Immune response to influenza after vaccination and infection

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"When you get, give.

When you learn, teach".

Maya Angelou (1928-2014)

To Frank, Nicolai, Birgitte, Christoffer and Mathias

Scientific environment

The present doctoral work is based on human clinical studies carried out at the Influenza Centre, Department of Clinical Sciences, University of Bergen and Haukeland University Hospital. Professor Rebecca Jane Cox, Professor Haakon Sjursen, and Ass. Professor Ingrid Smith provided supervision and guidance. The study on immunological responses to pandemic influenza infection was performed in collaboration with Akershus University Hospital and the Norwegian Institute of Public Health.

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Summary

Influenza causes great human morbidity and mortality from annual epidemics and pandemics occurring at irregular intervals. The socioeconomic impact of influenza is significant with 5% of adults and 20% of children infected, and 500 000 fatal cases globally each year. Elderly have the highest risk of fatal disease, while children are main transmitters of the virus and the youngest are most often hospitalized. Influenza is a vaccine preventable disease, but the vaccines require annual updating due to constant viral mutations, and they are only moderately effective. The immune system is vital in clearing influenza illness, and the mechanisms behind the complex immune response to the virus are not clear. Increased knowledge of these responses is required for the development of the much needed, improved future influenza vaccines. In this doctoral work we investigated the immunological mechanisms elicited in both vaccinated children and influenza infected adults. Two clinical studies were conducted and immune responses analysed. Firstly, we investigated the immunogenicity after vaccination with a live attenuated influenza vaccine (LAIV) in 55 children and controls. Evidence of early and durable LAIV induced responses was found in saliva, blood and tonsils, with responses both in the cellular and humoral immune compartments. This indicates a broad, protective response, where especially cellular responses are interesting. To our knowledge we are the first to show tonsillar responses after LAIV, and lasting cellular responses, up to one year. Saliva IgA is suggested as a possible, non-invasive correlate of immunogenicity after LAIV.

Secondly, we dissected the immunological responses in adults infected during the 2009 influenza pandemic. Patients in the acute phase showed low levels of $CD8^+$ T-cells compared to convalescent patients, and those with severe disease showed the highest levels of antibodies. $CD8^+$ T-cells are vital for viral clearance, however there are few reports on responses from naturally infected patients. Differences in T-cell subsets may define disease severity. The multifaceted immune response induced by vaccination or infection indicates that T-cells are important in the immune defence against influenza and are therefore ideal targets for future influenza vaccines.

List of publications

I. **Mohn K,** Bredholt G, Brokstad K, Pathirana R, Tøndel C, Aarstad HJ, Cox RJ Longevity of B & T cell responses after live attenuated influenza vaccination in children. *The Journal of Infectious Diseases 2014,* 15; 211(10):1541-9

II. **Mohn K,** Brokstad K, Pathirana R, Bredholt G, Jul-Larsen Å, Trieu MC, Lartey SL, Montomoli, C. Tøndel, H.J. Aarstad and R.J. Cox Live attenuated influenza vaccination in children induces B-cell responses in tonsils. *The Journal of Infectious Diseases 2016*, 214 (5): 722-731

III. **Mohn K,** Cox RJ, Tunheim G, Berdal JE, Hauge AG, Pandemic Group, Peters B, Oftung F, Jonassen CM, Mjaaland S Immune responses in acute and convalescent patients with mild, moderate and severe disease during the 2009 influenza pandemic in Norway, *PlosOne 2015,* PONE-D-15-30560R1

Related publications not included in this thesis

Papers related to pandemic patients:

I. Muthuri S, **Mohn K** (PRIDE consortium), Nugyen-Van-Tam JS et al**.** Effectiveness Of Neuraminidase Inhibitors In Reducing Mortality In Hospitalized Influenza A(h1n1)pdm09 Patients. *Lancet Respiratory Medicine* May 2014;2(5):395-404.

II. Muthuri, S, **Mohn K** (PRIDE consortium), Nugyen-Van-Tam JS, et al. Impact of neuraminidase inhibitors on influenza A(H1N1)pdm09-related pneumonia: an IPD meta-analysis. *Influenza Other Respiratory Viruses,* May 2016;10(3):192-204.

III. **Mohn K**, Lerum B, Skrede S, Cox RJ, Dyrhol-Riise AM, Simonsen HE, Langeland N, Aβmus J, Akselsen, PE, Sjursen H, Smith I. Mass vaccination of patient groups at risk and healthcare workers reduced hospital stay in patients with suspected Pandemic Influenza A (H1N1). *J Vaccines and Vaccination* Aug 2013, 4:6.

Papers related to LAIV vaccinated children:

IV. Panapasa J, Cox RJ, **Mohn K**, Aqrawi LA, Brokstad KA The expression of B & T cell activation markers in children's tonsils following live attenuated influenza vaccine, *Human Vaccines & Immunotherapeutics* 2015, 11:7, 1663-1672.

V. Marti GP, **Mohn K**, Brokstad K, Cox RJ, The Influence of Tonsillectomy on Total Serum Antibody Levels, Letter to the editor, *Scandinavian Journal of Immunology,* 2014, 80, 377–379.

Other Papers

VI. Mair-Jenkins J, Saavedra-Campos M, **Mohn K** (as part of Convalescent Plasma Study Group), et al. The Effectiveness of Convalescent Plasma and Hyperimmune Immunoglobulin for the Treatment of Severe Acute Respiratory Infections of Viral Etiology: A Systematic Review and Exploratory Meta-analysis, *The Journal of Infectious Diseases* August 2014, 211 (1): 80-90.

VII. Madhun AS, Akselsen PE, Sjursen H, Pedersen G, Svindland S, Nostbakken JK, Nilsen M, **Mohn K**, Jul-Larsen A, Smith I, Major D, Wood J, Cox RJ. An adjuvanted pandemic influenza H1N1 vaccine provides early and long-term protection in health care workers. *Vaccine.* 2010 Dec 16;29(2):266-7

Abbreviations

ASC	Antibody secreting cell
ACIP	Advisory Committee on immunization practices (CDC, USA)
APC	Antigen presenting cell
ARDS	Acute respiratory distress syndrome
BMI	Body mass index
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention (USA)
DC	Dendritic Cell
ECDC	European Centre for Disease Control
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
EU	European Union
FFU	Fluorescent Focus units
GBS	Gullian Barré syndrome
GC	Germinal centre
GCP	Good clinical practice
GMT	Geometric mean titre
HA	Hemagglutinin
HI	Hemagglutinin inhibition assay
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IIV	Inactivated influenza vaccine
IL	Interleukin
ILI	Influenza like illness
i.m.	intra muscular
i.v	intra-venous
LAIV	Live attenuated influenza vaccine
M1	Matrix protein 1
M2	Matrix protein 2

MAC	Membrane attack complex
MALT	Mucosal lymphoid tissue
MBC	Memory B Cell
MDV	Master donor virus
MHC	Major Histocompatibility complex
MKF	Macrophage
NA	Neuraminidase
NAI	Neuraminidase inhibitors
NK	Natural killer cells
NP	Nucleoprotein
NIPH	Norwegian institute of public health
PAMPS	Pathogen recognition receptors
PB	Polymerase protein basic
PBMC	Peripheral blood mononuclear cell
PHE	Public Health England
PRR	Pathogen recognition receptors
PR8	Influenza A/Puerto Rico/8/34 (H1N1)
QIV	Quadrivalent influenza vaccine
RCT	Randomized clinical trial
RNA	Ribonucleic acid
SAE	Serious adverse event
SARI	Serious airway respiratory infection
SEM	Standard error of the mean
SFU	Spot forming units
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cell
TIV	Trivalent inactivated influenza vaccine
TNF	Tumour necrosis factor
VAERS	Vaccine adverse effect registry system
VE	Vaccine effectiveness
WHO	World Health Organization

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Kristin G-I Mohn,
Bergen 24.06.16

Introduction

Influenza

Twice during the course of this doctoral work, we experienced severe influenza A/H1N1 infections at our hospital. First during the 2009 pandemic, and again in January 2016 when up to 50% of patients in intensive care units had acute respiratory distress syndrome (ARDS) related to influenza. The patients were in need of artificial ventilation, including the need for extra corporal membrane oxygenation (ECMO) ("mechanical lung"). Many suffered from secondary bacterial infections complicating their influenza illness, but only one died. Several patients survived with sequelae. Especially one female patient gave a vivid picture of what her breathing felt like while being given ventilatory assistance on the regular ward; "It is as if my lungs are full of cob-webs". These patients serve as an illustration of the potentially severe and fatal impact of influenza virus in man. While treating these patients suffering from severe influenza disease during the 2009 pandemic an interest in studying influenza immunology was ignited.

The work in this thesis has investigated the immune responses elicited after vaccination of children against seasonal influenza, and after natural influenza infection in adults during the 2009 pandemic. Influenza viruses have a unique ability to mutate and hence escape human immune defence mechanisms, necessitating annual vaccine updates. The most commonly used influenza vaccines are the inactivated influenza vaccines (IIV). Vaccination with a live attenuated influenza vaccine (LAIV) resembles natural infection, and immune responses after influenza vaccination and infection mirror each other. Therefore, studying the multifaceted immune responses to LAIV may aid in understanding the immune responses to influenza infection.

Influenza viruses are the worlds leading cause of respiratory illness, and are among the oldest viruses in historical records to cause severe epidemics and pandemics across the globe. In 2009, a novel influenza A H1N1 virus, defined as

A(H1N1)pdm09, emerged in Mexico and the world experienced its third pandemic since the devastating Spanish flu in 1918. Although less virulent than the feared 1918 virus, the 2009 pandemic caused extensive morbidity and mortality among young adults and the integrity of healthcare systems was threatened worldwide. This was the first pandemic in history to have countermeasures available in the form of both antiviral medication and a pandemic vaccine.

Influenza was a global focus of research for virologists, until HIV was identified in the 1980s. For decades after, HIV had centre stage and brought the field of immunology forward. With Ebola, the threat of highly pathogenic avian flu, and lately the novel Zika virus, virologists have major challenges to solve. After the emergence of the avian influenza A(H5N1), SARS coronavirus, the 2009 swine influenza pandemic, and later MERS-CoV, the global community has intensified influenza research and development of improved vaccines, with the goal of a "universal" influenza vaccine.

In 2012 a LAIV was licensed for children in Europe. This thesis will focus on the human immune responses to vaccination and influenza infection. The basis for this thesis is two clinical studies, one from children vaccinated with a seasonal LAIV, and the other from infected and hospitalized adult patients during the 2009 pandemic. This current doctoral work comprises issues on influenza immunology, vaccinology, public health care and considerations concerning vaccination and human clinical trials.

Influenza, history

The influenza virus was first isolated in the laboratory in 1932[1], making laboratory diagnosis of influenza possible. However, recordings of the illness caused by these viruses go back several hundred years[2]. The early influenza epidemics were described by their respiratory and systemic symptoms, as well as the typical acute onset of the outbreaks. The first scientific report of an influenza *epidemic* is a case report from Dublin, in the winter of 1693[1]. However, historical writings from the Middle Ages and as far back as Hippocrates in 412 BC describe a plague of respiratory illness

appearing periodically[1-3]. These reports of the sudden onset of fever, myalgia, arthralgia, as well as fatigue describe clinical features of influenza-like illness as we know them today [1].

Although there are reports of possible *pandemics* from 1173, the first recorded evidence of a pandemic dates back to 1510[2], so at least 13 pandemics caused by influenza A viruses have occurred at intervals of about one generation[2]. The "Spanish flu" emerging in 1918, killed an unprecedented 30-50 million people in two years, being the most lethal infectious disease outbreak ever recorded[1]. Detailed clinical case reports from 1918 vividly describe the desperate situation and the doctor´s attempts to treat these young adults with convalescent serum from pandemic survivors[4,5]. The illness was first detected in prisons and military camps in the USA and spread through deployment of military troops during the first world war[3]. In 1997 scientists succeeded in sequencing the original 1918 H1N1 virus from a lung tissue sample from an American soldier who died from pneumonia in Fort Jackson in South Carolina[6,7]. This 1918 H1N1 virus has since then been rigorously studied. In 2016, scientists demonstrated a peak in antibody responses to the novel 2009 H1N1 virus in survivors who were more than 10 years old in 1935, thus concluding that the 1918 virus was an H1N1 virus [8].

Although pandemics are considered a major threat, seasonal influenza is an annual challenge with a far greater public health impact than pandemics on morbidity and mortality[9]. However, during pandemics, morbidity and mortality rates are higher in younger age groups, causing larger burden on society and healthcare structures, inducing a global health emergency over a short period of time.

The epidemiology and global burden of seasonal influenza

Annually influenza causes global fatality rates of 250 000 to 500 000 people, and an estimated 3-5 million hospitalizations[9-11]. These numbers are extrapolated from US estimates into the global population of influenza serious airway respiratory infection (SARI), and a major underestimate of the burden of disease (Julia Fitzner, WHO, personal communication, Paris, January 2016). In Norway, numbers have been

extrapolated to 900-1200 deaths annually, however, these numbers contain a degree of uncertainty[12]. Following the 2009 H1N1 pandemic, influenza surveillance was improved to aid a more accurate estimation of the burden of severe disease in each country. Globally, the WHO has initiated these efforts, and several working groups have been established (EuroMomo, CONSISE, GLaMOR) [13-15].

The public health impact of seasonal influenza viruses depends on their transmissibility and virulence. In temperate climate zones, influenza circulates during the winter months, in the northern hemisphere from October to April, and in the southern hemisphere from May to September. In the tropical regions, there is an all year-round background of influenza with less obvious seasonality, although increased transmissibility has been observed during the rainy season. The WHO estimates that about 5-10% of the adult population and 20-30% of children are infected annually with influenza[9]. Average incubation time is 2 days (range 1-4 days), and adults shed virus during symptoms, whereas children shed virus longer, up to 14 days, due to lack of preexisting antibodies[16]. Children are hence the most important transmitters of the virus in the community[17,18]. Since children have most interpersonal contact, the measures taken to prevent influenza transmission focus on children, with school closures. In the UK, childhood influenza vaccination campaigns have shown signs of providing herd immunity[19,20]. Adults with comorbidities, with immune suppression or who are hospitalized also shed the virus for longer[21]. However, when pre-existing antibodies are lacking in most of the population, such as the case with the novel A(H1N1)pdm09 virus, no differences were found in the length of shedding between adults and children [16,22].

Clinical features and complications of influenza disease

Influenza is characterized by abrupt onset of respiratory and systemic symptoms and severe prostration, which help differentiate this virus from the countless other respiratory viruses. Another difference is the lack of prodromal respiratory symptoms, such as a congested or runny nose, which typically do not precede influenza, and many people without prior influenza experience wrongly self-diagnose

a common cold as influenza. However, those who have experienced influenza illness can usually name the year and the place where they fell ill.

Pulmonary complications to influenza illness include: bronchitis, viral pneumonitis, secondary bacterial pneumonia and ARDS, which have a high risk of fatal outcome[23]. Systemic, and extra-pulmonary complications may affect any organ system, as well as complications during childbirth[24]. In children, otitis media, febrile seizures and rare cases of viral myocarditis and meningoencephalitis occur[25,26]. During this year´s seasonal influenza, there have been more cases of severe H1N1 influenza disease in Norway, than normal seasons, with periods where half of the ICU beds at our hospital were occupied with these patients. Adults with life-threatening disease, requiring the most advanced medical care available, remind us of the deadly potential of this returning virus.

Risk groups for severe influenza infection

Patient groups at risk of severe disease are defined in the WHO guidelines for the control of seasonal influenza[24,27]. A higher risk of increased morbidity and mortality after influenza infection has been consistently observed in young children (< 5 years), elderly (> 65 years) and pregnant women[11,24,28-30]. Other risk groups are people of all ages with chronic conditions such as: asthma and other pulmonary or cardiac diseases, immunosuppression (by either medication or disease), diabetes, metabolic, liver, kidney and neurological/neuromuscular disorders[24,27,28]. Decreased neonatal birth weight, prematurity, perinatal mortality and fatal cases have been experienced in pregnant women infected with influenza, and they are therefore the WHOs number one priority for seasonal vaccination[24,27,29]. Obesity has been included as a risk factor after a post-pandemic study found that morbid obesity (Body mass index (BMI) >40) was an individual risk factor for hospitalization and fatal outcome (>7 times)[27,31] . Extensive research into host risk factors and viral virulence factors has been conducted to elucidate mechanisms causing severe human influenza disease.

The Influenza virus

The influenza virus is member of a distinguished small group of viruses capable of rendering otherwise healthy adults acutely ill and bedridden for up to one week. Although influenza causes severe morbidity and mortality each season, otherwise healthy individuals clear the infection without sequelae within 7-14 days. Through continuous mutations, the influenza virus avoids destruction by the human immune system, but stays virulent enough to cause massive spreading of virus progeny. In addition, influenza infects a vast diversity of species, from birds and bats to pigs, seals and horses, representing a huge viral reservoir[32]. This animal reservoir makes influenza eradication impossible. Therefore, influenza will continue to infect humans in the future.

Taxonomy

Influenza viruses belong to the Orthomyxoviridae family (Figure 1). They are single stranded RNA viruses and are further divided into four different types: A, B, C and D, by antigenic differences in their core proteins (matrix (M) and nucleoprotein (NP))[33]. Recently, a novel influenza D virus has been isolated from cattle, sheep, goats and swine, while cattle are thought to be the natural host, where it causes illness [34]. Types A and B are responsible for severe disease in humans, while influenza C infection only causes a mild respiratory illness and is not separately diagnosed in clinical practice. Only the influenza A viruses have pandemic potential, and are further classified into subtypes based on the two main surface glycoproteins located in the viral membrane, hemagglutinin (HA) and neuraminidase (NA). So far, eighteen different HA variants (H1-H18) and 11 NA variants (N1-N11) have been identified[33]. The influenza B viruses are divided into two distinct lineages: B/Yamagata and B/Victoria[35] and recently both lineages have been included in the quadrivalent seasonal vaccines. Currently two influenza A subtypes (H1N1 and H3N2) as well as both B strains co-circulate in humans. The WHO revised guidelines for the nomenclature of influenza are[33]:

- Type of influenza (A, B, C or D)

- The host of origin (e.g. chicken, equine, swine) if non-human
- Geographical area the virus was first isolated
- Strain number
- Year of isolation

In addition, for the influenza A viruses, the subtype is added in parenthesis. The scientists who sequenced the "Spanish flu" virus from a 1918 victim, proposed to name it: A/South Carolina/1/18(H1N1)[6]. Similarly, the primary isolate from the pandemic in 2009 arose from a human and was named A/California/07/2009(H1N1), abbreviated to A(H1N1)pdm09.

Viral structure

The influenza virus is mainly spherical in shape and consists of an envelope with embedded glycoproteins and a core containing the viral genome.

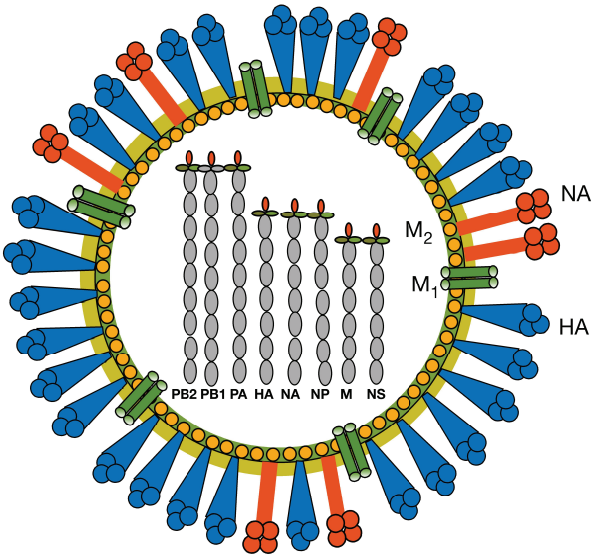


Figure 1. Viral structure: The segmented genome consists of 8 single stranded (ss), ribonucleic acid (RNA) segments, which code for the viral proteins. The viral polymerase consists of PB1, PB2, and PA and scaffolding protein, nucleoprotein (NP). The envelope is derived from the host cell plasma membrane during exocytosis of new virus progeny, and is lined by the matrix, M1 protein. The M2 ion channel is a transmembrane protein. HA and NA are the two most important glycoproteins of the virus and protrude from the viral membrane. They are targets for medication or specific antibodies. HA is more abundant compared to NA, appearing in a ratio of 5:1 on the viral surface. The influenza virus lacks proofreading mechanisms, when coping the genome, hence HA and NA mutations occur frequently. Figure made in collaboration with G. S. Johansen, UiB and inspired by Dr Karl Brokstad[36].

The haemagglutinin protein (HA)

The HA protein is a homotrimeric integral membrane protein which resembles a spike and protrudes from the viral surface. The HA consists of an immunodominant globular head domain (HA1) and a conserved stalk domain (HA2), which are linked together by a disulphide bond [37]. The globular head HA1 domain partially covers the HA2 stalk domain, making the head domain more accessible for antibodies. Antibodies that bind to the HA1 domain neutralize the virus, thus inhibiting viral entry into the target cell, and thereby protecting the host from infection. Antibodies that inhibit HA are measured in the Heamagglutination inhibition (HI) assay and represent a correlate of protection. The stalk domain is highly conserved and has low immunogenicity, possibly due to its low accessibility. The stalk domain allows A viruses to be divided into two groups, group 1 (e.g. H1, H2 and H5) and group 2 (e.g. H3 and H7). Antibodies to the stalk domain are broadly protective across a number of HA subtypes, and a potential target for future universal vaccine production[38,39].

Influenza ecology

The influenza viruses are remarkably infidel when choosing a host to infect. They infect more than twenty different animal species, mainly pigs, and different avian species (poultry and waterfowl) and bats[32], and represent a considerable zoonotic potential. The HA binds to sialic acid (SA) receptors on the host epithelial cell, permitting virus entry into the cell by endocytosis were further viral replication ensues (Figure 2). Human influenza virus bind to SA receptors with an α-2,6 linkage which is predominantly found in the upper respiratory tract of humans, limiting viral replication to this area[36]. In contrast, the avian influenza viruses bind to SA with α-2,3 linkage, and these are found in the alveoli of the lower respiratory tract in man. This is thought to be a mechanism by which the avian viruses have increased virulence, inducing viral pneumonia and respiratory collapse (ARDS). The epithelium lining the respiratory tract of pigs contains both α-2,3 and α-2,6 sialic acid receptors, hence pigs may be infected by both avian and swine influenza viruses[40]. Pigs are therefore referred to as the main “mixing vessels” of influenza subtypes and represent a constant reservoir from where novel influenza strains may emerge and

infect humans[40]. The majority of influenza subtypes (combinations of H1-16/N1-9) are found in aquatic birds, and they represent a mode of global viral dissemination. Only the subtypes H1, H2 and H3 are known to circulate in humans, although there have been zoonotic cases of H5, H7, H9 and H10 infections in man, there is no evidence of sustained human-to-human transmission. However, these subtypes are feared to have pandemic potential [41].

Viral life cycle

Influenza is transmitted to humans via virus in aerosols and droplets produced by sneezing or coughing in infected people or from infected animals.

