty in selecting the correct influenza B strain is illustrated by the fact that during the previous decade, a mismatch with the influenza B strain occurred in the US in five seasons out of ten (Belshe, 2010, CDC, 2011). This has raised interest in developing a quadrivalent influenza vaccine containing two influenza B strains, and the clinical trials are already on the way.

In addition to antigenic mismatch, other potential reasons may contribute to the lower effectiveness observed against influenza B viruses. Several studies have described that the immunogenicity of an inactivated vaccine against influenza B viruses is lower than the response seen against influenza A viruses (Englund et al., 2005, Neuzil et al., 2006, Vesikari et al., 2009). The adjuvant MF59 has been shown to enhance the immunogenicity of the inactivated vaccine, especially against influenza B viruses (Vesikari et al., 2009, Vesikari et al., 2011). Furthermore, LAIV has shown to elicit robust antibody responses also against influenza B viruses (Gruber et al., 1996, Belshe et al., 1998), and in the clinical trials the efficacy of LAIV against influenza A and B types has been comparable (Rhorer et al., 2009).

In Finland, the recommended dose of TIV is 0.5 ml (containing 15 μg HA of each of the three included strains) for everyone, regardless of the age of the recipient. In most other countries, a half dose (0.25 ml) is usually recommended for children <3 years. The higher dose used in our study might have contributed to the effectiveness. The immunogenicity of a higher dosage in children has been studied previously. Gross et al. found a high-dose vaccine containing 60 μg HA of influenza B significantly more immunogenic than the dose of 7 μg, without an increase in reactogenicity (Gross et al., 1982). In another study among adults and elderly people, higher antigen doses evoked stronger antibody responses against influenza B but not against influenza A strains (Palache et al., 1993). Recently, Skowronski et al compared 0.5 ml and 0.25 ml doses in children aged 6-23 months and found out that in infants 6-11 months of age, a 0.5 ml dose was significantly more immunogenic against H3N2 and B strains (Skowronski et al., 2011). In this study, the children were carefully followed up for adverse events, and no significant differences were observed between the groups. In our study, we were unable to record any adverse events, as the children were vaccinated as part of the national vaccination program in their own health care centers. However, the passive surveillance conducted by the National Institute for Health and Welfare (THL) reported only 38 adverse events for 63,048 children aged 6-35 months vaccinated as

part of the national vaccination program (Nieminen and Tikkanen, 2008). A high-dose vaccine containing 60 μg HA of each of the three strains has been approved for elderly people ≥65 years to enhance the immunogenicity (Fiore et al., 2010). The results of our study, combined with the detailed immunogenicity and reactogenicity data reported by Skowronski et al., suggest that the adoption of a 0.5 ml dosage for young children should be considered in other countries as well.

Our study was not an RCT, and consequently it is subject to limitations that apply to all non-randomized studies. The parents made the decision about having their child vaccinated, and different selection for vaccination is a potential source for confounding by risk factors. However, 24% of the children in our cohort were fully vaccinated, which was comparable to the overall vaccine coverage (27%) in this age group in our region. Age was the only difference we could observe between the groups. This difference can be largely explained by the Finnish national vaccination program that offered free vaccinations to children up to 35 months of age. Consequently, vaccine uptake was very low in children aged 36-40 months who were included in our study. To control for the potential bias caused by the age difference, we performed subgroup analyses by age and found out that the vaccine was effective also in the subgroup of children 6 months to 2 years of age. In addition, we performed a case-control analysis to address the potential confounding caused by children who never visited the study clinic during the study period. The VE estimates derived from this analysis that was limited to children who made at least one visit to the study clinic were in line with the results of the primary incidence analysis. We therefore believe that our results are well generalizable to child populations of similar age and unlikely to be significantly affected by any unknown biases.