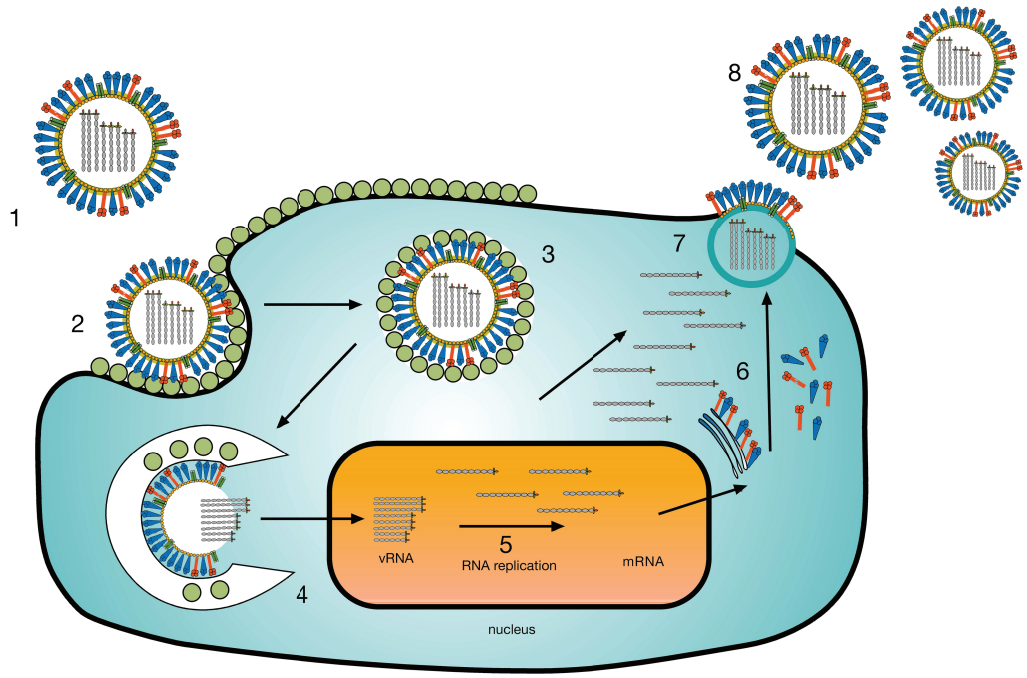


Figure 2: Schematic illustration of the influenza virus life cycle The HA glycoprotein on the virus (1) binds to the membrane of the host cell through a viral receptor, (sialic acid) (2). The virus enters the cell by receptor-mediated endocytosis (3). HA undergoes a conformational change resulting in the fusion of the viral envelope with the endosomal membrane (4) Viral vRNA is released into the cytoplasm, and translocated to the nucleus where it is transcribed into mRNA (5). The new viral gene segments are transported to the cell surface where they are assembled with the membrane proteins (HA, NP, M2) (6). The new virus progeny bud through the host cell membrane by exocytosis, which is dependant on the membrane protein NA (7). The new viral envelope is derived from the host cell membrane. NA is a target for antiviral medication, the neuraminidase inhibitors (NAI). The inhibition of NA prevents viral progeny from exiting the cell, and hence reduces viral shedding and symptoms. The NAI drugs will not prevent infection, and their effect is most potent when taken within the first 48 hours after illness onset. Figure made in collaboration with G. Johansen, UiB and inspired by Dr Karl Brokstad UiB.

Antigenic drift

The influenza RNA polymerases lack accurate proofreading mechanisms, causing point mutations in the viral genome coding for the surface antigens (HA, NA). This constant change enables the virus to escape attack by the immune response, such as antibodies. The newly mutated virus is unrecognizable to host antibodies and therefore has selective advantage and becomes the dominant circulating strain. This mechanism of antigenic drift is responsible for the annual epidemics or outbreaks, which in turn necessitates annual update of vaccine[42]. Mismatch between circulating strains and vaccine strains is a problem for the efficacy of seasonal vaccines. Such mismatch of the H3N2 strains had large consequences in the 2013-14 influenza season leading to excess mortality [43,44].

Antigenic shift

Antigenic shift represents the mechanism behind the appearance of novel influenza viruses with pandemic potential. Only Influenza A viruses are capable of antigenic shift. This occurs when two or more influenza viruses, of human or animal origin, infect a cell simultaneously and there is reassortment of the gene segments. This gives rise to a novel virus with altered genetic composition. If the novel virus circulates in an immunologically naïve population, the virus may spread rapidly and have the potential to herald a new pandemic. If the antigenic shift has resulted in a virus with increased virulence, the consequences may be disastrous[45].

The Swine flu pandemic in 2009

Pandemics arise at unpredictable intervals of 10-50 years, and since 1918 the world has witnessed pandemics, in 1957, 1968 and the latest in 2009 [2]. Before 2009, the emergence of a pandemic had long been anticipated, however it was the avian H5N1 influenza that was most feared. Hence, the global scientific focus had been on highly pathogenic avian influenza (H5N1) and increased surveillance of man and poultry in Asia. Therefore, when Mexico alerted the world to the novel influenza virus of swine origin, the world was taken somewhat by surprise. The first, dramatic reports evoked fears of a “worst-case scenario” pandemic with the potential of 1918 devastation.

Later the pandemic was classified as mild, with severity similar to seasonal influenza[15]. Nevertheless, many otherwise healthy young adults succumbed to the disease. Recent work has documented large differences in mortality across the globe and confirmed that Latin America had the highest mortality, with a more than 20 fold increase in severity compared to Europe[15]. These differences cannot be explained by differences in socioeconomic factors or availability of advanced healthcare systems alone[15]. The heterogeneity in pandemic influenza disease severity, as well as the geographical differences has occurred in most previous pandemics[15]. In a search for pandemic predictors, Simonsen et al studied pandemic outbreaks and found that an unusual shift in age mortality pattern was often a first sign of a pandemic, the younger people succumb while the seniors are spared[15]. This fact became evident after the 2009 pandemic where 85% of all laboratory confirmed A(H1N1)pdm09 deaths occurred in people < 65 years old[15], although the individual case fatality rate was the highest in the elderly[46] .

In 2009 the northern hemisphere was warned of the clinical implications of a novel H1N1 pandemic. Pandemics may have catastrophic potential, however, this time we had preparedness plans in place prior to the first pandemic wave and the pandemic was less severe than first expected. We had extensive surveillance systems, global communication, influenza vaccines and antiviral medication for prophylaxis or treatment of the virus, and antibiotics, modern diagnostics and advanced medical care available. Nevertheless, the integrity of the healthcare systems was challenged and in the future, increasing antibiotic resistance may leave future doctors with fewer weapons to combat influenza complications. Faced with a novel influenza virus in a population without pre-existing antibodies, the individual immune system is our most important defense strategy beside antiviral medication, before a the arrival of a new vaccine.

Influenza and the immune system

Innate immuity

The human immune system consists of a sophisticated network of cells, molecules and tissues designed to protect from external pathogens. This multifaceted system is comprised of the innate and the adaptive arms of the immune system and is illustrated in Figure 3. The innate immune system (Figure 3.1) is our first line of defense against pathogens and is constantly activated[47]. It is present at birth and provides an immediate generic response for attacking damaged cells or pathogens, which penetrate the initial barriers.

A vital factor determining the outcome of an influenza infection is the host´s initial adeptness to detect and restrict viral replication and spread of progeny virus at the site of infection entry. Antigen presenting cells (APCs) (Figure 3.2) recognize the virus as a general pathogen via pathogen recognition receptors (PRRs) capable of recognizing various pathogen-associated molecular patterns (PAMPs) exposed on the influenza virus. Activation of the PRR initiates intracellular signaling cascades, converging in the activation of pro-inflammatory cytokines which induces viral resistance in uninfected neighboring cells, as well as recruits other immune cells such as macrophages (MKF), neutrophils and natural killer (NK) cells (Figure 3.1) which kill the virus directly, and limit viral spread[48,49]. The recruitment of immune cells to the site of infection, leads to activation of the dendritic cells (DCs) which are a specialized type of APCs which link the innate and adaptive systems[47,50].

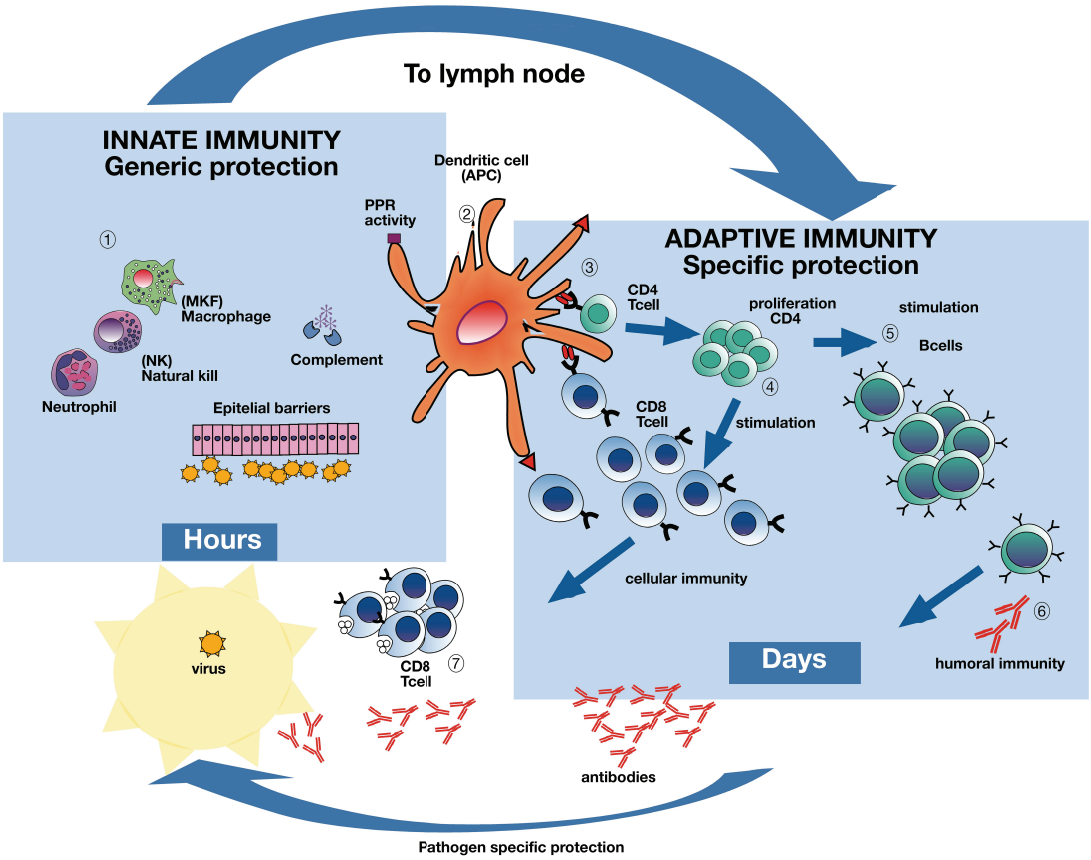


Figure 3. The innate and adaptive immune responses to influenza. The figure illustrates the different cells and their interaction during the immune response to influenza. The innate immune system consists of three main parts [47] 1) The physical and chemical barriers of the skin and mucosa including antibacterial secretions such as tears, saliva, digestive enzymes and mucus. 2) The blood and tissue immune cells, (phagocytes - monocytes, macrophages, neutrophils NK, DCs, basophils and eosinophils) which are constantly ready to combat pathogens if they breach the first barrier. 3) The blood proteins: complement factors, antimicrobial peptides and cytokines[47]. Complement factors are an important part of the innate immune system and consist of several proteins, which are cleaved and react together resulting in the membrane attack complex (MAC), causing cell apoptosis (death). Single proteins of the complement system may bind to the pathogens (opsonization)[47], further facilitating killing of the pathogen by specialized cells. T-cells recognize the foreign peptides presented by the DC through the T-cell receptor (TCR), which subsequently activates CD4+ and CD8+ T-cells and B-cells. DCs activate $CD4^+$ T-cells, which stimulate $CD8^+$ T-cell and B-cell proliferation resulting in influenza specific antibodies, and activated T-cells. Figure made in collaboration with G. Johansen (UiB) and inspired by Abbas and Ozbiosciences [47,51].

The adaptive immune system

The adaptive immune system plays a crucial role in the defense against influenza, and DCs initiate and conduct the subsequent adaptive immune response (Figure 3.2) [47]. The DCs are considered the most professional of the antigen-presenting cells (APCs),

due to their ability to present viral structures to the cells of the adaptive immune system both through the major histocompatibility complex (MHC) classes I and II (Figure 3.3). The highly specialized adaptive immune response requires several days to become fully activated. Although slower than the innate system, the hallmark of the adaptive immune system is that it has memory and the capacity to recognize an unlimited number of antigens with great specificity as opposed to the generic recognition by the innate system. The B-and T-cells are the most important cells of the adaptive immune system and further divide the adaptive immune system into the humoral and cellular compartments (Figure 3.4). The CD4+ T-cells orchestrate the immune response by assisting B-cell differentiation into antibody producing plasma cells or memory B cells and by secreting cytokines, which activate $CD8^+$ T-cells, and macrophages, that destroy virus infected cells (Figure 3.7). The activated B and T-cells proliferate and induce memory cells which are able to mount a rapid immune response with increasing specificity upon repeated infection with the same pathogen[52]. The ultimate goal of all vaccination is to achieve long-term, protective immunological memory. In the later years, the research focus on influenza immunity has shifted from protective antibody responses to understanding the cellular immune response to influenza infection[53-56].

Cellular immunity

Cellular immunity is based on two main lineages, the $CD4^+$ and $CD8^+$ cytotoxic subtypes of T-cells (Figure 3). Activation of T-cells by a viral infection is a dynamic and complex immune reaction where T-cells migrate between the blood and tissues[55]. $CD4^+$ T-cells represent a key factor in limiting severity of influenza disease due to their cytotoxic destruction of infected host cells and stimulation of B-cells and $CD8^+$ T-cells (Figure 4). $CD4^+$ T-cells are activated and differentiate into several different subtypes of T- helper cells, including Th1, Th2 and Th17 helper T-cells, as well as regulatory T-cells (Figure 4). The activated T-cell subclasses migrate to the local lymph nodes and secrete pro-inflammatory cytokines, which lead to an autocrine activation of the T-cell as well as stimulation and recruitment of immune cells to the site of infection, thereby controlling and regulating the adaptive immune response[57].

Th1 T-cells secrete interleukin-2 (IL-2) and interferon-γ (INF-γ) cytokines. IL-2 is an important growth factor for T-cells, stimulating expansion and differentiation of the activated T-cells into effector and memory T-cells[47]. INF-γ is a central cytokine, which has many effects, triggering phagocytosis by macrophages as a central effect.

Activated $CD8^+$ T-cells detect and cause a direct cytotoxic lysis of the influenza infected cell (Figure 4)[47]. $CD4^+$ T-cells activate MFKs and increase their ability to ingest virus-infected cells. $CD8^+$ T-cells cannot inhibit infection *per se*, however, they play an important role in limiting disease severity and reduction of virus shedding by killing of virus infected host cells and clearing of the infection.

The attention to cellular protective responses has increased after $CD8^+$ T-cell subsets were linked to less severe pandemic infection in 2009[53]. An important milestone on the way to develop a "universal " influenza vaccine will depend on developing a vaccine capable of inducing broad T-cell responses, which could provide protection from different viral subtypes[58]. $CD4^+$ and $CD8^+$ T-cells have both been linked to reduced severity of influenza disease, and different T-cell response patterns have been found in severely infected and mildly infected or vaccinated people [59]. LAIV has shown to induce T-cell responses [60,61](and **paper I)**, and T-cell responses are the goal of future seasonal influenza vaccines[54]. T-cell responses will be further discussed in the results and discussion section. A schematic overview of the roles of the adaptive T and B-cells in respiratory viral infection is presented in Figure 4.

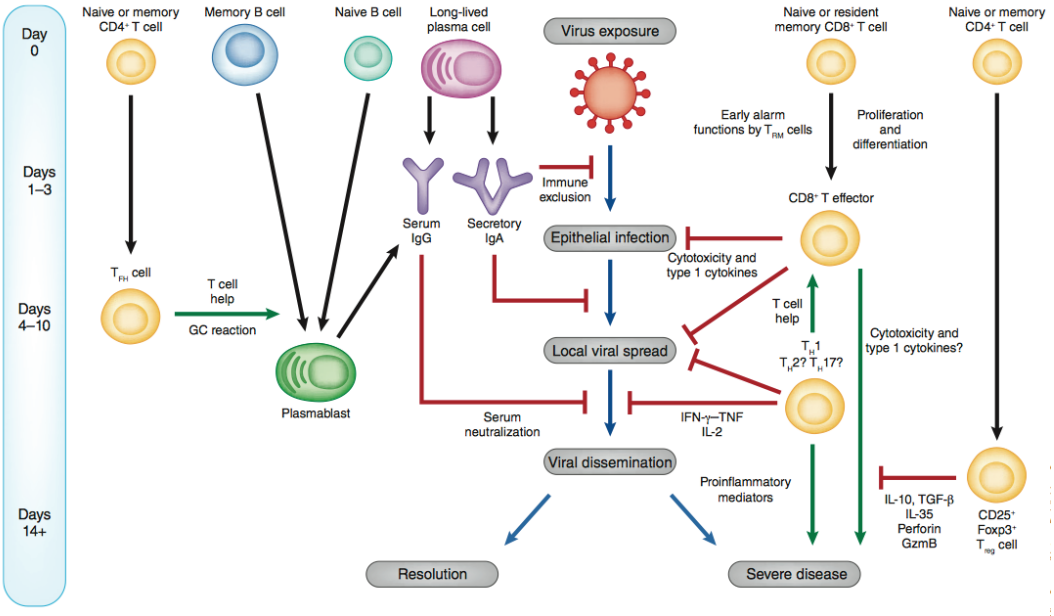


Figure 4. The roles of the adaptive T and B-cells in a respiratory viral infection. Plasma cells produce virus specific antibodies, which inhibit infection of epithelial cells, providing sterilizing immunity to influenza. T-cells protect after initial infection, and limit further viral spread- Activated CD4 + T-cells stimulate B-cell activation and CD8 + T-cells which kill virus infected cells, important for clearing of the influenza infection and avoiding severe disease. Reprinted with permission from Macmillan Publishers Ltd: [Nature Immunology] (Chiu, C. and P.J. Openshaw, Antiviral B cell and T cell immunity in the lungs. Nat Immunol, 2015. 16(1): p. 18-26, copyright (2016)[56].

Humoral immunity

Humoral immunity has been the focus of influenza researchers for decades[8,62] and an HI titer of 40 is a correlate of protection after influenza infection or vaccination[63]. Strain-specific antibodies provide sterilizing immunity to the priming strain, however they have short effect due to the constant viral antigenic drift[64] (Figure 4). Humoral immunity refers to the effector mechanisms mediated by B-cells and their antibodies. B-cells develop in the bone marrow, and migrate to the lymphoid tissue where maturation and activation occurs upon antigen re-encounter. When antibody-specific binding to an antigen occurs, the pathogen is neutralized or opsonized, and the activation of the complement system with antigen destruction ensues[47].

Naïve B-cells are located in the local lymph nodes and activated by antigen presented by the DCs or by capturing the antigen via the B-cell receptor[47]. Naïve B-cells

express IgM or IgD on their surface, and after activation they mainly produce IgM antibodies. Upon antigen presentation and priming, the B-cell is activated and differentiates into either an antibody-secreting plasma cell (ASC) or memory B-cell (MBC) (Figure 4)[56,64].

ASCs produce strain specific antibodies and are measurable in the blood one week post-vaccination or infection[65,66]. There are two types of ASCs; short-lived plasma cells residing in the lymph nodes and long-lived plasma cells, which home to the bone marrow and represent a form of immunological memory[47,67]. In the lymph nodes, the B-cells proliferate and undergo affinity maturation and class switching, resulting in the production of antibodies with higher affinity to the priming antigen[47]. IgG is the most abundant circulating antibody against influenza. IgA is important in initial influenza immune defense by virus neutralization at the site of entry in the respiratory mucosa, and is induced by LAIV administration[62] and (paper II).

In contrast, the MBCs circulate and upon antigen re-encounter, quickly proliferate and differentiate into plasma cells producing an increased amount of specific antibodies [47]. This swift secondary immune response ensues increased specificity and quantity of the antibodies. It is the main role of the MBCs and the rational behind a prime, boost vaccination regime used for many vaccines[47]. MBCs may survive for a long time in humans (more than 50 years), circulating between the bone marrow, peripheral blood and the lymphoid organs, continuously waiting to be re-stimulated by their specific antigens[68,69].

Tonsils

Tonsils are secondary lymphoid tissue, located at the site of entry of the upper respiratory tract, draining the oral and nasal cavities[47]. The tonsils are part of "mucosa associated lymphoid tissues" MALT, consisting of: pharyngeal (adenoid) and lingual tonsils and two tubal and palatine tonsils (referred to as tonsils). The tonsils and other lymphoid tissues, which surround the entrance to the respiratory tract, form the "Waldemeyer´s ring". Deep crypts maximize the epithelial surface area exposed to antigens where specialized Langerhans and M cells transport luminal antigens into

the tonsillar tissue[70-72]. Tonsils elicit mucosal immune responses against respiratory pathogens[73] , and are an important induction site and reservoir for B-and T-cells in lacrimal, salivary glands and airway mucosa[71,74,75]. Earlier work has shown that IIV induces rapid humoral immune responses as early as 2 days post vaccination in tonsils[76]. Delivery of LAIV via the intranasal route is perhaps the most efficient way of boosting mucosal immunity at the site of viral entry and was studied in **papers I + II.**

Influenza prevention and treatment

Vaccination continues to be the most effective intervention to mitigate transmission, illness, and fatal outcome of influenza. Although vaccines are cost-effective and considered the pillar in the defence strategy to combat influenza, vaccine effectiveness and supply during a pandemic is limited. Therefore, other important, non-pharmaceutical, public health measures to reduce viral transmission are essential[77]. This includes information campaigns, informing the public and promoting good hygiene. Frequent hand washing, self-isolation if ill, facemask usage, and respiratory etiquette are all actions that reduce virus transmission and attack rates[78,79]. In school and hospital settings, these measures are promoted during seasonal outbreaks. Since children are the main transmitters of the disease in the community, school closures and other confinement measures have been shown to slow down viral spread and reduce peak demand on healthcare systems[78,80]. These actions are rapid to implement, and in a pandemic setting they may buy time before more direct measures are in place, such as mass distribution of antiviral medication or vaccines[81]. However, as was experienced during the 2009 pandemic, the highly transmissible novel virus spread with unprecedented speed, spreading worldwide in a matter of six weeks. In the past pandemics, six months was needed for the virus to spread equally[82,83]. Norway was in a privileged situation, being one of the first European countries to receive the new pandemic vaccine, due to a pre-order contract, and having a stockpile of antiviral medication.[84]

Antiviral therapy

There are two main groups of antiviral medication, the M2-ion channel inhibitors, the adamantanes, and the neuraminidase inhibitors (NAI). These antivirals may reduce illness symptoms by one or two days, ameliorate severe disease and hence improve survival. Prior to 1999, the M2 ion channel inhibitors were the only available antiviral medication for treatment and prophylaxis[85]. They interfere with the process of viral endocytosis and release of viral RNA into the host cell cytoplasm, and thereby inhibit replication[85,86]. A major limitation in the use of the adamantanes was

the rapid development of resistance, psychological and neurological side-effects and lack of effect towards the influenza B virus (which has no M2 protein). The side-effects (e.g. confusion, anxiety, hallucinations and nightmares) occurred mostly the elderly who have increased elimination half time[85,87].

The licensing of NAI for treatment in 1999 has been a major improvement. Since then, the NAIs zanamivir (Relenza®, used as an oral inhalation) and oseltamivir (Tamiflu® available as oral capsules and suspension) have been the most widely used for influenza treatment and prophylaxis[85,88,89]. The NAIs block the exocytosis of newly synthesized virus progeny, thereby reducing viral replication and transmission for both influenza A and B viruses. There are few side effects, renal impairment being the most important[90]. The US, UK and Norwegian public health authorities recommend treatment with NAI in patients who are seriously ill with influenza or at risk of serious disease[91,92].

There has been a heated debate over the effects of NAI in influenza treatment. A Cochrane review of randomized clinical trials (RCT) conducted between 1997-2004 of oseltamivir treatment in previously healthy adults found a reduction of symptoms of illness by one day and a significant reduction in self-reported pneumonia[93]. Later, an individual meta-analysis and a systematic review confirmed the findings of reduced symptoms. In addition, they found fewer cases of lower respiratory tract infections and hospitalizations with influenza in oseltamivir treated patients [90,94].

The PRIDE group (Post-pandemic Review of anti-Influenza Drug Effectiveness)[95,96] (appendix paper I + II) conducted a global data meta-analysis of observational data on an individual patient level of more than 29 000 hospitalized patients during the pandemic, including a contribution from our hospital (appendix paper I + III). The Cochrane review and the PRIDE publication differ in methodology and patient group analysed. Where the RCTs considered healthy adults at a population level, with seasonal influenza treated in the community, the PRIDE group included individual data on hospitalized patients with pandemic influenza only. They found a reduction in mortality in NAI treated patients compared to non-treated (18% overall, 50% in

pregnant women) and a greater reduction if NAI treatment commenced early, within 2 days of symptom debut, (52% overall, > 80% in pregnant women and 35% in ICU patients). However, no differences were found in mortality reduction risk in the child population, perhaps due to differences in viral load or dosage[95]. Later Muthuri et al. published a follow-up study on influenza pneumonia cases and found that NAI significantly reduced the need for ventilator support and mortality[96]. The PRIDE findings support the clinical observations from experienced doctors attending this selective group of severely ill, hospitalized patients. An editorial in the Lancet recommended initiation of treatment with NAI if a patient was hospitalized with suspected influenza, and later termination if a different cause of illness was found[97,98,99].

NAI resistance

During the 2009 pandemic, Japan recommended early treatment with NAI in all patients with clinical illness, reported the lowest fatality rate of any developed nation, and was the only country reporting no maternal deaths [100,101]. Japan has the highest use of neuraminidase inhibitors per capita in the world, with >70% of global consumption[102], and has put in place resistance surveillance measures. In the pre-pandemic influenza season of 2007-08, several countries, including Norway, reported an increased number of oseltamivir resistant A(H1N1) strains in patients without NAI exposure[103,104]. Japan found low-level human transmission of resistant virus strains in NAI treated patients, but no reduced NAI susceptibility or increased resistance[102]. Japanese NAI resistance surveillance covering seasons 2008-2013, found sporadic A(H1N1)pdm09 resistant to NAIs, but no community spread, nor any reports of serious illness by resistant strains was detected[101,105].

In the Scandinavian countries the use of NAIs has been low, although increasing the last years. A report from 2008 found oseltamivir resistant strains in Norway; however there was no difference in symptoms or need for hospitalization, and the resistance was not associated to prior use of NAIs[103]. Since the 2009 pandemic, the WHO has monitored H1N1 strains closely, and 4 resistant isolates have been reported from Europe, one from Denmark[106], however none have been associated with increased

severity of disease or posed a threat to public health[106]. There is a continued need to monitor resistance since evidence of increased transmissibility or pathogenicity might not be evident in individual case reports[106]. The future management of influenza disease will perhaps necessitate the combined use of two or more NAIs as well as vaccination to avoid serious challenges if transmission of resistant strains becomes common. Similar to the increasing problems of antibiotic resistance, the future use of NAI will require much clinical wisdom, limiting the use to needy cases and avoiding abuse of a common resource, which could later have detrimental effects on public health.

Other treatment options

Due to concerns over resistance problems with NAI and the long time required for vaccine production, additional treatment options are being explored. Passive immunization with sera from influenza survivors has been used since 1918 and up until the last pandemic with variable but promising results[4,5,107,108]. Research into broadly neutralizing monoclonal antibodies is growing and could represent a novel set of antivirals[107]. A recent study found that monoclonal antibodies isolated from convalescent A(H1N1)pdm09 patients, reacted to conserved parts of the HA molecule, protecting mice and ferrets from lethal challenge [109,110]. These results have paved the way to upcoming human clinical trials and could aid the future design of the desired "universal influenza vaccine", providing protection across different subtypes of influenza viruses[54,77].

The stalk of HA is less exposed to the immune system compared to the head domain and remains antigenically fairly stable. A novel approach to combat influenza disease is the production of monoclonal antibodies to these conserved viral epitopes of the stalk of HA[111]. These antibodies may induce cross-protection to several viral subtypes, improving treatment options. However, although there have been optimistic findings in animal trials, human studies have not yet been conducted[112-114].

There are reports of improved survival rates after passive transfer of antibodies to severely ill patients from convalescent subjects during the 1918 pandemic and during

the H5N1 outbreaks in China[4,5,108]. Other treatments such as anti-inflammatory drugs may modulate the innate immune response to influenza, and corticosteroids have been used in severe influenza cases, in an attempt to reduce inflammation. However, two recent systematic reviews found that systemic corticoid treatment of influenza complications significantly deteriorated the patient´s outcome with increased mortality, complication risk and morbidity[115-117]. The immunosuppressive effects may have promoted increased viral replication, and the authors discourage the use of systemic corticoid treatment in influenza infections, unless systemic shock or organized pneumonia permitted indication of usage[118].

Influenza vaccines

The first influenza vaccines were developed in the 1940s and contained crudely purified, inactivated whole virus. Modern IIVs are chemically inactivated and come in various versions, whole virus, split virus (the virus is split using detergent or ether), subunit or virosomal (Figure 5, different vaccine types). The subunit vaccines consisting of purified viral surface antigens (HA and NA) have low reactogenicity. The current influenza vaccines are well tolerated and considered safe, with more than 140-170 million doses distributed annually in the US the last 5 years [119].

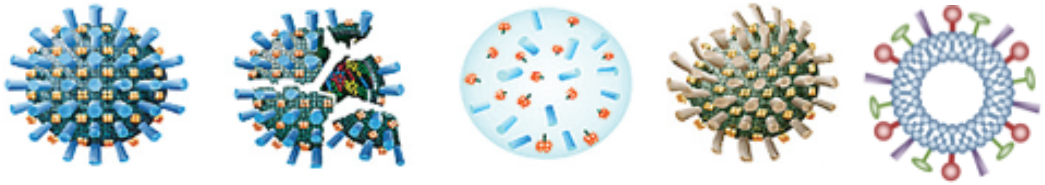


Figure 5. A shematic respresentation of different influenza vaccine formulations. From left to right, a whole intact virus, a split virus, the active subunit antigens of a virus, and a live attenuated (weakened) virus. Whole virus vaccines can be either inactivated or live attenuated. The split and subunit vaccines are chemically disrupted viruses and lack the natural virus structure. The spilt virus contains viral components, while the sub unit vaccines only contain purified surface glycoproteins HA and NA. Virosomes resemble the whole virus, and consist of an envelope but lack the genetic material. Reprinted with permision from The International Federation of Pharmaceutical Manufacturers & Associations http://www.ifpma.org/resources/influenza-vaccines/influenza-vaccines/about-influenza-vaccine.html and the virosome from the delivery perspectives of influenza vaccines[120].

Adjuvants

Adjuvants (from Latin adiuvare= aid) are chemical components designed to shape or intensify vaccine antigen specific immune reactions, and have been used for more than 90 years[121] (reviewed in[122]). Modern vaccines are produced with highly purified components with excellent safety profiles, however, due to their purity, their immunogenicity is low. In order to achieve a sufficient immune reaction, an adjuvant is incorporated into the vaccine [123], however development of new adjuvants are considered essential for future vaccine development.

Although adjuvants have been used for many decades, several modes of action have been uncovered just recently[124]. They contribute by ameliorating the uptake and presentation of weak antigens by APCs, hence activating the innate immune system and improving vaccine responses[121]. Adjuvants increase the magnitude and breadth of the antibody response, enabling a more rapid immune response as well as sparing the amount of antigen required to trigger a response. All these factors are important in achieving a sufficient immune response in the elderly, immunocompromised and young, as well as in a post-prophylaxis setting. In addition, dose sparing provides expanded supply and reduced costs[125]. An essential role of new adjuvants is to enable T-cells to optimize antibody responses as well as induce $CD4^+$ and $CD8^+$ effector cells. There have been many attempts at producing new adjuvants with promising results in animal models however, the hurdle to cross from mouse to man has proven difficult to overcome. The only adjuvants licensed for use in humans are aluminium based, oil-in-water formulations MF59 and AS03, AS04 (alum and monophosphoryl lipid A combination). Standard seasonal influenza vaccines used in Norway, do not contain adjuvants. Alum has been used for decades and is considered safe, however for influenza vaccines, the oil-in-water emulsion adjuvants (MF59 and AS03) have been more effective than alum. Both MF59 and AS03 were used in the monovalent pandemic vaccines in 2009.

Seasonal influenza vaccines

Implementation of influenza vaccines commenced 70 years ago, and despite their shortcomings, they have ever since been the most important and cost-effective counter measure to combat influenza. The vaccine is recommended to the population at risk of severe disease and to healthcare professionals and pig farmers[27,126]. In the US and the UK, the influenza vaccine is recommended to children and has been implemented in the childhood vaccination campaign[19]. The currently used influenza vaccines are divided into two classes, inactivated influenza vaccines (IIV) and live attenuated influenza vaccines (LAIV). The seasonal influenza vaccines, both IIV and LAIV, have traditionally been trivalent, i.e. consisting two influenza A subtypes and one B virus. However, due to co-circulating B lineages, the vaccine now includes both B lineages, hence both trivalent and quadrivalent vaccines are available. The ever-changing virus requires biannual updating of the vaccine strains in order to provide protection in the northern and southern hemispheres. The vaccines have since the beginning in 1945 been produced in embryonated hen´s eggs, and this remains the most important production platform for vaccines today. Egg supply and vaccine yield achieved from each egg, are the most important factors limiting influenza vaccine production. Production takes roughly six months, and involves consensus on the choice of vaccine strains to include in the vaccine, decided by the WHO each February for the northern hemisphere[27,127]. The choice of vaccine strains is based on sampling of circulating influenza viruses by a global influenza network of laboratories and followed by amplification of seed viruses, vaccine manufacturing and distribution. A mismatch between chosen vaccine strains and actual circulating strains does happen, although not frequently. A consequence of a vaccine mismatch is reduced vaccine efficacy as was seen in the 2014-15 season, resulting in excess mortality in several countries[43,128]. Production of quadrivalent vaccines is an improvement, reducing the risk of mismatch of B strains[129].

The time constraint is a serious hurdle to overcome in the case of a pandemic. Despite the pandemic vaccine in 2009 being made in record time, most of the world´s population did not receive the vaccine in time[125]. New production methods based on

cell cultures, plants and viral vectors are being explored in order to increase production speed and quantity as well as ease the burden of egg-dependent biannual vaccine production[130-132].

Risk groups and recommendations for influenza vaccination

The WHO has published guidelines for the recommendation of seasonal influenza vaccines to the population at most risk of severe or fatal disease. The European centre for disease prevention and control (ECDC) supports these guidelines, but each country makes national decisions, and Norway does not have the same recommendations as the UK or Finland. The UK introduced seasonal influenza vaccination in their childhood vaccination program (4-11 years old) in 2013[129]. Since it´s implementation, there has been published evidence of a reduction in morbidity in both the child and adult population, indicating an effect of herd immunity[19,20]. For the 2015-16 influenza season, the Center for Disease Control and Prevention (CDC) and the Advisory Committee on Immunization Practices (ACIP) in the USA, recommend annual influenza vaccination for all persons aged 6 months or older who do not have contraindications[127]. However, Norway and the ECDC recommend influenza vaccination for patients at high risk of serious influenza disease and it´s complications[133] [126]. Below are two tables with recommendations for influenza vaccination. Whilst the WHO focuses on costs and feasibility of vaccination, The Norwegian public health (NPIH) authorities do not consider these issues.

Elderly > 65 years old	
Inhabitants of nursing homes	
Children and adults with:	Chronic lung diseases
	Cardiovascular diseases
	Chronic lung diseases
	Diabetes (Type 1 and 2)
	Impaired renal function
	Impaired liver function
	Chronic neurological disorders
	Severe obesity, BMI > 40 kg/m^2
- Pregnant women in the 2nd and 3rd trimester	
- Healthcare workers with patient contact	
- Veterinarians and employees in the swine industry	

Table 3 List of recommended people for vaccination towards influenza according to NIPH.

The Table is based on the information from the Norwegian Institute of Public health (NIPH) [126].

Priority	Group	WHO reasoning for the recommendation
1	**Pregnant women**	Increased risk of severe disease and mortality in mother and child, secondary protection of child the first 6 months, globally feasible, pregnant women have contact with health institutions
2	**Healthcare workers**	Increased exposure to disease reduces morbidity and mortality among patients, protects integrity of healthcare system, feasible to implement
3	**Children < 2 years**	Carry main burden of serious disease, are greatest transmitters of disease, difficult to conduct
4	**Children 2-5 years**	Carry large burden of serious disease, respond better to vaccine
5	**Infants < 6 months**	Cannot receive vaccine, should be protected by vaccination of mother and close contacts
6	**Elderly > 65 years**	Cannot receive vaccine, should be protected by vaccination of mother and close contacts
7	**Patients with chronic disease**	Highest risk of severe disease identification of individuals, is effort and resource demanding

Table 4 List of prioritized groups for influenza vaccination according to the WHO. The table is based on WHO´s position paper on influenza vaccines and takes into consideration the risk posed for each group, the cost effectiveness and the feasibility of conducting vaccination in each group [18].

Live attenuated influenza vaccine (LAIV)

The Russians were the first to use a LAIV and have used it for over 40 years[134]. The vaccine was licensed in the USA for healthy people aged 2-49 years old in 2003, and in Europe in 2012, for healthy children 2-17 years old[19,135]. LAIV is administered as a nasal spray with one spray into each nostril, and requires replication of the live virus in the mucosa of the upper airways to induce protection. An illustration of the immune responses elicited by the LAIV vaccine is pictured below in Figure 6. By mimicking a natural infection, but without causing disease or onward transmission, the vaccine elicits both humoral and cellular immune responses[60,136]. An important mechanism for providing protection against influenza is that the effector cells or molecules are present in the mucosa, the site of viral entry. LAIV has shown to induce mucosal IgA antibodies which provide protection upon subsequent challenge (Figure 6) [62,137].

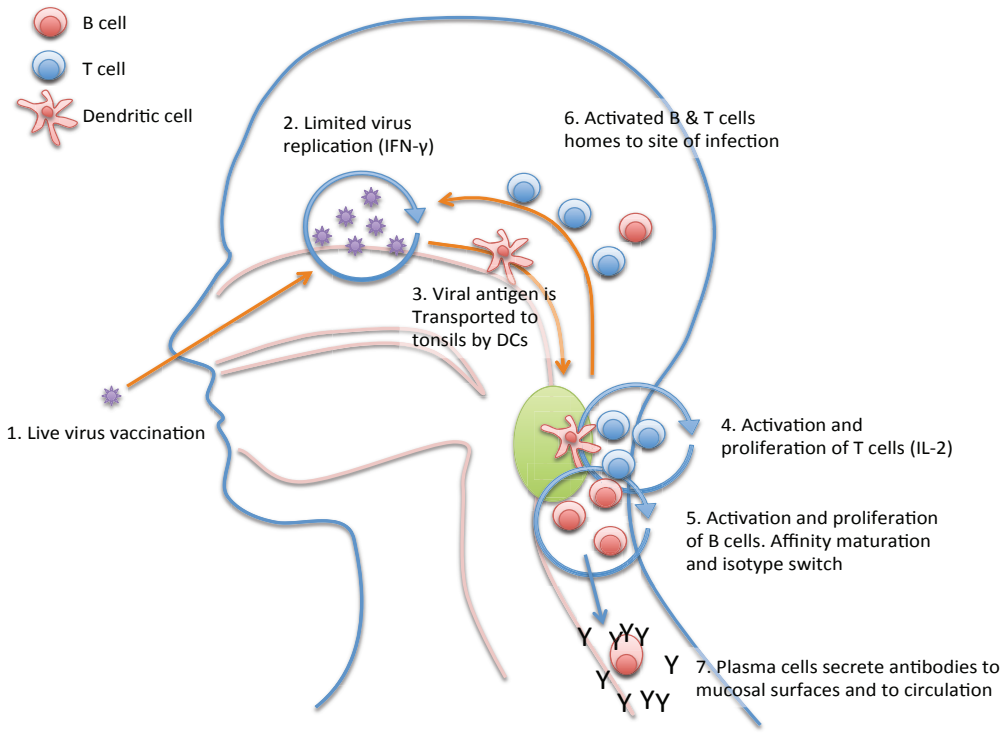


Figure 6 A schematic figure illustrating how LAIV induces an immune response. Figure kindly provided by Dr Karl A Brokstad and printed with permission[54].

For production, one master donor virus (MDV) is used for each A and B strain. The cold adapted variants were previously achieved by repeated passage of the virus in eggs at gradually decreasing temperatures[138]. Modern LAIV vaccine strains are produced by reverse genetics, using the HA and NA genes for the WHO recommended vaccine with six gene segments from a cold adapted, temperature sensitive and attenuated virus backbone with the desired mutations[139]. The virus is therefore restricted to replicate in the upper airways (Figure 6), where the temperature is lower and inhibited from descending and replicating in the warmer, lower respiratory tract (>33°C). The vaccine strains have been found safe, genetically stable, and have not been found to revert to the wild type viruses[139-141]. Usage of LAIV permits vaccine sparing compared to IIV, and one egg provides approximately 10 vaccine doses, compared to only one dose of IIV. LAIV is recommended for children due to the low immunogenicity of inactivated vaccines in this age group and the needle-free administration. LAIV has shown better immunogenicity than IIV in children [142], and only LAIV has been shown to induce T-cell responses in children[60,61,143]. Cochrane reviews have found approximately 80% efficacy in young children (<6 years old) and lower (40%) in adults to matched strains[144,145].

The vaccine is easy to administer and well tolerated with mostly local side effects observed the first 2-3 days after vaccination, such as runny or congested nose, and shedding of the vaccine virus is age dependent[146]. An increased hospitalization rate due to wheezing was found in children < 2 years old[142], and LAIV is therefore contraindicated in these children. It is also contraindicated in immunocompromised individuals, pregnant women and in children with severe asthma, or receiving salicylate therapy (risk of Reye´s syndrome with salicylate and wild-type influenza infection). However, a recent multicentre study of the safety of LAIV in young people (2-18 years) with egg allergy and asthma, found that the vaccine was well tolerated and there was a low risk of systemic allergic reactions in those with egg allergy[147]. Several studies have confirmed LAIV to be safe in children with intermittent wheezing and stable asthma, including children 18 months of age[147,148]. In our study, we included children with mild and moderate asthma and did not experience serious side effects or increased asthma[136]. In the US and UK, vaccination

against influenza is recommended to all people older than 6 months, whereas in the Nordic countries the LAIV is not widely used. In Norway vaccination is recommended for the elderly, > 65 years old, risk groups, and healthcare workers and pig farmers, and not children in general [19,126,127].

Studies have shown that the LAIV provides protection in animals challenged with heterosubtypic influenza strains[149]. In addition, children were protected against a drifted H3N2 variant virus, which occurred naturally during the study, and was not contained in the vaccine[60,149-151]. The LAIV has the potential to confer broader protection than the IIVs, especially in children when used in school settings[60,135,152]. The benefits of herd immunity have also been observed after LAIV vaccination of the child population in the UK, and T-cellular immune responses are considered to provide this observed effect[153,20]. It has been suggested that using the LAIV in the child population could be an important step in the protection against a new pandemic[154].

Pandemic vaccines

The 2009/10 pandemic vaccine was based on the initial isolates from the two first children diagnosed in California, USA (A/California/7/2009 (H1N1)-like virus). The vaccine was produced and licensed in several different formulations, both as an IIV (with and without adjuvant) and a LAIV. In Norway the Pandemrix® vaccine a low dose (3.75µg HA per adult dose or 1.85µg per paediatric dose) oil in water emulsion (AS03) adjuvanted vaccine was almost exclusively used. Even though the pandemic vaccine in 2009 was produced in record time, most of the world´s population did not receive the vaccine in time[125]. A total of 30.8 million doses of Pandemrix® were used during the pandemic in Europe[155]. The first batches of pre-ordered vaccine were received October 18th 2009, three weeks prior to the first wave in Bergen. This pandemic vaccine was highly immunogenic. A clinical study in healthcare workers conducted by our research group showed that one dose was sufficient to elicit protective HI titers (≥40)[156], and the levels were maintained for several years[157]. The initial findings were part of the evidence base concluding that one dose of vaccine

was sufficient, even though the Norwegian government had stockpiled two vaccine doses per person.

The first case of pandemic influenza in Norway was identified in April 2009 in people returning from affected areas [158]. During the summer, Norway experienced a wave of rhinovirus infections, which was followed by the first and only wave of pandemic influenza in October / November 2009. By then, the Norwegian medical community had preparedness plans in place.

Future influenza vaccine development

The need for annual seasonal vaccine update and the costly and cumbersome production as well as the late arrival of the 2009 pandemic vaccine has further motivated research into developing a “universal influenza vaccine”. Such a vaccine, would ideally afford protection against all influenza strains, a "one shot fix all” approach. The key to such a success lies in identifying conserved epitopes that exist for multiple influenza virus subtypes followed by developing a vaccine, which elicits an effective immune response to these antigens[114,159]. Several conserved epitopes are currently in focus; the extra cellular component (ectodomain) of the influenza A M2 channel protein, the stalk region of the HA[39,160], and the globular head of NA (since NA has lower antigenic drift rates than HA). In addition, vaccines capable of inducing cellular responses would be a major improvement[161]. Cellular responses to internal proteins such as Influenza A nucleoprotein (NP) and matrix protein M1 have shown promising T-cell activation when co-administered with TIV in a human phase I clinical trial[162]. The main challenge with all these common and conserved epitopes is their poor immunogenicity, which triggers the need to develop better adjuvants and vaccine production platforms[163]. Lately, the most promising results are in trials that supplement the current IIV with novel vaccines containing antigens from internal viral proteins; hence elicit protective immune responses from both B-and T-cell subsets. Although promising research has been achieved in mice and ferret models, as well as non-human primates, several years and considerable efforts are needed before any replacement of todays IIV and LAIV in man will occur[164].

Vaccine safety and controversies

Vaccines are given as prophylaxis to a healthy population and often to children. The tolerance for side effects is hence very low, and safety is of the utmost importance. Both live, attenuated and inactivated influenza vaccines are well tolerated and considered safe. However, there have been several controversies connected to adverse effects. The public questioning of safety issues has influenced vaccine acceptance rates ever since 1976, when recipients of a swine influenza vaccine in the USA were affected by an unexplained increase in the risk of Guillain-Barré syndrome (GBS)[165]. Later, the Vaccine Adverse Events Reporting System (VAERS) was established in the US in 1990. A study of the trends of GBS reported to the VAERS after influenza vaccination from 1990-2003 found a possible causal association between the vaccine and GBS[166]. Later studies confirmed this association, however, they found several magnitude greater risk of GBS after influenza like illness (ILI) than after influenza vaccination[167]. A post-pandemic study of the risk of GBS following pandemic vaccination in 2009, suggested a small increased risk of 1-3 additional cases per million vaccinated individuals[168]. This study emphasized the potential confounders, and that clinicians and public health officials must weigh the benefits of influenza vaccination against possible risks.