6.5 Future considerations

Despite the accumulating knowledge on influenza in children, several issues need to be assessed in more detail. The key issue in oseltamivir treatment is minimizing the delay from the onset of symptoms to the initiation of treatment. Hence, it would be important to explore different ways to tackle the logistical challenges associated with the initiation of early treatment. In addition, a proper cost-effectiveness analysis on oseltamivir treatment would be highly valuable in guiding the treatment recommendations both in children and in adults.

A great majority of influenza-related research has focused on influenza A, and after the recent pandemic an overwhelming amount of data on 2009 pandemic H1N1 viruses has been published. This is understandable because of the pandemic potential and higher morbidity associated with influenza A. However, influenza B viruses cause a substantial share of overall influenza morbidity, especially in children and adolescents (Olson et al., 2007). Therefore, further research into the diagnostics, treatment and prevention of influenza B virus infections is warranted.

The efficacy of different types of influenza vaccines in children needs to be evaluated in different settings and during several seasons to enable better assessment of the overall impact and long-term cost-effectiveness of the vaccination. However, performing placebo-controlled trials may be ethically difficult in the future, especially

in countries where influenza vaccination is officially recommended or included in the vaccination program (Fiore et al., 2010). Carefully performed observational cohort studies with virologically confirmed endpoints are able to provide detailed data on the field effectiveness of the vaccine and the impact of vaccination campaigns in different settings. As the efficacy of influenza vaccination remains suboptimal in all age groups and the constant antigenic evolution continues to complicate the selection of vaccine strains, the development of more effective influenza vaccines is needed. In children, the current hot topics are the use of adjuvanted vaccines to boost immunogenicity and the potential importance of the heterosubtypic immunity against strains not included in the vaccine. In addition, the discussion still continues whether healthy children should be vaccinated against seasonal influenza in the first place. The ultimate aim and challenge of vaccine development is still the development of an universal influenza vaccine that would elicit a broad immune response and that would be effective against all strains, without being affected by the continuous antigenic evolution.

SUMMARY AND CONCLUSIONS

The purpose of this study was to evaluate (I) potential signs and symptoms predicting influenza infection in children, (II) the performance of different diagnostic methods during the early stage of the illness, (III) the efficacy of early oseltamivir treatment, and (IV) the effectiveness of TIV in young children.

In Study I, we found out that fever was the only symptom predicting influenza in children, and the predictive capability increased with incremental elevations in the child's temperature. A few other symptoms, such as an impaired general condition, gastrointestinal symptoms and pharyngitis, increased the likelihood of influenza but did not reach statistical significance in the multivariate analysis. Because even during confirmed influenza epidemics, influenza causes only a minority of children's febrile respiratory infections, these results suggest that laboratory diagnostics are required for a reliable diagnosis and the rational use of antivirals in children.

In Study II, the conventionally used laboratory methods (viral culture, TR-FIA, RT-PCR) were found sensitive already during the early stage of the illness, as they could detect influenza viruses in 92% of the children in whom the influenza infection was eventually confirmed. The sensitivity of the influenza rapid test used was 90% for influenza A and 25% for influenza B. From this study, we can conclude that influenza can be reliably diagnosed in children already during the early stage of the illness.

Early oseltamivir treatment initiated within 24 hours from the onset of symptoms was found effective against influenza A by shortening the duration of the illness by 3.5-4.0 days. No efficacy on the duration of the illness was observed in children infected with influenza B viruses. Early oseltamivir treatment reduced the incidence of AOM, but only when initiated within 12 hours from the onset of the illness. These results confirm the benefits of early oseltamivir treatment in children with influenza A and emphasize the importance of initiating the treatment as early as possible. The number of children infected with influenza B viruses was too low in our study for firm conclusions to be made, but our results suggest that the efficacy of oseltamivir against influenza B might be significantly lower compared to influenza A.

The effectiveness of TIV against well-matched influenza A viruses was 84%, while no efficacy was observed against lineage-level mismatched influenza B viruses. Importantly, the vaccine was also effective among children <2 years of age in whom previous evidence for its effectiveness is scarce. Our findings provide strong evidence for the effectiveness of TIV also in young children, if a good match between the vaccine and circulating strain is achieved.

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