Much research has been conducted to find the causative agent behind the tragedy of increased narcolepsy cases in children after pandemic vaccination[169], although the full mechanism has not yet been revealed. Initial reports linked the disease to the AS03 adjuvanted pandemic vaccine (Pandemrix ®)[170-173], and cases in Nordic countries have been linked to a specific HLA haplotype[172]. The latest research has found a link between vaccine nucleoprotein (NP) content and subsequent immune response to sleep receptors in the brain[174]. In this study, vaccine-induced antibodies cross-reacted with both influenza NP and the hypocretin receptor 2, suggesting a causative link between pandemic vaccination and later narcolepsy in predisposed individuals[174]. Massive media coverage and later monetary compensations to affected children has increased vaccine scepticism at a population level. To illustrate this, we had a patient with risk factors with severe influenza in the ICU who had declined the influenza

vaccine recommended by her GP in fear of side effects. In order to inform the public and uphold confidence in the vaccine advice given, surveillance systems of adverse events linked to vaccination are of vital importance. Public health communication will play an increasingly important role, since the doctors´ advices is are mostly given on a one-to-one person basis, while information in the media reach many more. While teaching, the safety and scepticism to influenza vaccines is a hot topic and regularly debated, illustrating that even among highly qualified medical workers, the need for updated information is critical.

Correlates of protection and vaccine effectiveness

To measure the immune effect of an influenza vaccine, the only agreed correlate of protection is the HI. This measures indirectly the amount of virus specific antibody in the blood. Traditionally an HI titer of 40 or above indicated protection, and has been used for evaluating and licensing of inactivated influenza vaccines. However, while conducting LAIV efficacy trials (clinical trials measuring the number of ILI symptoms with a positive PCR in vaccinated people) has shown that the HI titer underestimates the protection achieved[175]. In these trials, the vaccinated individuals were protected from PCR confirmed infection however, the HI titers did not indicate protection [175].

Estimates of vaccine efficacy vary, depending on the outcome measure and the method used. Kavanagh et al. showed that the pandemic vaccine had an efficacy between 34%-60%, with different methods used[176]. The efficacy numbers vary for the LAIV, but are higher in children than adults[135,177,178]. The best efficacy numbers are obtained for the subset of individuals with influenza symptoms and virology confirmed illness. The IIV and LAIV induce immune responses in different ways, and hence it does not seem appropriate to use the same correlate of protection to measure their efficacy. This has been taken into consideration for the licensing requirements for LAIV. The former statement requiring obtaining a HI titer of > 40 has been removed[179]. The LAIV induces both humoral and cellular immune responses and there is an urgent need for new correlates of protection in order to evaluate these vaccines in the future as well as develop new vaccine.

Aims

- Main objectives of the **pediatric vaccine trial** using a seasonal LAIV
  - To investigate systemic humoral and cellular immune responses in children after LAIV vaccination **(paper I)**
  - To investigate local mucosal and systemic immune response in saliva, tonsil tissue and blood in children after LAIV vaccination **(paper II)**
- Secondary objectives
  - To evaluate safety and tolerability of the vaccine in children, including asthmatics **(paper I)**
  - To evaluate the use of saliva sampling **(paper II)**
  - To study the effect of priming on the immune response **(paper II)**
  - To compare B-cell responses in tonsillar tissue to B-cell responses in peripheral blood (**paper II**)

- Main objective of the **pandemic patient study** in infected adults **(paper III)**
  - To describe the humoral and cellular immune responses after pandemic influenza infection in patients with different disease severities
- Secondary objectives
  - To investigate differences in frequencies of T-cell subsets using specific influenza peptides
  - To compare immune responses in acute and convalescent patient groups

Methods

The work in this thesis is based on patient material collected during two clinical studies and analysis of the immunological and clinical responses to influenza disease and LAIV vaccination. In the following, the study design for the pediatric vaccine trial and the pandemic patient study will be explained. Lastly, the laboratory methods conducted with the help of colleagues at the Influenza Centre and the Norwegian institute of public health (NIPH) are described. Below is an overview of the two studies and the patient material used in the published papers.

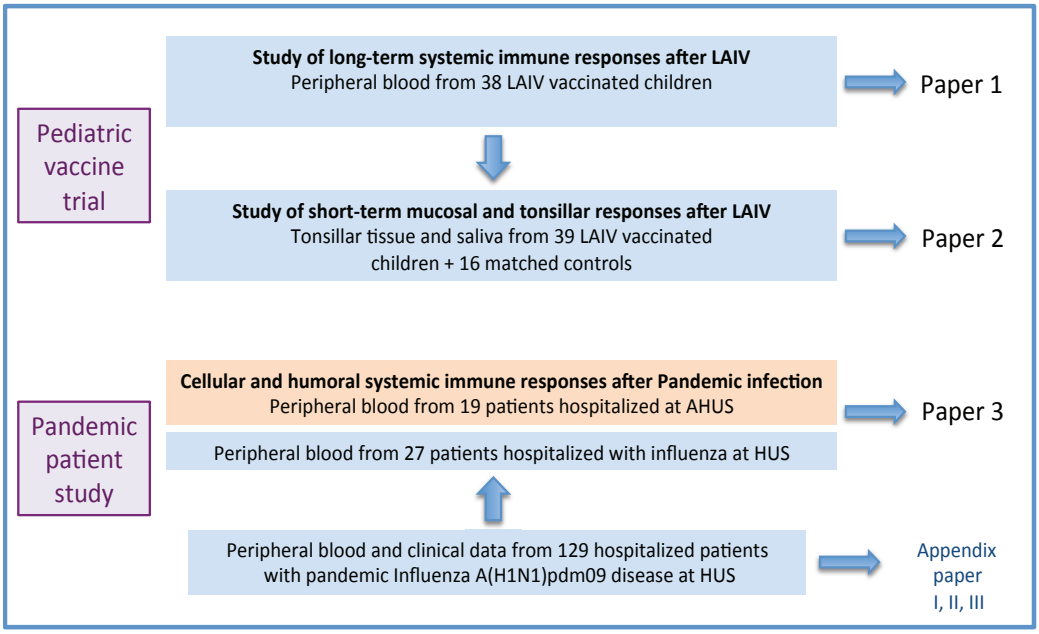


Figure 7. Overview of the 2 studies conducted during this thesis

Pediatric vaccine trial, the subjects and the recruitment (papers I and II)

Papers I and II are based on immunological and clinical findings in a pediatric LAIV vaccine trial during the influenza season 2012-13. This trial was conducted in collaboration with the Pediatric clinical trials unit and the Ear Nose and Throat (ENT) department at Haukeland University hospital (HUH). The children were recruited from the operation list for elective tonsillectomy at the ENT outpatient clinic. The

recruiting procedure involved several steps of information. Firstly by phoning the parents in advance, followed by a formal information letter by mail. If the parents lived separately, they were individually informed. Lastly, after the ENT doctor confirmed the indication for tonsillectomy, recruitment and further information was given in person at the hospital. If only one parent had signed the document, email acceptance from the other parent, was considered sufficient for consenting. Both parents and children > 12 years provided voluntary, written informed consent before enrollment in the study.

Data was collected on baseline demographics, medical and influenza vaccination history, risk factors for influenza infection as well as temperature, height, weight, and relevant vaccination and medical history. The children were randomly assigned into subgroups with vaccination 3, 7, or 14 days prior to tonsillectomy based on the date of operation provided by the surgeon. A suitable time for blood and saliva sampling and subsequent vaccination was scheduled at the pediatric trial unit according to the parent´s preference. The second dose of vaccine, and subsequent visits with blood and saliva sampling, were all conducted at the pediatric trial unit. On the scheduled day of tonsillectomy saliva was sampled prior to the operation. Blood sampling was limited in volume (8-30 ml) and conducted while the child was under anesthesia, and the tonsils were collected and brought to the laboratory immediately upon removal. The inclusion and exclusion criteria used for the trial were:

Inclusion criteria: Otherwise healthy children 3-18 years old with a signed, inform consent letter by parents and children > 12 years, no fever or ILI symptoms the last 7 days, stable mild to moderate asthma (with daily use of inhaler) and non-pregnant (pregnancy test conducted if indicated).

Exclusion criteria: Children with serious, chronic medical conditions, serious or unstable asthma, recent influenza or fever, pregnancy, use of acetyl salicylic acid (ASA), immunosuppressive therapy, allergy to the vaccine or it´s components and children under governmental custody.

The LAIV vaccine

The vaccine used was Fluenz® (Astra Zeneca) $10^{7.0 \pm 0.5}$ fluorescent focus units (FFU) of live attenuated influenza virus of each of the following three strains, A/California/7/2009 (H1N1)pdm09-like strain, A/Victoria/361/2011 (H3N2)-like strain and B/Wisconsin/1/2010 strain. The vaccine was administered intranasally as 0.1ml per nostril. At the time of the study, the vaccine was not licensed in Norway. We imported the vaccine from Finland and the UK for trial purposes. Children 3-9 years old received 2 doses of vaccine at a 4-week interval, and children ≥10 years old received a single dose of vaccine as recommended by the manufacturer. The children were immunized during the influenza season from October-January 2012-13.

Pediatric vaccine trial - study design and samples

The study design used in **papers I and II,** is illustrated below (Figure 8). Blood (PBMCs and plasma), saliva and tonsils were collected from the recruited children. Samples were collected pre-vaccination (day 0) and on the day of tonsillectomy (Days 3, 7 or 14) in addition to days 28, 56, 180 and 360 post-vaccination.

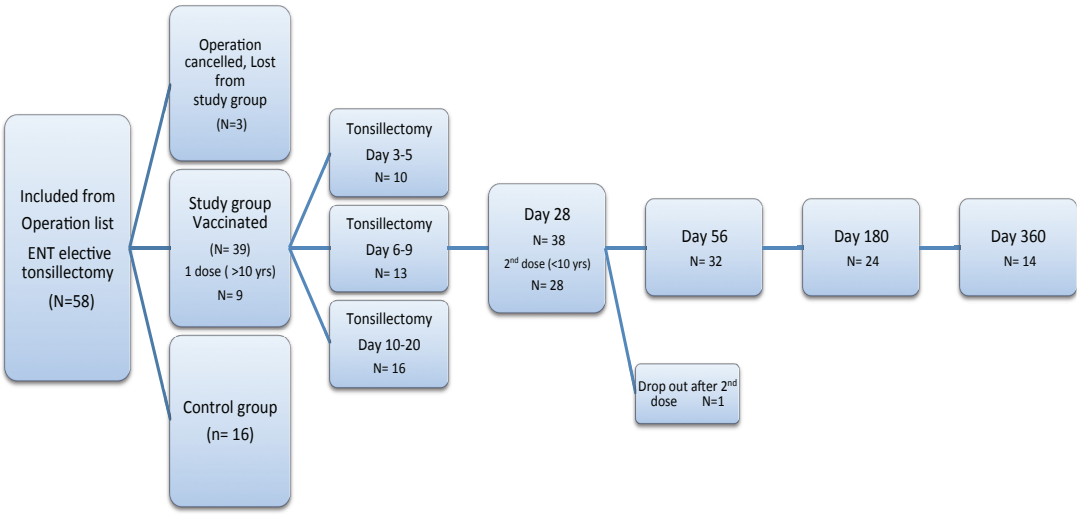


Figure 8. Overview of Study design in papers I and II: The children were recruited from the ENT list of operations for elective tonsillectomy. Upon enrollment, the children were vaccinated with LAIV at 3 (range 3-5), 7 (range 6-9) or 14 (range 10-20) days prior to the date set for tonsillectomy. The children were followed up with consecutive sampling of blood and saliva up to 360 days. Blood and saliva were collected at all time points.

Blood sampling was limited since the children were small and blood loss during the procedure was expected. Prior to the blood sampling, local anaesthetic cream was applied to the skin to ease discomfort. Children received prizes after sampling; however, parents received no monetary compensation for participation in the trial, only reimbursement of transport or parking costs.

Soliciting of Side-reactions

Side-reactions were solicited by collecting a self-reported questionnaire of local and systemic side-reactions after each dose of vaccine during follow-up visits. The parents were also asked about side-reactions or if the child had symptoms of influenza like illness (ILI) when they returned for sampling at the pediatric trial unit. Parents were also provided with the phone number to the medical staff if they had any questions regarding symptoms or side-reactions during the trial. An overview of the study design can be found in Figure 8. The following local and systemic side effects were solicited:

Local: Congested or runny nose and airway symptoms

Systemic: Fever >38°C, lethargy, reduced appetite, headache and myalgia

The solicited side effects were recorded for 7 days post-vaccination and were graded 0-3. (0= no reaction, 1= mild reaction, which did not affect daily life, 2= mild/moderate reaction affecting daily life and 3= reaction which required doctors consultation). The results are illustrated in Figure 2 in the supplementary information page100.

Pandemic patient study - study design and samples (paper III)

In **paper III** 46 patients were recruited from Haukeland University Hospital (HUH, Bergen) (n=27) and Akershus University Hospital (AHUS, Oslo) (n= 19). The immune responses in the acute and convalescent phase of pandemic disease were studied and the patients were divided according to disease severity based on the clinical findings in appendix paper III [180].

- mild (no hospitalization) (n =6)
- moderate (hospitalization ≤ 2 days) (n = 20)
- severe illness (hospitalization > 2 days (n= 20)

These 46 pandemic patients formed the basis for **paper III** where the study design is illustrated in Figure 1 [181]**.** Blood samples (serum and PBMC) were collected from a subgroup (n= 27) of patients hospitalized for >24 hours at HUH in the acute phase of pandemic influenza illness as described in appendix paper III[182]. All patients met the clinical case definition for A(H1N1)pdm09 disease (modified CDC case definition), as previously described [180]. Nasopharyngeal swabs were collected from patients at inclusion for confirmatory viral diagnosis. For comparison 19 patients with ILI symptoms (hospitalized and out-patients from AHUS), were recruited in the convalescent phase.

Laboratory assays

For the pediatric vaccine trial and the pandemic patient study, the sampling methods and laboratory assays conducted to analyze immunological responses were identical. However, some assays conducted consecutively on fresh samples were only run during the pediatric vaccine trial. These assays were performed with the help of colleagues at the Influenza Centre. The different laboratory assays focus on the adaptive immune responses elicited by the vaccine. The assays measure immune response in the humoral or cellular immune compartment as illustrated in the figure below.

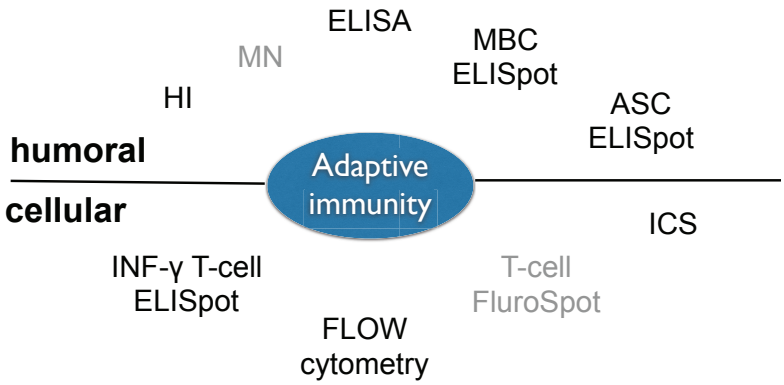


Figure. 9 Overview of the different laboratory assays and the immune responses they measure in the humoral and cellular parts of the adaptive immune system. T-cell FluroSpot (Fluorescent ELISpot measuring cytokines secreted by T-cells (IFN-γ and IL-2)), and Micro Neutralization (MN) assays were conducted but are not part of this thesis.

Sample handling; Blood, PBMC and Plasma (papers I, II and III)

Fresh PBMCs were separated immediately by density gradient centrifugation using Cell Preparation Tubes (CPT, BD, USA)[183], according to the manufacturer's instructions and went directly into assays for the pediatric vaccine trail and the pandemic patient study. Cells were stored in liquid Nitrogen, and plasma samples were aliquoted and stored at -80^{o}C before use in the HI assay.

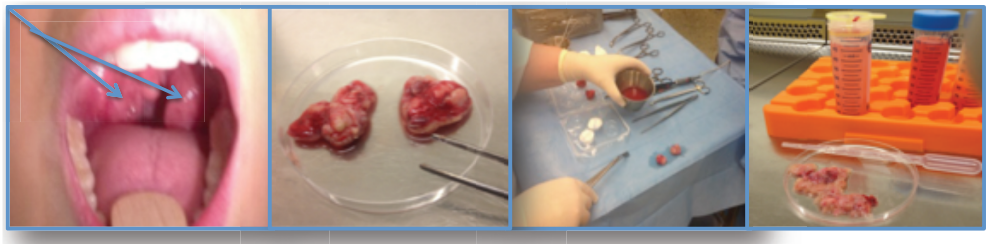
Saliva (paper II)

In the pediatric vaccine trial, the saliva samples were collected using an absorbing swab (Salimetrics®, USA) at each time point prior to blood sampling. The swab was held against the lower buccal mucosa for two minutes, until moist, placed in a tube and kept on ice until centrifuged before storage at -20°C.

Tonsils (paper II)

Immediately following tonsillectomy, whole tonsils were collected (in 0.9% NaCl) to isolate the tonsillar mononuclear cells (TMCs) using Lymphoprep (Stemcell tech. UK). The tonsils were manually disrupted in a petri dish using forceps and a scalpel, and filtered before the lymphocytes were separated by density gradation

centrifugation. Lymphocytes were used fresh in T-cell, ASC and MBC ELISpot assays [76]. The pictures below illustrate tonsil hypertrophy and tonsil handling.



Supplementary Figure 2. Pictures illustrating tonsils From left: Visibly enlarged tonsils bilaterally in a patient prior to operation, and during collection in the operating theater.
Photo: Kristin G-I Mohn with permission

Hemagglutination inhibition (HI) (papers I, II, III)

Plasma samples from each individual were tested at the same time, in duplicate, in the HI assay using 8 Hemagglutination units of the homologous virus (H1N1, H3N2 and B vaccine strains) and on A(H1N1)pdm09 for patient study as described earlier [156]. HI titres were defined as the reciprocal of the highest serum dilution inhibiting hemagglutination, the lowest detectable titer was 10, and negative titres were assigned a value of 5 for calculation purposes. The assay is based on the ability of antibodies to bind to the virus, and inhibit agglutination between the viral hemagglutinin and red blood cells (RBCs). An illustration of the HI assay from a single well is shown below. A larger picture of a plate from the HI assay run during the study is shown in supplementary information.

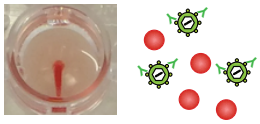
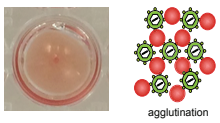


Figure 10: Antibodies present, inhibiting agglutination



Antibodies not present, agglutination of RBCs

The HI is the commonly used immunological assay to measure influenza specific antibodies elicited by infection or vaccination. The assay is not MHC specific and an

HI titer of ≥ 40 is defined as a correlate of protection, with a 50% reduction in the risk of contracting influenza infection [63].

ELISA (papers II and III)

The enzyme linked immunosorbent assay (ELISA) is an immunological assay based on antigen-antibody interactions. This method uses a detector antibody conjugated to an enzyme and a colorimetric substrate to detect and quantify the presence of antibody in a body fluid, most commonly serum or saliva.

We conducted the ELISA to measure the influenza specific IgA in saliva after LAIV vaccination in **paper II** and in serum in **paper III** as previously described [184]. The ninety-six-well ELISA plates (maxisorb, Nunc) were coated with 2μg/ml HA of each split influenza virus antigen (H1N1, H3N2 or B strains), or anti-human IgA and allowed to bind overnight at 4˚C. After blocking, the plates were subsequently incubated with the patient samples, allowing the specific antibodies to bind to the coated influenza antigens. A secondary IgA capture antibody (Biotin-conjugated goat anti-human IgA) was used to detect the antibodies. Purified, human IgA was used to create a standard curve. The absorbance values are measured by using an automated ELISA plate reader with the Ascent software Version 2.6. An absorbance value between 0.5- 2.5 was considered positive in relative to the standard curve obtained. A schematic illustration of the indirect ELISA method is shown in the supplementary information.

B cell Enzyme linked immunosorbent assay (ELISpot) (paper II)

Antibody secreting cell (ASC) response by ELISpot

The ELISpot is a recognized immunological method to quantify immunological cells secreting antibody (B cells) or cytokines (T-cells). An antibody secreting cell (ASC) ELISpot quantifies the number of cells spontaneously secreting antibody. The influenza specific IgG, IgA and IgM ASC response after vaccination was determined by ELISpot using fresh PBMC according to Cox et al. [185]. ELISpot plates were coated with influenza split virus antigens (H1N1, H3N2 or B) for tonsils or a mixture of the

three viruses for blood. The patient´s PBMCs or TMCs were added and the antibodies secreted by the ASCs bound to the antigen. The imprint of each ASC was developed using an insoluble substrate. An illustration of the ELISpot assay is shown in supplementary information page102. The plates were read using the Immunoscan™ reader. The negative control (uncoated well) was subtracted from the influenza specific response. The results were presented as ASCs or MBCs per million PBMCs/TMCs.

Memory B-cell (MBC) response by ELISpot

The antigen-specific IgG, IgA and IgM memory B cell (MBC) responses after vaccination were quantified by ELISpot as originally described by Crotty et al. [186,187]. Fresh PBMCs were plated in 24-well plates and supplemented with an optimised mix of polyclonal mitogens and cultured for 6 days at 37°C in 5% CO_2 as earlier described [187]. The negative control were PBMCs incubated in medium alone. The polyclonal stimulation of PBMCs, allowed MBCs to develop into ASCs, which can be detected by the ELISpot assay. At day 5 ELISpot plates were coated with virus antigen or PBS alone (negative control) and incubated overnight at 4^{o}C. The cells were blocked, plated in duplicates in 2-fold dilutions onto ELISpot plates and incubated for 16 hours at 37^{o}C, in 5% CO_2. A conjugated goat anti-human IgG, IgM and IgA (Southern Biotech) was used as detection antibody. Results are presented as virus-specific IgG MBC per 1×10^6 PBMC. Influenza specific Ig secreting cells per million PBMCs were presented as percentage of total IgG, IgA and IgM respectively, as earlier described [187]. A figure illustrating the ELISpot assay is added in the supplementary information.

T-cell ELISpot (papers I and III)

T-cells produce IFN-γ, which forms the basis for the detection of T-cells by the ELISpot assay. The ELISpot detects specific T-cells secreting IFN-γ at the single cell level, and we used an available commercial kit (Mabtech AB, Sweden). Pre-coated anti- IFN-γ plates (96 well) were used according to the manufacturer´s instructions. PBMCs were stimulated with influenza antigens (split virus vaccine of each strain;

(H1N1, H3N2 or B), specific peptides for $CD4^+$ or $CD8^+$ T-cells or medium alone (negative control) as described in **papers II and III**. Plates were incubated overnight at 37°C in 5% CO_2 and developed the following day. A picture from the reading of IFN-γ for T-cell quantification is illustrated below.

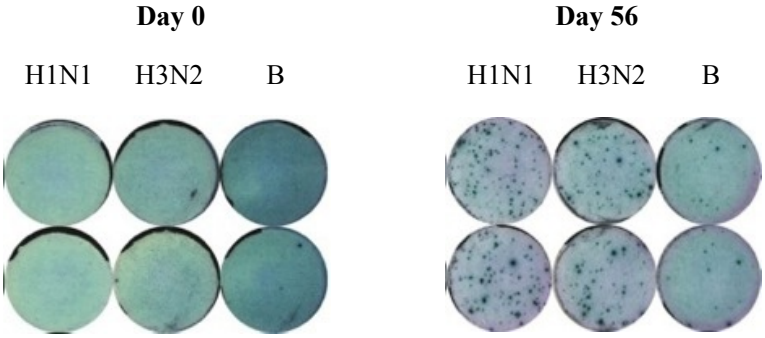


Figure 11. Photos from the Elispot assay. Each spot represents an influenza specific, IFN-γ producing T-cell. Day 0, there are almost no spots, (child is naïve to these strains). The increase in number of spots specific for each of the three virus strains (H1N1, H3N2, B) is visible after vaccination with 2 doses of LAIV (day 56). The picture was kindly provided by Dr Geir Bredholt.

Intracellular cytokine staining (ICS) of $CD4^+$ T-cells (papers I and III)

To measure the T-cell specific responses, PBMCs were stimulated with a premixture of the three split virus antigens in the vaccine (H1N1, H3N2 and B) for LAIV vaccinated children or A(H1N1)pdm09 for infected adults from the Pandemic patient study. After overnight stimulation the cells were permablized and stained for CD3, CD4 and the influenza specific Th1 cytokines (IFN-γ, IL-2 and TNF-α) and analysed by a BD LSRFortessa flow cytometer (acquiring $\geq 3x10^5$cells) for the expression of IFN-γ, IL-2 and TNF-α as described earlier[188]. Medium alone was used to detect the basal cytokine production in non-stimulated cells. The gating strategy is illustrated in supplementary figure 2 in **paper II** and in the supplementary information.

Statistics

Statistical analyses were performed in GraphPad Prism version 6 for Mac and Prism 6 for windows (GraphPad Software, USA) and SPSS version 17 (for paper I only). Comparison of intracellular cytokine (ICS) production was done, using student´s t-test. Other results were tested using Non-parametric analysis of variance (ANOVA)

and adjusted when multiple comparisons were made (Mann-Whitney test, Kruskal-Wallis or Wilcoxon test (paired group). Whenever possible, paired tests were used. Correlation analysis was performed using Spearman correlation. For all statistical tests a p-value <0.05 was considered significant.

Methodological considerations

Collaboration with ENT and pediatric clinical trial unit

We chose to conduct the pediatric vaccine trial for several reasons. Firstly, the license for LAIV was first obtained for Europe in 2012, hence there existed limited European research data concerning this vaccine. The LAIV had been used for a decade in the US, and we chose to vaccinate the children scheduled for elective tonsillectomy because this was a mean of obtaining valuable tonsil tissue. Our research group had also earlier conducted similar studies in collaboration with the ENT department at HUS using the IIV; hence we knew the trial would be feasible due to the proximity of the laboratory and the clinic. In addition, these pediatric patients are often ill with upper respiratory tract infections, (URTIs) and would benefit from the vaccine during the influenza season. The LAIV was proven safe and efficacious, hence with minimal risk to our child trial subjects. However, influenza vaccination is only recommended for high-risk individuals in Norway, hence the LAIV would not normally been offered to these children.

Importantly we chose to follow up for one year since earlier efficacy studies focused on the immunological effects of LAIV after one or two doses. Few studies focused on the long-term immune responses[62 189]. The earlier studies found early immunological responses 7 days post vaccination, and formed the basis for the study design. We wished to study an earlier and a later time point in addition to the standard 28 and 56 days post vaccination. Lastly, there were no studies conducted post-pandemic or with tissue (tonsil) samples from children post LAIV vaccination, hence novelty at a global level was possible. The limitations were the clinical and laboratory capacities to simultaneously recruit, collect, document and run the immunological assays with limited staff available. A large degree of flexibility and dedication was essential.

Since this was a pediatric intervention study, we wished to collaborate with the pediatric clinical trial unit. The trial was monitored and conducted according to Good clinical practice (GCP). The staff was of invaluable help, both in the logistics as well as follow up vaccination and sampling.

Generalizability and representativeness

The pediatric study population is considered fairly representative, both in gender and age for the patients who undergo elective tonsillectomy. However, we focused on the majority of children operated as day patients, i.e. able to be dismissed the same day, which were the vast majority. We did not include high-risk children to minimize trial complexity and risk. These estimated 10 children had serious comorbidities, requiring a higher level of care, and were operated in the main hospital theaters. Excluding these children may have affected the generalizability. However, they would perhaps have had contraindications for receiving the vaccine, and therefore not possible to recruit.

The children were largely Norwegian, Caucasian with chronic tonsillitis or tonsillar hypertrophy, thus care must be taken when extrapolating these results to a completely healthy child population or to individuals with other ethnic backgrounds. In the pandemic patient study, recruiting was from hospitalized patients in Bergen and Oslo, and the sampling was random, decided and limited by staff capacity alone.

Selection bias

The term ”selection bias” refers to a systematic flaw in study design for the selection of patients, which would influence the generalizability of the results obtained. A possible selection bias is that all subjects required tonsillectomy. Although healthy and free of infection when vaccinated and operated, there is a possibility that this influenced our results. However, we included a non-vaccinated control group for comparison of tonsil results. This is further discussed under the topic of controls.

Recruiting was limited to a single site hospital, and to the operation list for elective tonsillectomy. This could cause a selection bias. However, we recruited from the entire list of children set up for elective tonsillectomy and the majority consented (approximately 65-70% acceptance rate), reducing selection. In addition, most of the parents who chose not to participate, allowed their children to be in the control group. The parents consented to have their child immunized with an influenza vaccine, which is not routinely recommended for healthy children in Norway. However, we do

not consider this to affect the results. The main reason not to participate was difficulty for parents to take the required time off from work. A minority voiced their skepticism towards vaccines in general. The children were then randomized into the subgroups based on the date of scheduled tonsillectomy.

For the pandemic patient study, the selection was dependent on the doctors' limited time and hospitals resources in general. Our hospital is a tertiary, public teaching hospital as well as local hospital. Since Norway does not have private hospitals for acute illnesses, all ethnic and socioeconomic groups are admitted to our hospital, and the population is not diversified prior to admission. The vast majority of patients asked to participate consented, as all that was required was medical information and an extra blood sample. The high turnover of patients was the main factor limiting the number of patients included with a signed informed consent letter. We believe the patients included represent the Norwegian population hospitalized during the pandemic.

Statistical methods

Traditional statistical methods have been applied in this thesis using Graphpad Prism 6.0 software for Mac OS X. Since results from biological samples tend to be shifted to the right, and do not follow Gaussian distribution, we have used non-parametric tests, with the ICS results being an exception as mentioned. In our analysis of immune responses over time, we corrected for multiple testing using Bonferroni, where the obtained p-value was multiplied by the number of tests conducted. When using the Kruskal-Wallis test, Dunn´s correction was used. In general, the low numbers and lack of statistical power limit our trial, making conclusions difficult. In addition, observational studies are considered hypothesis generating, and not confirmative, which poses a limitation to our studies.

Subgroup analyses

Subgroup analysis is considered hypothesis generating only, due to the high risk of unexpected findings. This is especially true for observational studies. Our subgroup analysis of the immune responses after pandemic infection in **paper III,** must

therefore be interpreted with caution. Although largely descriptive, the findings are of value due to the difficulty in obtaining biological samples from infected patients during the pandemic. The results may therefore provide the basis for more hypothesis driven studies in the future.

Ethical considerations and legal aspects

For the pediatric vaccine trial, the recruitment procedure, sample and data handling were performed according to Good clinical practice (GCP), the Declaration of Helsinki, Norwegian law on health research and approved by The Regional Ethical Committee of Western-Norway and the Norwegian Medicines Agency in October 2012 (www.clinicaltrials.gov, NCT01866540 and EUDRACT-number 2012-002848-24). The trial was conducted during the two subsequent influenza seasons 2012-13 and 2013-14, with a follow up of one year for each cohort. All patients and children older than 12 years old, included in the current thesis took part voluntarily, without monetary compensation. All patients were personally informed about the study by a colleague or myself and provided signed informed consent. The recruiting doctor was not responsible for the child´s treatment at the ENT clinic, hence removing any feeling of pressure to participate.

In view of the valuable long-term results, we regretted not asking for ethical permission to follow up the children longer than one year. Future applications should include a possibility of a trial extension.

The H1N1 Pandemic patient study **(paper III** + appendix papers I, II, III**)** received ethical approval from the Regional Ethical Committee of South-East and Western-Norway, in September 2009 (REC number 2009/1853 and 2009/2295). In the patient study, the recruiting doctor was part of the team of doctors treating the patients, and although precautions were taken, it is not possible to exclude a patient´s possible feeling of the need to participate. However, participating did not involve any additional procedures or treatment for these patients. The extra blood samples for study purposes were drawn at the same time as the regular samples in the majority of cases. The study was a collaboration between our hospital and the Norwegian

institute of Public Health and Akershus University hospital and illustrates the importance of combining data sets in difficult or rare clinical situations.

Confounding

Confounding by indication is the most important confounding factor in observational clinical studies, which is avoided in RCTs due to the randomization. It is therefore not possible to conclude on associations observed during observational studies. In the pandemic patient study, our hospital is small enough to have been able to include all hospitalized patients from the local population. However this was not possible due to the limited research resources. The patients were therefore included when capacity permitted, without selection. Colleagues performing the assays were not blinded to the severity of influenza illness of the hospitalized adult or to the child being vaccinated or a control. Blinding was not prioritized due to the limited resources, but would have been optimal, although perhaps less important since this was not efficacy trial.

As previously discussed under a possible selection bias, there is a possibility of an unknown influence of the background of chronic tonsillitis or hypertrophy on the immunological responses. We have not subdivided the findings based on the indication for tonsillectomy but chose to analyze by number of days post-vaccination. Later we have also divided by age. When conducting the immunological assays, we were blinded to the knowledge of reason for the procedure.

Recruiting process

The recruiting procedure for the pediatric vaccine trial was time consuming, to meet the parent´s need for information. The time spent was considered necessary, particularly in view of the general scepticism towards influenza vaccination in Norway. We believe the high percentage of parents enrolling their children in the study relied heavily on the information being given in person by the responsible doctor. There were several obstacles to recruitment, including cancellation of operation and children who were ill (naturally higher numbers in the winter season) and therefore not able to join the study or no longer requiring tonsillectomy. These

logistical challenges required tremendous flexibility from participating staff. In our clinical trial protocol, we aimed for group sizes of 10 children at each time point after vaccination for tonsillectomy. The vaccinated children were randomly assigned to one of three subgroups vaccinated 3, or 7 or 14 days prior to tonsillectomy. This allowed the comparison of the early immune response in the tonsils to the controls and between time points, as we could not collect repeated blood samples within such a short space of time to the operation. For safety reasons, Day 3 inclusion was the earliest time point accepted by the anesthesiologist due to the risk of side-reactions to the vaccine either delaying tonsillectomy or complicating interpretation of possible adverse events after the surgical procedure of tonsillectomy. The side effects of vaccination usually manifested themselves by day three. Approximately 5 children had their procedure postponed due to fever on the operation day. It was often not clear if the fever was vaccine induced or the debut of a cold, often occurring during the winter months. Due to the complexity of this pediatric clinical trial, we chose to expand the groups of 7 and 14 days post vaccination. Additionally, if the operation time for “a day 3 child” was cancelled, we attempted to reschedule to day 7 or 14 post vaccination.

Missing samples at certain time points occurred when there was an insufficient blood volume. Assays were then prioritized resulting in missing time points for some assays. The blood samples were taken while under anesthesia, however, this proved to be challenging on several occasions. Anesthetic cream was used to ease discomfort on the follow-up visits, and although special pediatric needles were used, dry taps occurred. For successful tonsil sampling, many requirements were essential. The child had to be infection free, vaccinated without side effects, fasting from the day before and calm in order for pre operative saliva sampling. Dry mouth and an anxious child resulted in missing saliva samples.

The first season, we managed to recruit 55 children of which 24 of the 39 vaccinated children were followed up for one year but only 14 provided sufficient samples for all time points. The number of dropouts became an issue when we realized the importance of the long-term kinetics of the immunological responses.

Prior to the second year of clinical trial, changes were made in the verbal information upon inclusion, and in addition, we expanded and included adults for later comparison of immune responses as well as study the influence of ageing. The importance of obtaining the last sample 12 months later was emphasized. This emphasis and management of expectations upon inclusion reduced the number of dropouts the second year, proving the importance of correct and detailed information from the start. The second year of trial (2013-14), a total of 55 children and 44 adults were included, following the same protocol as the first year and 34 children provided samples at 12 months. These studies are ongoing and are not part of this thesis.

The use of controls

The study design involved recruitment of children into either a vaccination or control group based on the wishes of both parents and the child if over 12 years old. As both tonsils were removed at tonsillectomy, they could only be collected at a single time point. Therefore, 16 matched non-vaccinated control children were included to provide a background (non-vaccination level) immune response in the tonsils. Hence, the control group was included as a comparator for tonsillar B cellular responses from the vaccinated children. The control children had samples collected while they were in hospital for the tonsillectomy procedure. They were recruited from the same patient population and in parallel to the study subjects, throughout the influenza season during the winter of 2012-13. We made an effort to match controls in age and gender within the constraints of the pediatric population requiring tonsillectomy. We found similar IgA antibody titers (in serum and saliva) in controls and pre-vaccination (day 0) samples from the vaccinated children, justifying their use as a control group. The controls have not been used in subsequent analysis of later time points post-vaccination since there was no need. Concerning the vaccinees, the repeated follow up samples ensured that they represented their own controls; comparing day 0 (pre) to the subsequent post-vaccination time points.

Saliva sampling

We chose to sample saliva for local antibody testing instead of the more commonly used nasal washing, due to the ease of the sampling method, as opposed to the discomfort of a nasal wash, in an attempt to have as many children (and parents) as possible return happily. The use of the Salimetrics® swab for saliva sampling the first year, proved challenging to get to the laboratory quickly while on ice. For the second season (2013-14) the method was changed to using a different swab (OraSure®, USA). This method was logistically easier, did not require ice, and the swab and tube were pre-manufactured, smaller and easier to use, and accepted by even the three year olds. Change of sampling method is not optimal during a study, and hence results based on saliva samples will not be comparable between the two years.

Pandemic patient study, Sampling and recruiting process

The main challenge in running the patient study was conducting it during the heat of the pandemic when all resources in the clinic and the laboratory were stretched to a maximum. The importance of a good clinical team became evident in order to inform and recruit eligible patients. Although there were meetings to plan the study, the key to success was the determination and ownership as well as idealism from involved partners to pull the study through to results. The lack of good research during outbreaks and patient material for research purposes has been emphasized at conferences by heads of public health, the WHO and science institutions. There is a large room for improvement.

Correlate of protection (COP)

HI is the only accepted COP for influenza, and the agreed level of antibody titer considered to provide protection is 40 in healthy adults. This is probably not valid for child antibody responses. In addition, HI is considered to underestimate the protection achieved by LAIV and to date a robust COP has not been established for LAIV. Hence, there is an urgent need for a new COP for evaluating the immunogenicity induced by LAIV in children. While conducting our LAIV trial we

have used several different methods to estimate antibodies elicited by the LAIV vaccine. However, the methods measure different antibodies specific for different antigens and are therefore not comparable. An even larger hurdle in antibody comparisons is the great inter-laboratory differences in protocols used. The WHO has aided in establishing expert groups committed to help standardize the protocols used for influenza immune response analyses to reduce the variability.

Measuring protection after LAIV

During the trial, we collected information on self-reported ILI symptoms at each visit, which none reported. All parents reported that their children were of much better health that winter, after the tonsillectomy and vaccination. We are not able to define which intervention provided them with better-perceived health, however in these children, the tonsillectomy probably played the major role. However, had these children been infected with influenza as well, they were at risk of experiencing a great deal of morbidity due to their age and being prone to infections.

However, we did not measure protection after vaccination by swabbing the children and testing for influenza. This was a financial decision since the trial was investigator funded. In hindsight our trial and later publications would have benefited from proving that the children were protected from PCR positive infection, although our sample set was too small to consider efficacy, and none reported ILI symptoms.

Laboratory assays

For all assays conducted, standardized laboratory assays were used and an optimized standard operation protocol (SOP) followed. Samples were given a study number. In general, if enough sample material was available, we detected virus specific responses (H1N1, H3N2 and B). We had sufficient amounts of plasma samples and tonsillar cells for strain specific assays to be conducted. However, there were severe limitations on the number of PBMCs separated from blood samples, hence the assays using PBMCs were strictly prioritized and conducted using a mixture of the viral antigens. The results when using the mixed viral antigens therefore represent the

immune responses to the vaccine in general and are not strain-specific and some time points are missing.

Enzyme linked immunosorbant assay (ELISA)

The ELISA assay results must be interpreted based on the chosen antigen. For the salivary IgA, we detected specific antibodies to each strain (H1N1, H3N2 or B) and the antibodies measured are those that react to the split antigen, predominantly antibodies binding to the HA and NA. To further dissect the response, purified protein of HA could have been used to differentiate the NA and HA specific responses. For serum IgG, inactivated pandemic specific virus was used as antigen.

Hemagglutination inhibition assay (HI)

HI antibodies bind primarily to the globular head region of the HA, close to the receptor binding site, and other antibodies with potential protective effect (antibodies to the NA, stem of HA or to the M2 protein) will not be measured. The measurement of serum HI is not fully suitable, for determining protection for the elderly and the young, and for certain vaccines such as LAIV. Although not optimal, the HI has been used for decades, and was regarded as the best available parameter for measuring vaccine immunogenicity. However, revised EMA guidelines for seasonal and pandemic influenza vaccines have omitted the earlier HI requirement due to the uncertainty and focus on demonstration of efficacy only for LAIV[190].

In primed children, HI responses are not boosted after vaccination, and therefore HI is not a suitable COP for LAIV. A suitable serum control is important to minimize the variations in HI responses and to differentiate non-specific binding from virus specific binding. As mentioned earlier, there is an urgent need for the defining a suitable COP for measuring the elicited immune responses after LAIV.

ELISpot

The ELISpot method enables quantification of cells secreting antibody and requires optimization. The optimal setting includes a concentration of antigen, which gives the lowest background but highest number of spots. However, prior to our vaccine trial,

the assay had mostly been used for adult samples. The method had been optimized during earlier trials run by the Influenza Centre, and the method was optimized for this study based on control children. Due to the limited number of PBMCs from each child, the MBC and T-cell ELISpots were run with a mixture of the viral antigens, hence they represent a vaccine response.

The last literature search was performed April 22nd 2016.

Results and discussion

Immunological responses to vaccination and infection.

Knowledge concerning the human immunological response to pandemic influenza disease is limited, and the literature on early mucosal and long-term immunological responses after seasonal LAIV vaccination in children is scarce. Both children and infected, hospitalized patients are difficult to study due to their age, the costs and the nature of pandemic influenza outbreaks. This doctoral thesis is based on researcher initiated clinical studies in these two patient populations. The studies and results in the **papers I-III** will be discussed below and cover issues related to influenza immunology, vaccination, virology, public health concerns as well as ethical considerations.

The longevity of the LAIV induced systemic immune response

Results from our study indicate that LAIV elicits both early and durable immune responses in children in both the humoral and cellular immune compartments. In **paper I**, we detected a significant and long-term increase in strain-specific HI, systemic antibody titers after vaccination, lasting up to one year. In addition, we found significantly increased numbers of IgM MBCs as well as T-cellular responses, which were maintained for up 6 months in most children, and up to one year in some children. Induction of long-term immunological memory is the goal of all vaccination and therefore an important finding. Our results show that the antibody levels were significantly increased in naïve children and were higher in children with pre-existing antibodies. This indicates that LAIV induces protection in naïve children and that in primed children, the LAIV perhaps helped maintain the response. Others have found that after IIV, the ASC response increased in naïve subjects, and IIV induced stronger MBCs responses than LAIV [191]. Other studies have shown long-term serum antibody responses (HI) or protection after LAIV, but not MBC or T-cell responses [62,189]. To our knowledge, our study is the first to show the durability of the cellular and

humoral immune response in children beyond the most common time points of 28 and 56 days after vaccination used in vaccine trials.

One of the most striking findings throughout the entire study was the inter-strain variation in immune response and kinetics. The B-strain induced the highest antibody responses both locally (saliva + tonsils) and systemically **(papers I and II)**. The H1N1 strain showed the highest antibody levels, which were maintained, but without further increase. Similar observations with the strongest responses to the B strain in young children have been noted by others [192,193], and the reason for this is not clear. It may be due to lack of pre-existing immune responses to the B strain, but it could equally be due to differences in the infectivity of the LAIV strains. One or two doses of LAIV did not affect the durability of the antibody response, however as mentioned levels were higher in children with pre-vaccination antibodies. Whilst the majority of the study children were exposed to the H1N1 strain through vaccination or infection during the 2009 influenza pandemic, they were largely naïve to the H3N2 and B strains. In contrast, the H3N2 strain circulated to a much lesser degree in Norway during the study period and the B strain had limited circulation [194]. Despite being mostly naïve to the B-strain, the majority of the children reached protective HI titers as early as 14 days post-vaccination **(paper II)**, indicating a rapid induction of protective antibodies. The kinetics of the circulating ASC response was slower after LAIV vaccination compared to IIV, with the highest response observed two weeks post-vaccination as opposed to 7 days for IIV. The extended ASC response after intranasal immunization could be due to longer persistence of the vaccine viruses.

Differences in dosage could not explain the observed inter strain differences, since the three strains are equally represented in the vaccine ($10^{7.5}$ FFU per strain). However, differences in infectivity could provide an explanation. Indeed, the year after the study, in 2014, the US experienced surprisingly low efficacy of the H1N1 strain after LAIV [195,196], which led the USA advisory committee on immunization practices (ACIP) to withdraw it´s earlier preferential recommendation of the LAIV to children [197,198]. The manufacturer stated that the H1N1 strain had a temperature

sensitive mutation rendering it heat instable, possibly explaining the lack of protection observed in the community[198].

In our study, the vaccine was given under strict trial regulations, with the cold chain intact; hence the vaccine was not subject to heat exposure. The problem of viral instability applied to the US and not to Europe. If our H1N1 results were affected, it is less likely that heat exposure alone was the cause. Our study was conducted post-pandemic. We therefore initially speculated that pre-existing antibodies, or T-cells, inhibited viral replication of the H1N1 vaccine strain in the nasal mucosa, eliciting a lower level of humoral response. However, the later results of a defect strain provided an additional possible explanation. At the time of writing this thesis the manufacturer has updated the H1N1 strain used in future LAIV vaccines from 2016, and future efficacy studies will indicate if this resolves the problem.

Kinetics of the local and systemic immune responses after LAIV

The early kinetics of the mucosal immune response was the focus in p**aper II.** We found significant increases in influenza specific salivary IgA after only 14 days, lasting up to 6 months (and one year in 7 children, unpublished results). Others have found increases in mucosal IgA from nasal wash, persisting for up to 6-12 months, however we do not know of others measuring saliva IgA after LAIV in children, although early responses have been found in adults[62,97,138]. Similar to the antibody responses, the highest responses in saliva were directed towards the B strain, which has been observed in two other studies as well [192,193]. The reason for this is not clear, but it could be due to the B virus being better adapted to replicate in humans, since the B strain showed induction of both antibodies and T-cells, while the A viruses mostly induce T-cells and to a lesser degree antibodies. No increase in IgA was observed for the H1N1 strain, consistent with the other immunological parameters we measured.

Our study found that the salivary IgA results correlated with the HI titers for all three vaccine strains. This is an important finding, linking systemic and local antibody

responses, and is supported by a study, which found a correlation between nasal IgA and HI titers [192], perhaps reflecting vaccine immunogenicity.

Mucosal antibodies are vital in protecting the upper airways, the site of viral entry, while serum antibodies generally protect the lower respiratory tract. LAIV elicits secretory IgA, which plays a major role in protection towards influenza infection, being the predominant antibody secreted at the mucosal surfaces [62,189,192,199]. Nasal IgA has been found to be associated with protection from influenza illness in young children (< 3 years old) and with resistance to experimental challenge with influenza A in adults [192,199]. A placebo-controlled, double blind study in children, found that both serum and nasal IgA antibodies correlated with LAIV induced protection, but that nasal IgA was the stronger correlate [200]. Other studies have demonstrated that despite the lack of a robust serum antibody response, the LAIV provides protective immunity [175,201-203]. The induction of mucosal antibodies may hence be the most important effect of LAIV [36]. Boyce et al. suggested that nasal IgA could be the sole indicator of vaccine take in seropositive children, showing that these children were 4.5 times more likely to have a rise in nasal IgA compared to HI [193]. Indeed, a review comparing the immune responses to LAIV and IIV indicated that induction of mucosal antibodies could be a superior indicator of immunogenicity of LAIV compared to serum antibodies [54]. Our findings of long-term salivary IgA responses after LAIV indicate that the mucosal immune response is well developed in young children and that the LAIV may provide local protection in the nasal and oral cavities.

Unlike the HI, there exists no correlate for mucosal antibodies[54] . Mucosal antibodies from nasal wash have not been found a suitable correlate of protection (COP), due to challenges in sampling, assaying of mucosal antibodies as well as great variations in quality and quantity of nasal secretions [54,192,199]. Perhaps a more consistent collection of saliva may overcome these problems.

Since the established HI assay has proven suboptimal for children or for measuring immunogenicity after LAIV, there is a global interest in finding an early predictor of

vaccine immunogenicity. Given the multifaceted response dependent on age, priming status and comorbidities, perhaps a single COP for LAIV will not be feasible, however a partial COP could be of great value. We suggest that the non-invasive sampling of salivary IgA could be used as a new possible indicator of vaccine immunogenicity in children.

Tonsil responses

In **paper II,** the focus was on the short-term systemic and mucosal effects of the LAIV. We found that the LAIV induced early (7-14 days post-vaccination) B-cellular responses (ASC and MBC) in the tonsils, in addition to the earlier discussed systemic antibodies (HI). Our results show that MBC levels increased significantly post-vaccination in blood and tonsils in naïve children, while primed children maintained their high levels for up to one year in blood. MBC levels correlated with systemic antibodies (HI), indicating that responses in the tonsils are reflected in the peripheral blood. This has earlier been observed for ASC numbers after IIV [76]. We found that after tonsillectomy the levels of systemic antibody did not differ in the short or long-term, indicating that tonsillectomy does not compromise antibody production and the possibility of other lymphoid tissue in the area compensate [204] (appendix paper IV).

The tonsils may play a significant role in mediating protection against influenza. As tonsils only could be collected at a single time point, a non-vaccinated, well-matched control group was used to show pre-vaccination tonsillar B-cellular responses. The control group had similar antibody (HI) and saliva IgA titers as the study subjects pre-vaccination, justifying their use as controls. To our knowledge, we were the first to demonstrate tonsillar responses after LAIV. However the uniqueness of our study is the collection of pediatric tissue samples, in addition to blood samples, at the early time points (3-14 days) post-vaccination. The trial study design was based around the distribution of ASC in the peripheral blood, which peak at approximately 7 days post vaccination, but which have been detected as early as 2 days post vaccination [76,205]. We hypothesized that the response to LAIV may have different kinetics in the tonsils than the blood, and chose to sample the children at one earlier (day 3) and one later (day 14) time point than the proposed peak of ASCs, 7 days post vaccination[66]. We

did not find significant increases in tonsils day 3 post-vaccination, but tonsillar responses were dominated by unswitched IgM ASCs. This indicates activation of naïve B-cells and we found that they increased at day 7, prior to the increase in blood. Similarly a study using IIV found a rapid response in both systemic and tonsillar IgA and IgM ASCs in young children[76]. The systemic ASC response after LAIV was prolonged (peaking day 14) compared to after IIV (peaking day 7), perhaps due to the local application and virus replication in the mucosa providing a longer stimulation period versus the injected IIV [76].

The earliest time points were difficult to sample and are limited, hence there is a possibility that early responses are not detected due to small numbers. The side effects presented from day 2, indicating virus replication, and "vaccine take" at this time. An early immune response should therefore perhaps be detectable the following days. Indeed one child did show an increase in HI from day 0 to 3 and several children to day 7 post-vaccination towards the H3N2 and B strains (supplementary figure **paper II**)[206].

One of the questions arising about our study population is if it is possible to regard the population as one, when the tonsils are sampled at different time points. However, we do not believe that the tonsillectomy *per se* would interfere with the immune response to the vaccine. To find support for this, we analyzed the different immune responses based on dividing the groups by day of tonsillectomy and did not find statistically significant differences. We therefore chose to study the group as one. We have however not analyzed the data based on the reason for tonsillectomy, and we could have done this to strengthen our study. The limitations of small numbers and missing samples, due to insufficient sample volume, make it difficult to draw strong conclusions. Although considered important, the tonsils role in eliciting immune responses after antigen delivery by intranasal vaccination is not widely reported. Our trials offer increased understanding of immune responses after intranasal vaccination, and may provide the basis for larger studies.

Correlates of protection (COP) after LAIV

In **paper I** we found that HI increased significantly to the H3N2 strain after one dose. The antibody level was maintained for one year above a titer of 80 with the majority with levels above 160 in the 14 children for whom we had one-year results. In a clinical trial with > 4000 children, Black et al suggested that an HI response of 110 or 330 for H3N2 could be more appropriate for protection in children [207]. There has been global consensus that an HI titer of 40 or more, has been a practical COP and is used in licensing of new vaccines today. However, it is based on adult responses to infection and the cut-off is debated. The HI underestimates the protection obtained by LAIV since LAIV induces other immune responses, which are not detected by HI (e.g. local IgA, T-cell). An additional challenge is the large inter-laboratory differences in running this classical assay, and international collaborative studies are making efforts to standardize the method. Despite ten years on the global market, and in contrast to the IIVs, there exists no COP for LAIV.

In a search of a suitable COP, a major experimental, efficacy trial in the community, was conducted by Forrest et al in 2008 with T-cell responses in > 2000 children vaccinated with LAIV [143]. They suggested a COP of the T-cellular immunity of 100 SFU (spot forming units) per million PBMCs. Unlike the HI titre, IFN-γ ELISpot responses are not yet an established correlate, however they have been used in several studies with different thresholds (range 20-100 SFU/ million PBMCs), and the suggested number of 100 has been considered arbitrary [53,152,208]. In support of this, we found in **paper I,** that LAIV induced IFN-γ secreting T-cells, above the suggested threshold of 100 SFU/million PBMCs. A major drawback for using T-cells as a COP is the number of cells needed, and the limited volume of blood, which can be sampled from children. In addition the need for laboratory processing and running of the ELISpot assays makes T-cell analysis laborious.

The decades since the influenza vaccines were introduced have been dominated by IIVs, which induce systemic antibodies specific towards the major surface proteins of the virus. It is therefore no surprise that the assays developed to measure this antibody response, in particular the HI, have been used to measure protection

achieved by vaccination. Research into detailed adaptive immune responses induced by LAIV is warranted, and will pave the way for new and easily measureable COPs.

T-cell immune responses after LAIV vaccination

In **paper I,** we found that T-cells increased significantly after one dose of LAIV, and were boosted after the second dose [136], and they remained above the proposed protective level of 100 SFU/million PBMCs [143] for six months. This is important, since it indicates a robust immune response. We observed the same inter-strain differences as with the serology results, with the highest T-cell responses to the B strain, followed by the H3N2 and H1N1 strains. Studies comparing the immune response after LAIV and IIV in children and adults, found that only children mounted a T-cellular response after LAIV [60,61]. Other important human studies have shown that $CD4^+$ and $CD8^+$ T-cells are important in limiting disease and may confer hetero subtypic immunity [53,152,208]. The first study was a human challenge study, and the authors related their findings of less influenza symptoms to pre-existing $CD4^+$ T-cells [208], while the second study was conducted during the pandemic and found the presence of $CD8^+$ T-cells to be important and associated with less severe influenza illness in patients [53]. While others have shown that T-cells are induced after LAIV [60,61], we wished to investigate the duration of the T-cell response. The previously mentioned studies used specific peptides to measure the $CD8^+$ T-cell responses. We did not have access to these peptides and used split virus as antigen, which does not differentiate between $CD4^+$ and $CD8^+$ T-cell responses**.** The split antigen represents an inefficient antigen presentation to stimulate $CD8^+$ T-cells. Hence our results are predominantly $CD4^+$ T-cell responses to the external surface glycoproteins. This explains why we did not detect specific $CD8^+$ T-cell responses. This is a limitation to our findings however, we have plans to analyse T-cell subsets using peptides in the future.

Interestingly, we observed that the 5 children, who did not respond serologically to H1N1, had significant increases in virus specific T-cell responses after vaccination. This indicates that although they were non-responders in the humoral compartment, they responded in T-cells, which may provide clinical protection. Our results suggest

that the humoral and cellular immune compartments respond independently. This could perhaps imply that if there is sufficient antibody protection, the cellular compartment does not respond, and vice versa, if there are no protective antibodies, the cellular compartment responds to limit infection. Such a theory could be tested by vaccinating groups of unprimed and primed children and analysing the different humoral and cellular responses.

T-cells are critical for the control of viral infections, and may protect from severe illness or fatal outcome. There are no vaccines today, which are licensed on the basis of limiting severe disease. In the future, a reduction in disease severity may be a valuable approach when developing a "universal" influenza vaccine. Vaccines eliciting T-cell responses have been a major focus in recent years, and there are a number of influenza vaccines in clinical trials aiming at inducing T-cells, recently reviewed by Sridhar [54]. If the LAIV provides partial protection through activated T-cells it could prove valuable by reducing severe illness and buying time before a specific vaccine is available, if facing the threat of a novel virus.

The effect of priming on the subsequent immune response

To demonstrate the effect of priming, we stratified the children according to their pre-vaccination antibody titers (HI). In **paper II,** we found that naïve children mounted a significant increase in B-cell responses (ASC and MBC) after LAIV, while primed children had higher pre-vaccination numbers, which did not boost, indicating a possible threshold level. When we dissected the tonsillar immune response we also found that primed children had higher MBC levels, pre-vaccination which did not boost. Memory B cells responses are rapid, producing more high affinity antibodies than naïve B-cells. The finding that primed children had higher MBC numbers in both blood and tonsils could indicate that a more mature immune response is achieved in these children. This is supported by earlier studies, which show that serum IgG levels correlate with resistance to infection [199]. This could imply that repeated vaccination with a LAIV could provide a broader resistance to influenza.

Proof of an immunological threshold has been found in mice, where the MBCs had a propensity to differentiate to terminally differentiated plasma cells, and not increase in numbers upon re-stimulation [209]. The authors suggested that this phenomenon would avoid accumulation and overexpansion of a specific MBC clone as well as secure a rapid expansion of cells producing antibodies [209]. Several studies support this theory with different vaccines such as MMR and Hepatitis B with findings of persistent levels of MBCs despite declining antibody titers with time [210,211]. Sazaci et al. found that after vaccination (LAIV or IIV), influenza specific IgG MBCs increased during the first 4-5 years of life, and then reached a plateau level, with children 5-9 years old showing similar levels to adults [66]. They hypothesized that circulating MBC levels remain fairly constant over time. Biologically, such a threshold seems logical, the human body strives to achieve homeostasis and waste is avoided. Therefore, it is probably adequate for the immune system of the children in our study, with sufficient MBC present in the circulation, to not increase the level of MBCs to H1N1 after LAIV. Whereas the significant rise in children naïve to H3N2 and B strains, secures a pool of MBCs able to rapidly provide copious amounts of specific antibodies when needed. This is an interesting immunological finding, however it will not have implications on vaccination practices, since children are not vaccinated based on measuring their pre-vaccination priming status.

However age is an important factor for the subsequent immune response after vaccination, and priming is an indirect measure of age, since younger children will have had fewer influenza exposures. The adaptive immune system develops primarily after birth and is linked to exposure to pathogens after the child is born. Memory B-cells and effector T-cells increase with antigenic challenge and reach adult levels at 10-15 years of age [212]. The link between age and a stronger immune response to one influenza strain is commonly regarded as “original antigenic sin”. In influenza research this is the observation that the individual´s first influenza A infection dominates the subsequent antibody responses to influenza. This theory was originally suggested by Dr. Thomas Francis Jr in 1960[213]. He also suggested that new strains could emerge when an immunological gap in antibody protection occurred with “the disappearance of immunological veterans and their replacement by inexperienced

youth"[213]. This suggests that new dominant viral strains occur with intervals of about one generation, and is supported by history with pandemics arising with 20-40 year intervals. He ends by suggesting that the "sin" can be compensated by "the blessing" of a tailored vaccination eliciting specific antibodies which are lacking in the young.

Although individual variation will influence the subsequent immune response, the overall results from our study may be interpreted to reflect the differences in immune responses in primed children (majority previously exposed to H1N1), and a naïve population (majority without prior H3N2 and B strain exposure). These differences in infection history rendered our study population unique, but also make extrapolation of our results to children who have been regularly immunized difficult, since children are not routinely influenza vaccinated in Norway.

Clinical aspects Pediatric vaccine trial patients

In **paper I,** we solicited adverse reactions in all children for 7 days, and experienced few side effects, mostly runny and congested nose, as expected. "No side reactions" was reported in 46% of children after the first dose, and in 90% after the second. Most side effects presented by day 2 and waned by day 3-4 post-vaccination. These findings were in line with earlier studies [214]. Since the vaccine had been used for a decade in the USA, investigating the side effects was not the primary goal of our study. However, our trial was the first to use the LAIV in children in Norway, and we included 6 children with mild to moderate asthma. Although our subjects were younger (mean age 4 years), we did not find increased side reactions or asthma symptoms among asthmatic children, in line with findings in a large study with > 2000 older asthmatic children (6-17 years) [215]. On the contrary, the parents reported their children to be "healthier than ever". This is probably due mostly to the effect of tonsillectomy. The children in our study suffered frequent ear and throat infections, with impaired hearing and delayed speech development and were therefore recommended for tonsillectomy. A study has indeed found a reduction of otitis media after seasonal (IIV) influenza vaccination of children aged 1-3 years, adding scientific proof to the parents' subjective reports [216].

Importantly, an increased rate of medically significant wheezing has been found in children 6-23 months receiving LAIV[142], and LAIV is therefore contraindicated in children younger than two years. Asthmatic children are at risk of severe influenza disease and are recommended for annual vaccination however; studies have found that 75-90% are unvaccinated [215]. Few clinical studies have included children with asthma, since LAIV is not recommended in children with high-risk medical conditions. Therefore, increased knowledge about the effectiveness and safety of vaccines in this risk group is essential.

Humoral immune responses after natural pandemic infection

In **paper III,** we studied the immune response in hospitalized patients with pandemic influenza, and we grouped the patients based on the length of hospital stay. We found differences in the immune responses after pandemic infection in acute and convalescent patients, as well as between patients with severe and mild infection. Firstly, we explored the humoral response and found that 3 weeks post-infection, the convalescent patients recovering from severe disease had significantly higher antibody (HI and IgG) levels compared to the patients with mild disease. The antibody titers declined with time, but remained higher in the severe group and above the protective threshold in 67% of patients 8 months post-infection. This is in agreement with studies that found a correlation between antibody levels and disease severity[217], and that pandemic infection elicited high antibody titers up to 15 months post-infection[218]. The patients in the acute phase of disease mounted high antibody levels, indicating a functioning immune system, perhaps contributing to their survival.

Studies of protective immune responses in naturally infected individuals are difficult to conduct since one cannot predict who will become infected, and baseline responses cannot be collected. This is as opposed to a human challenge model, where baseline and follow-up measurements are conducted. Since two of the convalescent patients were vaccinated, there is a possibility that the antibody responses in these individuals were not induced by infection alone. However, due to the timing of vaccination in Norway, the patients were probably vaccinated and infected almost concomitantly,

hence without the time to achieve a protective antibody response. However, national seroprevalence data indicated very low population levels (2%) of pre-existing antibodies to the H1N1 strain prior to the pandemic [219]. We therefore believe that the antibodies measured were only induced by infection, but we cannot rule out that vaccination may have contributed as a boosting in these two individuals.

We compared immune responses in patients with different disease severities, since we did not have pre-infection serological titers. A study on severe influenza disease, found that high antibody levels, were linked to extended high levels of viral shedding in the lower respiratory tract[220]. However, we did not find increased viral load or mutations of the viruses in our patients. Samples were taken from the nasopharynx upon admission only. Therefore, we cannot rule out that samples from the lower respiratory tract would have shown higher viral loads, or even mutant viruses. We experienced a similar clinical picture to most other western nations[46,221], and the situation and resources needed were well documented by colleagues from the southern hemisphere before the pandemic wave hit Norway [222-225].

Our current clinical guidelines indicate extended NAI treatment in patients with severe influenza disease (in ICU or on a ventilator) due to their prolonged viral shedding. The impact of the NAI treatment on the immune response in our patients was not measured during the pandemic. However, post-pandemic studies have found that those who received NAI treatment had a lower risk of mortality [95,96](appendix paper I, II). The vast majority of our patients (74%) received NAI treatment (appendix paper III) and the early start of NAI treatment may therefore have contributed to both their recovery and limiting of disease severity. The effect of NAI on immune responses in our study is unknown. However, the treatment with NAI started when the patient was admitted, with a median time from symptom onset to admission of 3 days (appendix paper III). With 3 days of virus replication, we assume little influence on antibody levels, supported by Bonduelle et al.[59]. However, a possible effect of reduced antibody titers would depend on early treatment since treatment would reduce viral load and attenuate immune stimulation.

T-cell responses after natural pandemic infection

T-cells are critical for the control of viral infections. The 2009 influenza pandemic posed the influenza scientific community with a unique, "natural experiment". The majority of the population was naïve to the A/H1N1pdm09 influenza strain, and those infected could be sampled while in hospital. When investigating the **cellular responses** in **paper III,** we observed that patients with severe disease had higher $CD4^+$ T-cells (ICS) compared to moderately ill patients. Whilst we only had acute ICS results, a study by Bonduelle et al. found that vaccinated and mildly infected individuals had similar immune memory profiles, which were distinctly different from severely infected individuals one year post-vaccination or post-infected [59]. Comparing immune responses in vaccinated, mildly and severely influenza infected people is interesting since the goal of vaccination is to mimic a mild/asymptomatic infection which provides future protection. In agreement with our observations, they found that the patients with severe infection had higher levels of antibodies and CD4+ T-cells compared to mildly infected or vaccinated individuals. Additionally, they observed that higher levels of CD4+ and CD8+ T-cells with lung homing and cytotoxic potential characterized vaccinated individuals [59]. CD8+ T-cells are essential in the defence against viruses since they kill virus infected cells. We found low levels of $CD8^+$ T-cells in patients in the acute phase, and higher levels in the convalescent group, perhaps representing a recall expansion after infection. Furthermore, Sridhar et al. found that CD8+ T-cells with cytotoxic markers were associated with mild pandemic H1N1 infections[53]. We did not study these markers on CD8+ T-cells in our study, however, interestingly the acute patients (severe and moderately ill) were characterized by low frequencies of $CD8^+$T-cells compared to both patients recovering from mild disease as well as from a healthy population[226]. The findings of Sridhar et al. with high levels of $CD8^+$ T-cells in mild cases [53] may suggest that our patients were severely ill due to low pre-infection $CD8^+$ T-cells, or alternatively this phenotype was depleted during the initial days of infection without managing to limit infection. Another possibility is that $CD8^+$ T-cells have left the circulation and migrated to the site of infection in the lungs. This would explain the low levels found in the PBMCs in the acute phase and higher levels found in the convalescent phase

and in healthy individuals. Migration has also been suggested as a reason for the finding of a rapid increase and subsequent decline in $CD8^+$ T-cells in naturally infected H1N1pdm09 patients by Hillare et al.[227]. If $CD8^+$ T-cells migrate to the lung, perhaps the $CD8^+$ T-cell response elicited in patients with severe disease was important for their survival? We did not find significant differences in $CD8^+$ T-cells frequencies between severe and moderately ill patients, possibly due to low numbers, and unfortunately there were no samples from mild cases for comparison. Our study, although limited by small numbers, may serve as a basis for future studies on the effects of T-cell subsets.

When further dissecting the immune response, we found that the $CD4^+$ T-cells predominantly recognized epitopes from the viral envelope in the acute phase. This is in contrast to our convalescent patients which had similar $CD4^+$/$CD8^+$ T-cell numbers predominantly recognizing conserved epitopes that have been associated with less severe influenza illness[228]. Importantly, the $CD8^+$ T-cells react towards conserved viral epitopes with the possibility of heterosubtypic protection. It will therefore be very interesting when further studying the LAIV response in the children, to elucidate if influenza specific $CD8^+$ T-cell are induced.

The small number of patients is a limitation to our study, however the strength is the uniqueness of the samples, obtained during the pandemic. The intrinsic difficulty in collecting such samples, and the lack of immunological information after the pandemic was highlighted by one of the independent reviewers of paper III. The results from our study are largely descriptive, but the findings might be of value later, as a basis for more hypothesis driven clinical trials during influenza outbreaks. Possible answers to the reason some patients develop severe disease might lie in the T-cells. Although different studies show a large heterogeneity in the immune responses to infection, our findings provide support for the importance of the individual's initial immune response in the clinical outcome of infection. Furthermore, it has been suggested that the immune response to influenza is related to the individual´s quality and magnitude of an immune response before infection[59]. In this sense, both age and the influenza exposure history will be important parameters.

Studying the detailed, natural immune responses occurring after infection in different disease severities as well as in vaccinated individuals is the laborious short-term task. The answers may help to reach the long-term goal, to construct improved influenza vaccines[59] also targeting T-cell responses[53].

Influenza vaccination strategies and vaccine effectivness

In **paper I,** vaccine effectiveness was not measured by laboratory confirmation of influenza, since the LAIV had a proven record of efficacy after decades of use before licensure in Norway, and recently reviewed by Caspard et al. [229]. LAIV has shown superior efficacy compared to IIV in young children in general varying from 70-90% to matched strains [135,230,231,232], as well as in those with a history of respiratory tract infections (RTI), asthma and HIV [215,231,233]. In addition, LAIV recipients with breakthrough influenza had less severe illness [230,231]. These studies are a couple of years old, and as mentioned, last years LAIV efficacy results from the US were discouraging. Follow up studies will provide further information and perhaps an explanation for the variation in efficacy studies.

The lack of laboratory confirmation is a limitation to our study, and in hindsight, perhaps we should have found the funds and personnel capacity to conduct this. We asked the parents about ILI symptoms during the trial. Although they did not report this, it is possible that the children had mild influenza. On the other hand, our study is an immunogenicity study, with numbers too low to consider efficacy and was not powered to detect this. With the vaccine scepticism in Norway, the proven efficacy of the LAIV facilitated the recruiting process.

Although the elderly represent most of the fatal cases of influenza, children are the main transmitters of the disease in the community, and most often hospitalized during influenza outbreaks; often without influenza as suspected diagnosis [234,235]. Seasonal influenza vaccination strategies vary between countries. The USA, Canada and UK and some European countries recommend influenza vaccination in children from 6 months old, and the UK has also implemented influenza vaccine in their childhood vaccination program. Norway and most other European countries only vaccinate the

at risk population [236]. A European consensus report from 2006 recommended children < 3 years old to be considered a high-risk population and recommended for vaccination [237]. Since then, Finland is the only Scandinavian country to recommend seasonal vaccination of healthy children in this age group[236]. Based on the trial experience and lack of focus on cost-effectiveness in the general population, it is unlikely that influenza vaccination will be implemented in the childhood vaccination programme in Norway, although countries like the UK have estimated large national cost savings by vaccinating children towards influenza with LAIV[238].

Limitations of human clinical studies

Numerous clinical and laboratory constraints provided limitations to our **pediatric vaccine trial**. Repeated and precise information as well as sufficient time with each patient were factors that increased the follow up percentage. The recruiting potential was limited to the list of children set up for elective tonsillectomy during the influenza season. Conducting pediatric clinical trials is complex and challenging, and the recruitment required assistance from a paediatric clinical trial unit. Due to the regulatory work, insurance and staff required, few clinical trials are conducted without support from the pharmaceutical industry. Our clinical studies are such exceptions. They were researcher initiated and intramurally funded, thus providing us with choices and subsequent limitations, most of which have previously been discussed under methodological considerations. In Norway, parents are not compensated for trial participation- it is an altruistic decision. It was therefore important to keep the child and parents content.

The **pandemic patient** study was conducted without professional trial help and the work required was overwhelming, hence limitations to the study are several; the limited number of patients, the lack of pilot study, and recruiting and sampling while in the middle of an outbreak. There were insufficient staffing capacity to recall the patients after discharge; hence patients were lost to follow up. Informing and recruiting patients rested with only a few doctors, and collecting study samples proved to be difficult. In addition, the Regional Ethical committee restricted us to

patients who could provide consent in person. All limitations considered, the lesson learned was that in order for a clinical trial to succeed under such challenging circumstances a clear leadership from the start was essential, and allocation of additional resources would have been beneficial. However, in such a situation, the treatment of all our patients had to be paramount. Although low numbers, our clinical data set proved to be well sorted, detailed and clean. We were therefore invited to join the global meta-analysis on the effect of NAIs, with the PRIDE consortium. In this collaboration, clinical data of more than 29000 patients across the world were gathered and analysed on an individual level (appendix paper I+II+III) [95,96]. Our contribution (n=129) proved to be the 7th largest sample set coming from a single hospital, illustrating that although our patient numbers were small on their own, they became valuable when combined with others.

Conclusions

The aims of conducting our two clinical studies were to investigate the immunological responses after seasonal LAIV vaccination in children, as well as studying the immunological findings after pandemic influenza infection. The findings provide insights into the immunological and clinical responses elicited after LAIV vaccination and pandemic H1N1 infection. In conclusion, the study aims in this thesis were answered in the following papers:

Paper I:

- The LAIV was well tolerated and easily administered
- The LAIV induced long-term systemic antibody (HI) and MBC responses, up to 6 -12 months post vaccination
- The LAIV induced long-term systemic T-cell responses, up to 6-12 months
- There were large inter-strain variations in immune response and kinetics with the B strain inducing the strongest responses
- Increases in T-cell responses were observed in children with no increase in HI titers, indicating that the humoral and cellular immune compartments respond independently

Paper II:

- The LAIV induced significant increases in local mucosal and systemic serum IgA antibodies 14 days post-vaccination to the H3N2 and B strains, but not to H1N1.
- LAIV significantly induced salivary IgA which was maintained up to 6 months, hence, salivary IgA may be a future measure of immunogenicity after LAIV
- Influenza specific IgA correlated with serum HI responses

- LAIV significantly increased Memory B-cell (MBC) levels in blood and tonsils in naïve children, but not in the primed
- There was a significant correlation between tonsillar and blood IgG ASC after LAIV vaccination
- The IgG and IgA ASC response occurred earlier (day 7) in the blood than in the tonsils, whereas the IgM response, occurred earlier in the tonsils

Paper III:

- Patients with severe influenza disease had significantly higher antibody levels 8 months post infection, than patients suffering from mild disease
- Patients sampled in the acute phase of influenza disease had low numbers of $CD8^+$ T-cells compared to convalescent patients
- There were significantly higher $CD4^+$ cytokine secretion in patients with severe disease
- The clinical parameters found in hospitalized patients reflected global findings
- Human biological samples for research purposes are intrinsically difficult to obtain during outbreak situations

Future perspectives

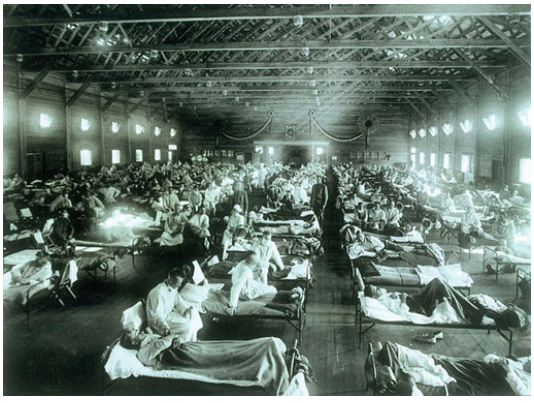
Our findings support European recommendations for LAIV immunization of children. Future studies may elucidate the ability of LAIV vaccines to induce cross-reactive antibodies and cellular immunity. In addition, better understanding of the tonsillar immune responses will aid the development of vaccination strategies aimed at enhancing local immunity against influenza. After finalizing the commenced studies and evaluating adults, we hope to explain the lower effectiveness of LAIV found in adults compared to children, and hope to advise on future vaccine strategies against influenza for children. The value of our largely naïve child population has been recognized, and we are collaborating with The University of Stanford on studying immune responses in children. Future studies in individuals with different immunological experience will aide in deciphering the complex human immune response to influenza.

Although alot is known about the viral structures and how they react with the host cell, the precise immunological events that ultimately lead to the production of long lasting and neutralizing antibodies or cross-reacting T-cells remains unclear. In order to design better vaccines, it is essential to gain better understanding of these complex immune responses induced by natural infection. Future research on the immune responses in naturally infected patients will help answer some of these questions. Similarly, some of the answers might be found by studying the immune responses in children after LAIV vaccination, since LAIV mimics natural infection. Studying the responses in naïve children may perhaps illustrate an immunological scenario in adults where the population lacks protective antibodies. Studying the responses in primed children could aid in elucidating the responses to seasonal influenza vaccines and perhaps provide useful information for improved future seasonal vaccines.

The personal costs of a narcolepsy diagnosis triggered by the pandemic vaccine casts a serious shadow on the otherwise protective effect the vaccines had. This severe adverse reaction will most probably make future mass-vaccination campaigns more challenging. Today, we have a competent expert panel on pandemic influenza.

However, given the experience in the recent years with devastating outbreaks of infectious diseases (Cholera, Ebola, Corona (SARS, MERS), Zika) and avian influenza, should pandemic preparedness perhaps be broader than influenza?

A review in Critical Care Medicine pointed out the lack of high quality clinical research after the pandemic and state that clinical research responses to outbreaks are often fragmented and too late [239]. This fact demonstrates the need for separate research teams in outbreak situations. Preparing a generic case report form (CRF), and having preformed "outbreak research plans", with a finished general protocol approved by the ethics committee could help us in a future outbreak. International consortiums have been established to improve and help future research during pandemic outbreaks [240]. Clearly clinical research is at its best when clinical and scientific expertise is combined. The question concerning a new pandemic is not *if* it will arise, but *when* and *from what corner of the world?* With pre-written research protocols and a planned use of research staff, high-quality human immunological and clinical research should be possible.



Emergency military hospital during influenza epidemic, Camp Funston, Kansas, United States Image: courtesy of the National Museum of Health and Medicine, Armed Forces Institute of Pathology, Washington, D.C., United States.)

Influenza ICU treatment, Haukeland University Hospital, Norway, January 2016. Photo: Kristin G-I Mohn, printed with permission

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Supplementary information

Side reaction form

Registreringsskjema for bivirkninger/ reaksjoner etter influensavaksinasjon
Vi ønsker å kartlegge evt. bivirkninger etter influensa vaksinasjon.

ID nr: _____ Fødselsdato:_____

Vaksinasjondag: ___/___/2012

Kryss av hvis ingenting å melde _____

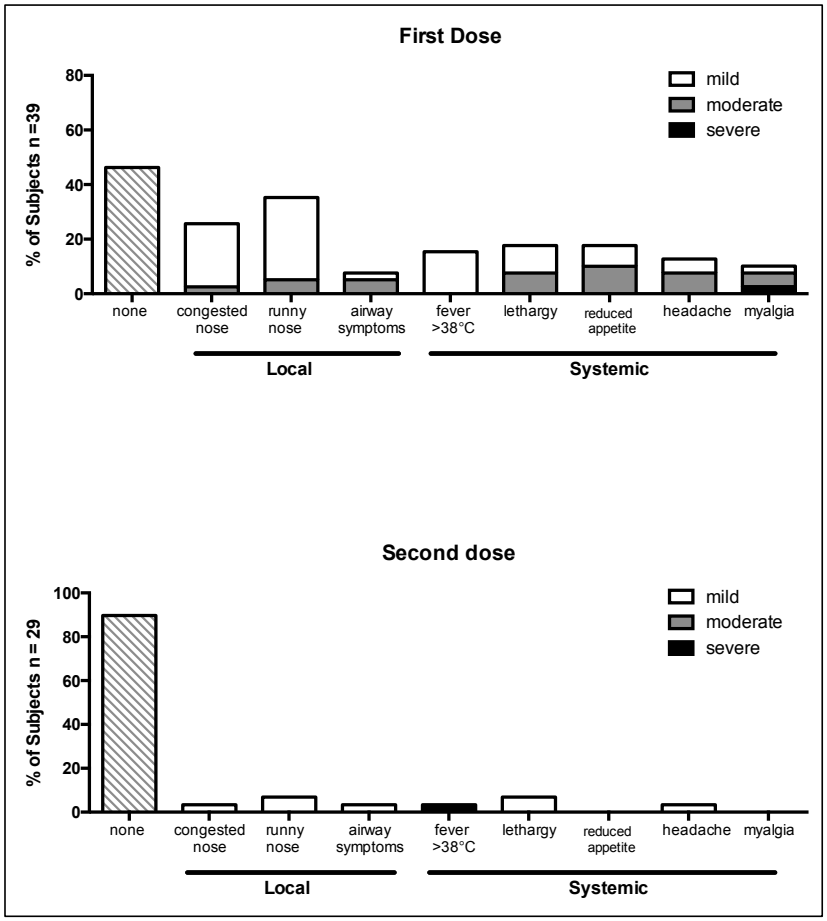
MERK AV I DE RUBRIKKENE SOM PASSER. Gradér fra 1-3
Tomt felt betyr at du ikke har noe å melde fra om,
1= Svake/ ubetydelige symptomer som ikke gir særlige plager.
2= Symptomer som er så besværlige at de påvirker den daglige aktiviteten, men som ikke krever legetilsyn,
3= Sterke eller vedvarende symptomer som i høy grad påvirker den daglige aktiviteten, eller som krever legetilsyn.

	Lokale symptomer			**Generelle symptomer**					
Dag etter vaksinasjon	**Nesetetthet**	**Rennende nese**	**Luftveis symptomer**	**Feber>38°C (temp)**	**Slapphet**	**Nedsatt appetitt**	**Hode pine**	**Muskel-verk**	**Andre hendelser (beskriv)**
0									
1									
2									
3									
4									
5									
6									
7									

Skjemaet returneres til Klinisk Forskningspost Barn, Barneklinikken, Haukeland Universitetssykehus. Tlf 55 97 57 20.
Dr. Kristin Mohn kan også nås på tlf 97 77 07 55

Supplementary table 1: Protocol documents, side reaction form. This form was given to parents and collected after each LAIV dose at the follow up visits.

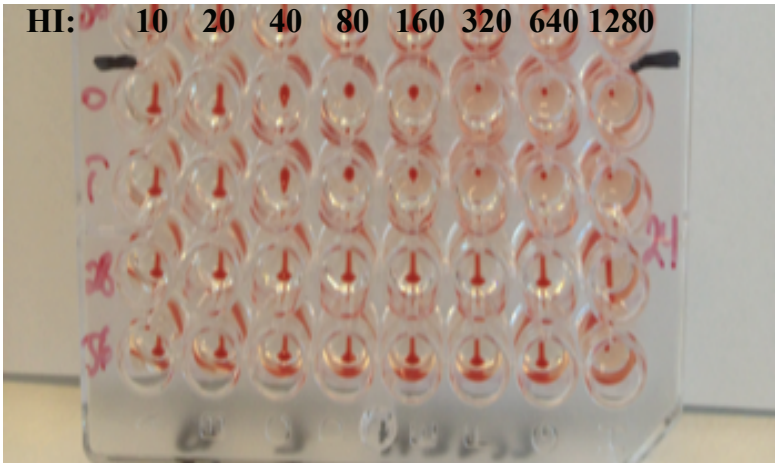
Side reactions



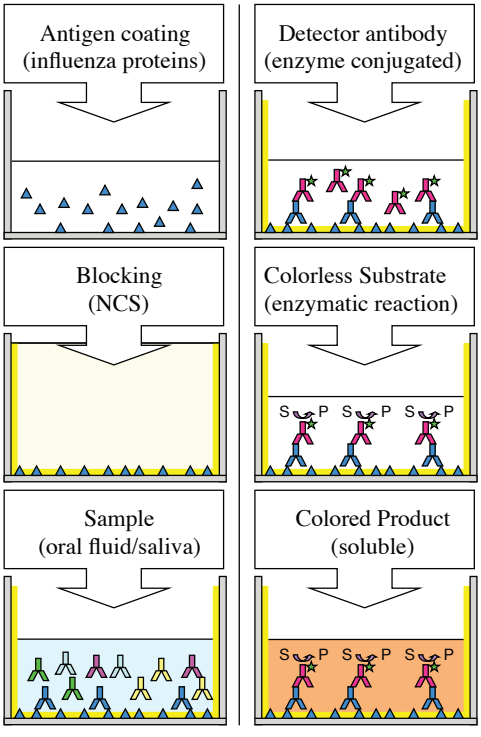
Supplementary Figure 1 Side reactions after first and second dose of LAIV. Side reactions were solicited and graded by increasing severity 0-1-2-3 as indicated on the form. Reactions were registered as local or systemic. After the first dose >40% reported "no side effects" and after the second dose this increased to 90%.

Laboratory assasy

HI assay

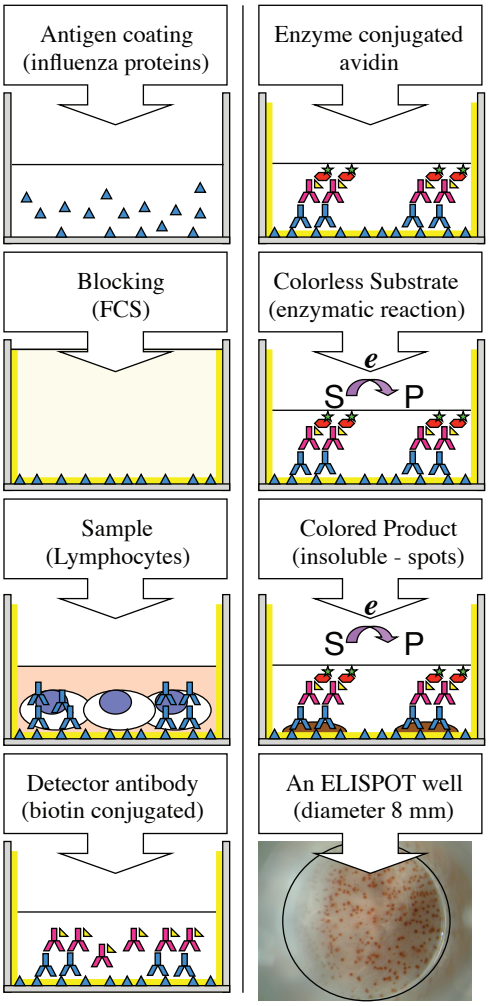


Supplementary Figure 2 The HI assay: An HI plate for patient (no 24) for days 0, T (tonsillectomy day), 28 and 56 is shown. The titer is read as the highest reciprocal titer of inhibition of hemagglutination. The serum was double diluted across the plate, starting with a 1:10 dilution. Child no 24 has antibodies present day 0 and day T (day of tonsillectomy), indicating a primed child with pre-vaccination antibody responses with a good response and an increase in antibodies at day 28, after one dose, which was maintained at day 56.
Photo: Kristin G-I Mohn



ELISA assay

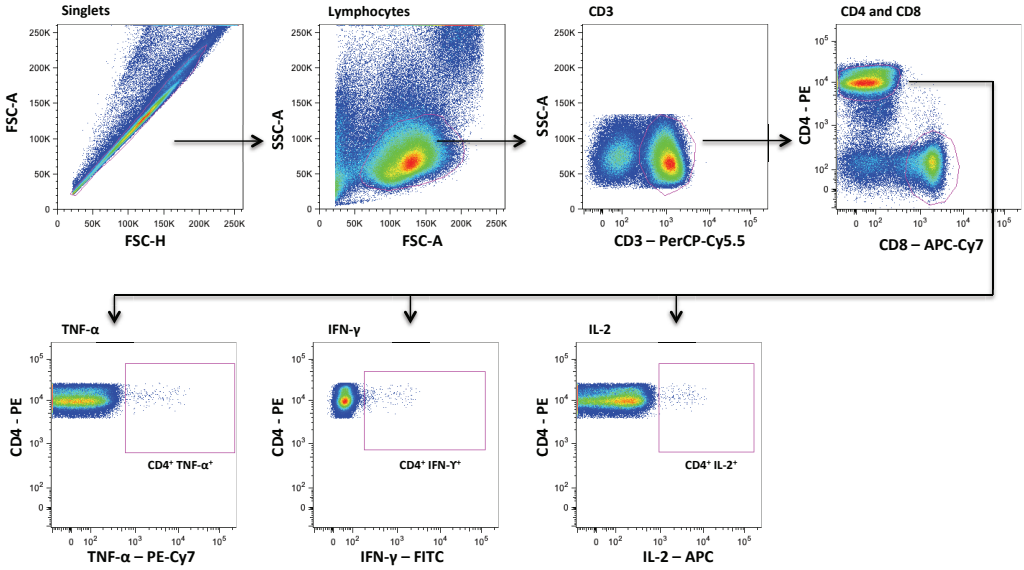
Supplementary Figure 3. Indirect ELISA was used to measure the influenza specific local IgA antibody levels in the saliva after vaccination with LAIV. The plate was coated with the specific influenza antigen (H1N1, H3N2 or B). The cells were blocked to bind non-specific antibodies. The plates were incubated with patient saliva samples. Biotin-conjugated goat anti-human IgA was used as a secondary capture antibody. Streptavidin peroxidase was added as the substrate and the soluble colored product was measured by spectrophotometry. The illustration was kindly provided by Dr. Karl A Brokstad and printed with permission.



ELISpot assay

Supplementary Figure 4 The ELISpot assay. The principle for analysing for influenza specific antibody secreting cells. Plates are coated with influenza antigen or anti-human IgG /IgA/ IgM. Lymphocytes are added and if they are producing specific antibody they leave an imprint. Each spot represents one single influenza specific B-cell secreting specific immunoglobulin. The illustration was kindly provided by Dr. Karl A Brokstad and printed with permission.

Gating strategy



Supplementary Figure 5: A representative gating strategy for quantifying intracellular Th1 cytokine responses by flow cytometry. The figure illustrates the gating strategy used to distinguish single cells (singlets), lymphocytes and subsets of T-cells (CD3, CD4 and CD8 T cells). Within the $CD4^+$ T cell population, cells were discriminated as follows: $CD4^+$ TNF-α^+, $CD4^+$ INF-γ^+, $CD4^+$ IL-2^+. Kindly provided by Dr. Rishi Pathirana and published as supplementary i **paper III**[181].

Papers I-III

I

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MAJOR ARTICLE

Longevity of B-Cell and T-Cell Responses After Live Attenuated Influenza Vaccination in Children

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Background. The live attenuated influenza vaccine (LAIV) is the preferred vaccine for children, but the mechanisms behind protective immune responses are unclear, and the duration of immunity remains to be elucidated. This study reports on the longevity of B-cell and T-cell responses elicited by the LAIV.

Methods. Thirty-eight children (3–17 years old) were administered seasonal LAIV. Blood samples were collected before vaccination with sequential sampling up to 1 year after vaccination. Humoral responses were evaluated by a hemagglutination inhibition assay, and memory B-cell responses were evaluated by an enzyme-linked immunosorbent spot assay (ELISpot). T-cell responses were evaluated by interferon γ (IFN-γ) ELISpot analysis, and intracellular cytokine staining of $CD4^+$ T cells for detection of IFN-γ, interleukin 2, and tumor necrosis factor α was performed using flow cytometry.

Results. LAIV induced significant increases in B-cell and T-cell responses, which were sustained at least 1 year after vaccination. Strain variations were observed, in which the B strain elicited stronger responses. IFN-γ–expressing T cell counts increased significantly, and remained higher than prevaccination levels 1 year later. Expression of T-helper type 1 intracellular cytokines (interleukin 2, IFN-γ, and tumor necrosis factor α) increased after 1 dose and were boosted after the second dose. Hemagglutination inhibition titers were sustained for 1 year. Vaccine-induced memory B cell counts were significantly increased, and the response persisted for one year.

Conclusions. LAIV elicited B-cell and T-cell responses that persisted for at least 1 year in children. This is a novel finding that will aid future vaccine policy.

Keywords. influenza; LAIV; humoral; T-cellular; longevity; pediatric; IFN-γ.

Annually, influenza virus infection has a large socioeconomic burden on society, with 500 000 fatal cases globally [1–3]. The World Health Organization estimates that 20% of children are infected with influenza virus each year, and they are the main source of spread of the virus and have a high burden of the disease [4]. Vaccination is the cornerstone of prophylaxis and is recommended for high-risk patients. The trivalent inactivated influenza vaccine (TIV) is safe and provides protection but may not be the optimal vaccine for young children, owing to their lack of previous infection. Since 2003, the cold-adapted live attenuated influenza vaccine (LAIV), administered as a nasal spray, has been approved in the United States for individuals aged 2–49 years. The vaccine was licensed in Europe in 2012 (for individuals aged 2–17 years) and was implemented in the British childhood vaccination campaign, beginning in 2013 [5]. In June 2014, the Advisory Committee on Immunization Practices preferentially recommended LAIV in healthy children 2–8 years old when it is immediately available [6].

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Despite many years on the market, there are no established correlates of protection for LAIV. Efficacy studies and observational data suggest that the LAIV provides higher levels of protection than TIV in children [7–11]. The hemagglutination inhibition (HI) titer is widely used as a surrogate correlate of protection after TIV receipt; however, the cutoff titer of 1:40 is based on adult trials. There is debate on both the protective HI level in children and the fact that an HI titer underestimates the protection obtained by LAIV [12–14]. Cellular immunity may be a better measure of protective immunity after LAIV in children [15]. $CD4^+$ T cells have the ability to act as effector cells and to direct and generate specific memory $CD4^+$ and $CD8^+$ T-cell subsets with diverse functions [16–18]. Animal and human studies have demonstrated protective cellular immunity after LAIV [10, 19–21]. Therefore, the duration and quality of the immune response in humans after LAIV needs to be studied.

We conducted a pediatric clinical trial to elucidate the immunological mechanisms induced by LAIV, with emphasis on the long-term, strain-specific cellular immune responses. Our study is unique, as we obtained sequential blood samples from young children up to 1 year after vaccination, allowing us to compare and analyze serum and cellular responses in the same child. Our results indicate that LAIV induces long-term humoral and cellular immune responses in children and that priming is important in determining the magnitude of the response.

MATERIALS AND METHODS

Patients and Study Design

Thirty-eight healthy children, consisting of 20 boys and 18 girls aged 3–17 years old, were recruited at Haukeland University Hospital (HUH) in Norway. From October 2012 to January 2013, children were immunized with the trivalent seasonal LAIV (Fluenz, Astra Zeneca, Birmingham, United Kingdom). Fluenz contained $10^{7.0}$ fluorescent focus units of attenuated reassortant of A/California/7/2009(H1N1)pdm09-like, A/Victoria/361/2011(H3N2)-like, and B/Wisconsin/1/2010 strains. The vaccine was administered intranasally as 0.1 mL per nostril. Children 3–9 years old received 2 doses at a 4-week interval, and children ≥10 years old received a single dose of vaccine as recommended by the manufacturer. The study was approved by the Regional Ethical Committee of Western Norway and the Norwegian Medicines Agency and was monitored by HUH (clinical trials registration, NCT01866540; EUDRACT registration 2012-002848-24).

Upon enrollment, parents and children aged ≥12 years provided voluntary, written informed consent. We collected data on baseline demographic characteristics, medical and influenza vaccination history, and risk factors for influenza virus infection. All procedures were conducted at the pediatric trial unit at HUH. Children with mild-to-moderate asthma (clinically stable with daily use of inhalators) or who had received prior influenza vaccination were included. Exclusion criteria are listed in Figure 1. A self-reported questionnaire of local and systemic side effects was completed after each vaccination.

Samples

Blood samples (volume, 8 mL) were collected after vaccination (Figure 1). Fresh peripheral blood mononuclear cells (PBMCs) were isolated using Cell Preparation Tubes (BD, New Jersey) [22]. Plasma samples were aliquoted and stored at −80°C before use in the HI assay.

HI Assay

Plasma samples from each individual were tested in duplicate by means of an HI assay, using 8 hemagglutination units of the

Figure 1. Study design. Healthy children scheduled for elective tonsillectomy were recruited from the Ear, Nose, and Throat Department, Haukeland University Hospital (HUH), Bergen, Norway. Thirty-eight children received live attenuated influenza vaccine (LAIV), and 29 children (all <10 years old) received a second dose. Blood samples were collected before vaccination and at 4 time points after vaccination. The number of subjects providing samples at each time point is shown. Not all children provided blood samples at all visits, owing to difficulty in obtaining blood samples with a sufficient volume (ie, 8 mL). Exclusion criteria were as follows: serious, chronic medical conditions; serious asthma; recent influenza; fever; pregnancy; use of acetyl salicylic acid (ASA) or immunosuppressive therapy; allergy to the vaccine components or earlier complications to vaccination; or under governmental custody.

homologous H1N1 and H3N2 vaccine strains and 0.7% turkey red blood cells [23]. HI titers were defined as the reciprocal of the dilution causing 50% HI. Negative titers were assigned a value of 5 for calculation purposes.

Interferon γ (IFN-γ) Enzyme-Linked Immunosorbent Spot (ELISpot) Assay

IFN-γ–precoated 96-well plates were used according to the manufacturer's instructions (Mabtech, Sweden). PBMCs (400 000 cells/well) in Roswell Park Memorial Institute medium plus 10% fetal calf serum were added to wells, along with negative control (medium alone) or influenza virus antigens (5 μg/mL of split virus vaccine of each strain; H1N1, H3N2, B). Plates were incubated overnight (37°C, 5% CO_2) and developed the following day. The plates were read using the Immunoscan reader and associated software (CTL-Europe). The negative control was subtracted from the influenza virus–specific response.

Intracellular Cytokine Staining (ICS) of $CD4^+$ T Cells

Expression of the influenza virus–specific T-helper type 1 (Th1) cytokines (IFN-γ, interleukin 2 [IL-2], and tumor necrosis factor α {TNF-α}) were measured using ICS of $CD4^+$ T-cells. PBMCs were stimulated overnight with a mixture of the 3 split-virus antigens in the vaccine (H1N1, H3N2 and B; 2.5 μg/mL of each protein) in the presence of brefeldin A, monensin, and anti-CD28 and anti-CD49 antibodies (BD Bioscience, San Jose). After overnight stimulation, cells were stained and analyzed on a BD LSR II flow cytometer for the expression of IFN-γ, IL-2, and TNF-α, as described earlier [24]. The antibodies used are provided in Supplementary Table 1, and the gating strategy is specified in Supplementary Figure 2.

Memory B-Cell Response, Determined by ELISpot

The antigen-specific immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin M (IgM) memory B-cell responses after vaccination were quantified by ELISpot, as described by Pathirana et al [25] and Crotty et al [26]. Influenza virus–specific immunoglobulin-secreting cells per million PBMCs are presented as percentages of the total IgG, IgA, and IgM responses, respectively.

Statistics

Statistical analyses were performed in SPSS, version 17, and GraphPad Prism, version 5 for Mac (GraphPad Software, San Diego, California). For all statistical tests, a P value of $< .05$ was considered significant. ICS results were compared using t test. For the remaining results, analysis of variance (by the Kruskal–Wallis test) with multiple comparisons testing was used.

RESULTS

Study Subjects

Thirty-eight healthy children, including 6 with asthma, received the LAIV (median age, 4 years). Ten children did not receive a second dose owing to an age of ≥10 years (n = 8), concurrent illness at the time of vaccination (n = 1), and withdrawal from the study (n = 1). Samples were collected before and after vaccination with sampling points as indicated in Figure 1.

Safety and Side Effects

The vaccine was easy to administer and well tolerated. Adverse events were solicited by questionnaires during the initial 7 days after vaccination, and 46% of children reported no side effects after the first dose. Reported side effects were mild and mostly local. Seven children (18%) reported runny/congested nose, and 4 (11%) reported systemic side effects (Supplementary Figure 1). Six children had mild or moderate asthma (clinically stable with daily use of local steroids and β2 agonists), of whom 5 reported no side effects after vaccination and 1 reported transient local side effects. Parents of the asthmatic children did not report asthma exacerbation during the trial. In general, reactions often started 2 days after vaccination and mainly lasted 1–3 days (data not shown). One severe adverse event required consultation but not treatment; this occurred in a healthy 17-year-old girl with nontypical influenza-like illness symptoms of arthralgia. After the second dose, 26 children (90%) reported no side effects, and 3 (10%) reported mostly local side effects (Supplementary Figure 1).

HI Antibody Response Against Influenza Virus A Strains Persists for 1 Year

Figure 2*A* and 2*B* show the HI response to the H1N1 and H3N2 strains before and after LAIV receipt. An HI titer of ≥40 was considered a protective response.

Before vaccination, the majority of children (25 [66%]) had protective antibody titers toward H1N1 (geometric mean titer [GMT], 71; 95% confidence interval {CI}, 40–125). Thirteen children did not have protective HI titers, of whom 9 had no detectable antibody (HI titer, < 10) to the H1N1 virus. An increase in HI titer occurred after the first dose (day 28; GMT, 95; 95% CI, 55–164) and after the second dose (day 56; GMT, 111; 95% CI, 64–194), when 27 subjects (84%) had a protective antibody titer (9% seroconverted). Eighteen subjects had HI titers of ≥40 to the H1N1 virus at 180 days, and 6 subjects had no detectable antibodies. At day 360, 11 of 14 children (79%) had a protective HI level (≥40), of whom 3 seroconverted, but 2 of these children had high prevaccination levels. Two children had no detectable antibodies. Four children without prevaccination antibodies remained seronegative throughout the study.

For the H3N2 strain, 14 (37%) of the 18 children (47%) with an HI titer of < 40 were seronegative (HI titer, 5; GMT, 37; 95% CI, 20–68; Figure 2). After the first dose, there was a significant increase in HI titers ($P < .0001$) in all children except 2, reaching protective HI levels (GMT, 286; 95% CI, 203–401). The increase observed after the second dose was significant, compared with the titer on day 0 ($P < .001$), as well as the titer on day 180 ($P < .01$),

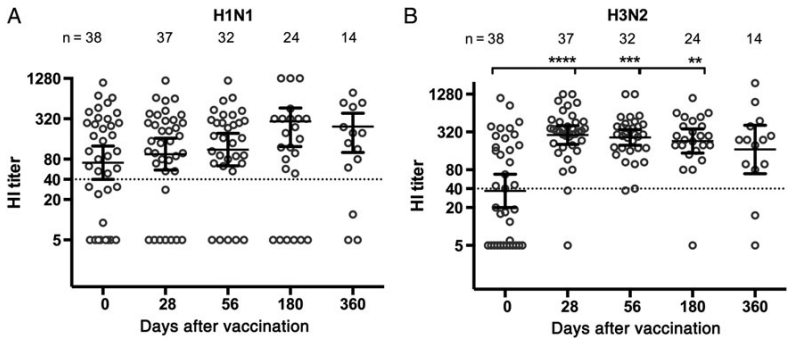

Figure 2. Hemagglutination inhibition (HI) antibody titers after vaccination. Children were intranasally vaccinated with 1 (for those aged ≥10 years) or 2 (for those aged <10 years; doses were administered at a 28-day interval) doses of live attenuated influenza vaccine. HI antibody titers to H1N1 (*A*) and H3N2 (*B*) were measured at the following time points: day 0 (before vaccination), day 28 (after the first dose), day 56 (after the second dose), and days 180 and 360 after vaccination. Each symbol represents the HI response of 1 child, with bold horizontal lines and whiskers denoting geometric mean titers and 95% confidence intervals, respectively. The dotted line represents an HI titer of 40, considered the protective level [27]. The statistical significance of differences from prevaccination levels was determined by analysis of variance, using the nonparametric Kruskal–Wallis test. $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

and 47% of the children seroconverted. One 4-year-old child had an HI titer of 40 after 2 doses but had no detectable titers at other time points. The antibody titers remained elevated 180 days after vaccination, with 96% of subjects (n =23) having protective HI titers (GMT, 229; 95% CI, 147–357). At day 360, 12 subjects (86%) had sustained a protective HI antibody response (GMT, 169; 95% CI, 69–410), while the titer in only 2 children remained <40. Of the 14 children evaluated at day 360, 8 (57%) seroconverted. There was no significant difference in the durability of the HI response for either strain in children receiving 1 or 2 doses of vaccine (Supplementary Figure 3).

Long-term Increased IFN-γ Response

We measured the IFN-γ response by using an ELIspot, and we observed interstrain variations. The highest numbers of specific IFN-γ–secreting cells after vaccination were towards the B strain, followed by the H3N2 strain, and the lowest numbers was to the H1N1 strain. Before vaccination, the majority of children (77%) had levels of IFN-γ–secreting T-cells of ≥100 spot-forming cells (SFCs)/10^6 PBMCs that were specific to H1N1, which is a suggested level of protection against influenza (Figure 3*A*) [15]. There was a significant increase in IFN-γ–secreting cells 28 days after the first dose ($P < .05$) and a further increase after the

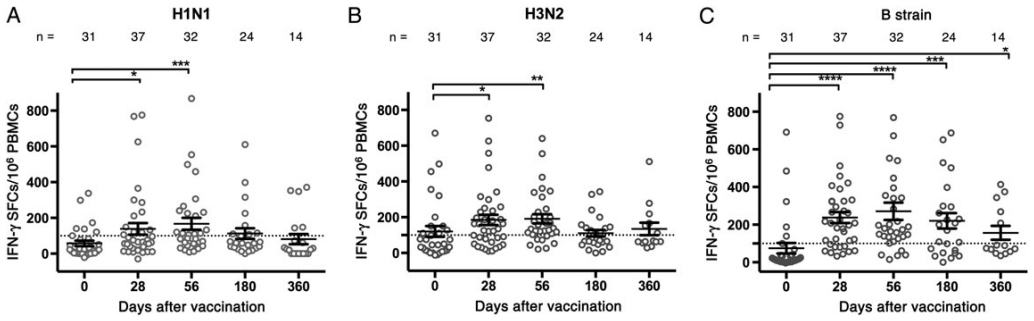

Figure 3. Long-term interferon γ (IFN-γ) immune response in blood after live attenuated influenza vaccination (LAIV). The long-term immune response was evaluated by measuring the number of IFN-γ–producing T cells, measured as spot-forming cells (SFCs)/10^6 peripheral blood mononuclear cells (PBMCs) after LAIV, using the IFN-γ enzyme-linked immunosorbent spot assay. Children were intranasally vaccinated with 1 (for those aged <10 years) or 2 (for those aged ≥10 years; doses were administered at a 28-day interval) doses of LAIV. Blood samples were collected at 0, 28, 56, 180, and 360 days after vaccination. Each symbol represents the influenza virus–specific SFCs/10^6 PBMCs for each child for each influenza strain in the vaccine (*A*, *B*, *C*), with bold horizontal lines and whiskers denoting mean values and standard errors of the mean, respectively. The dotted line represents 100 SFCs/10^6 PBMCs, considered the protective level [15]. The statistical significance of differences from prevaccination levels was determined by analysis of variance, using the nonparametric Kruskal–Wallis test. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

second dose ($P < .001$). The levels declined toward day 180, decreasing below the proposed protective level at day 360 after vaccination, but the mean value remained higher than prevaccination levels, although the difference was not significant. Six of the 7 children with ≥100 SFCs before vaccination had received the Pandemrix vaccine.

For the H3N2 strain (Figure 3*B*), 20 subjects (65%) had <100 SFCs/10^6 PBMCs before vaccination, and the numbers of H3N2-specific IFN-γ–secreting cells increased significantly after the first ($P < .05$) and second ($P = .01$) doses. Levels declined toward day 180 and increased slightly again by day 360, remaining above the suggested protective level of 100 SFCs/10^6 PBMCs, although the difference from prevaccination levels was not significant. Nine of the 11 children with ≥100 SFCs/10^6 PBMCs before vaccination had received the Pandemrix vaccine. There was no significant difference in IFN-γ response in children receiving 1 or 2 doses (Supplementary Figure 4)

The response was highest toward the B strain, with 25 subjects (80%) exhibiting <100 SFCs/10^6 PBMCs before vaccination, but it increased significantly after the first dose ($P < .0001$), with a subsequent boost after the second dose ($P < .0001$). By days 180 and 360, the levels declined, but the mean levels remained above the protective level and were significantly higher than at day 0 ($P < .001$ and $P < .05$, respectively).

The IFN-γ response at each time point after vaccination was plotted against the HI response at day 0 (the prevaccination effect) and day 28 (the postvaccination effect) after immunization. There was a significant correlation between the fold increase in HI titer for H1N1 and the fold increase in IFN-γ secretion (Spearman $r = 0.438$; $P = .036$), but the correlation was not observed for H3N2 (data not shown).

Further analysis of the 5 children who remained seronegative (HI titer, 5) to the H1N1 strain after vaccination (2 doses) found a significant increase in influenza virus–specific IFN-γ responses after vaccination ($P = .029$).

Increased Multifunctional CD4$^+$ T-Cell Response After LAIV Receipt

Figure 4*A* shows that the frequency of Th1 CD4$^+$ T cells that express a single cytokine (IFN-γ, IL-2, or TNF-α) increased after the first dose and was significantly higher after the second immunization, compared with prevaccination levels. Similarly, the percentage of multifunctional CD4$^+$ T cells expressing either 2 (IFN-γ and IL-2, or IL-2 and TNF-α) or 3 (IFN-γ, IL-2, and TNF-α) cytokines increased after the first dose and significantly increased after the second dose, compared with prevaccination levels (Figure 4*B*).

Increased Long-term Memory B-Cell Response After LAIV Receipt

We evaluated the long-term influenza virus–specific memory B-cell response (IgG$^+$, IgA$^+$, and IgM$^+$) after LAIV receipt (Figure 5).

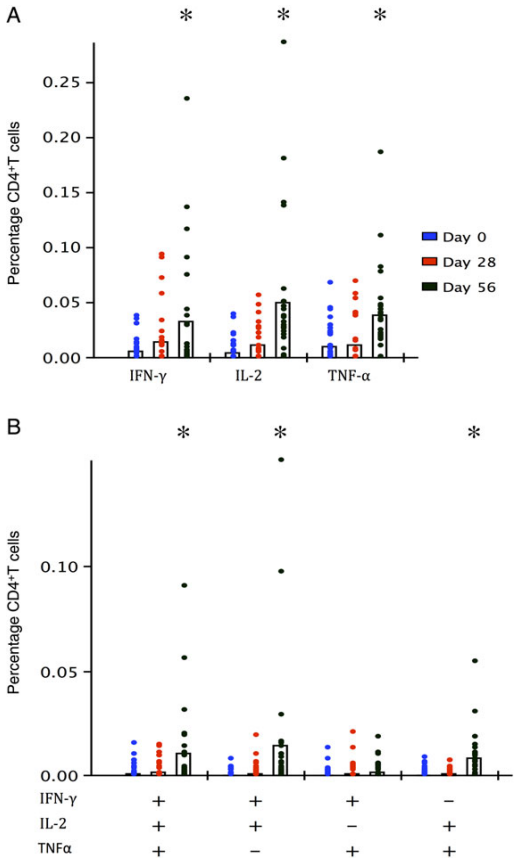

Figure 4. The CD4$^+$ T cell cytokine (T-helper type 1 [Th1]) response before and after vaccination. Peripheral blood mononuclear cells obtained before vaccination (day 0) and 28 and 56 days after vaccination were simulated overnight with split-virus antigen from a mixture of the 3 virus strains in the vaccine (H1N1, H3N2, and B). The percentage of CD4$^+$ T cells secreting either single (*A*) or multiple (*B*) Th1 cytokines was measured by multiparametric flow cytometry. *$P < .05$, by the Student *t* test, compared with the CD4$^+$ T-cell response before vaccination (day 0). Abbreviations: IFN-γ, interferon γ; IL-2, interleukin 2; TNF-α, tumor necrosis factor α.

Overall, the highest frequencies were measured toward the B strain, and the lowest were observed toward the H1N1 strain.

The levels of H1N1-specific IgG$^+$ memory B cells were high before vaccination and remained elevated at all sampling points after vaccination. The IgM$^+$ memory B-cell frequencies to H1N1 increased significantly at all time points after vaccination ($P < .01$), whereas the IgA$^+$ memory B-cell response was only significantly higher on day 360 ($P < .01$).

For the H3N2 strain, IgG$^+$ memory B cells increased after the first dose of vaccine, with a significant boost ($P < .001$) after the second dose. The frequencies of H3N2-specific IgG$^+$ memory B

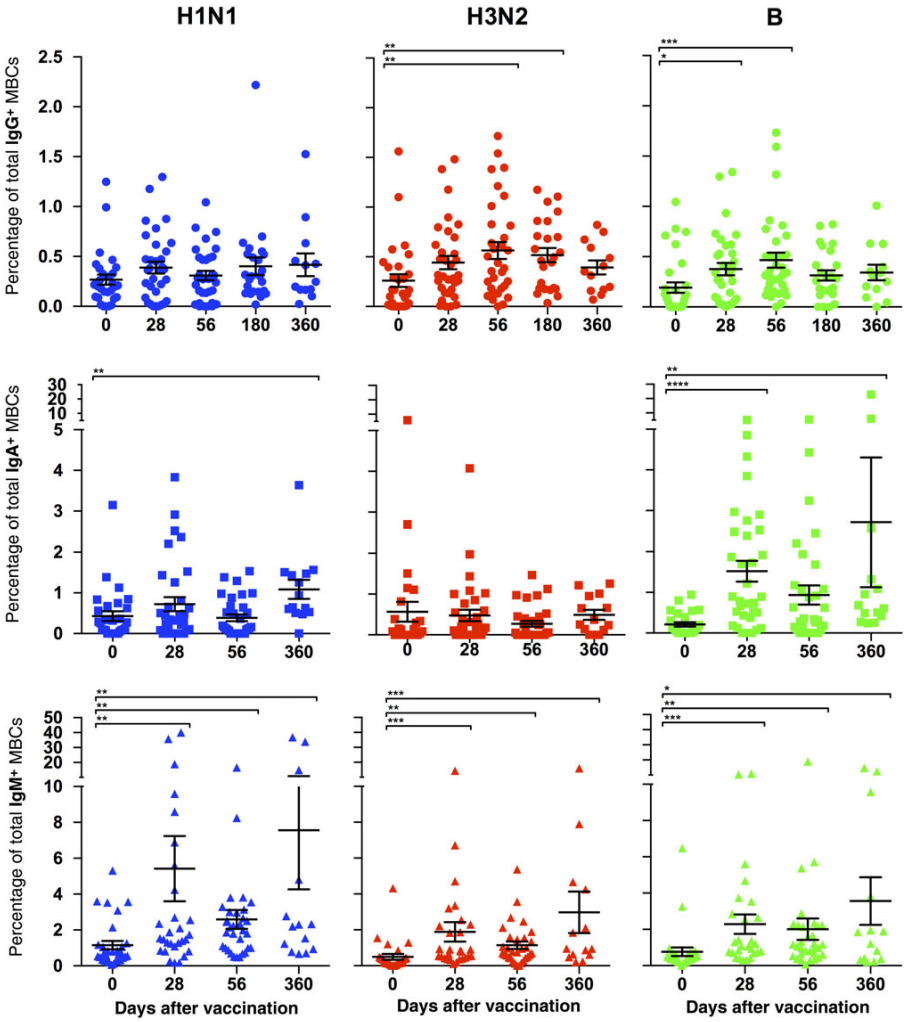

Figure 5. Long-term memory B-cell (MBC) responses after live attenuated influenza vaccination (LAIV). The frequencies of influenza virus–specific immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin M (IgM)–expressing MBCs before and after LAIV receipt. MBCs were stimulated to proliferate and differentiate into antibody-secreting cells by mitogens in vitro, and levels were subsequently measured by an enzyme-linked immunosorbent spot assay. The *y*-axis shows the percentage of influenza virus–specific MBCs. IgG^+, IgM^+, and IgA^+ MBCs were measured against the 3 influenza virus strains in the vaccine. Data are represented as the percentage of antigen-specific IgG^+, IgA^+, and IgM^+ MBCs among all IgG^+, IgA^+, and IgM^+ MBCs, respectively. Each symbol represents 1 child. IgA^+ and IgM^+ MBC frequencies for day 180 were not determined because of laboratory constraints. The lines represent mean values ± standard errors of the mean. The statistical significance of differences from prevaccination levels was determined by analysis of variance, using the nonparametric Kruskal–Wallis test. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$.

cells were maintained at day 180 ($P < .01$) and decreased at day 360, although they remained higher than at day 0. We did not detect increased levels of IgA^+ memory B cells to H3N2 at any time point after vaccination; in contrast, IgM^+ memory B cells increased significantly at all time points ($P < .01$).

For influenza B, the frequencies of all immunoglobulin classes of memory B cells increased significantly after 1 dose of vaccine, with *P* values of <.05 for IgG and IgA and <.001 for IgM, for which the highest numbers were observed. After the second immunization, both the IgG^+ and IgM^+ memory B cells

increased significantly ($P < .01$). At day 360, the frequencies remained higher than those observed before vaccination for all 3 immunoglobulins.

DISCUSSION

Clinical pediatric trials with LAIV generally focus on short-term responses after 1 or 2 doses or on efficacy studies performed during influenza seasons [9, 28, 29]. Less is known about the long-term immunological responses, but there are studies reporting the durability of serum and local antibodies and the efficacy of LAIV [30, 31]. The aim of this study was to evaluate the longevity of the humoral and cellular immune responses elicited by LAIV in young children, with a focus on the effect of priming. To our knowledge, our study is unique in reporting the long-term immune responses to LAIV in children, including those with asthma. We found that the vaccine was safe and easy to administer, with mild side effects after the first dose and even fewer side effects after the second dose (Supplementary Figure 1).

This study was conducted during a postpandemic period, when the H1N1 strain dominated. In general, the highest immune response was against the B strain, followed by the H3N2 strain, with the lowest response against the H1N1 strain. The total levels of serum IgG, IgA, and IgM were stable throughout the study period [32]. The serological response to LAIV was evaluated by an HI assay, with differences observed in the response to the H1N1 and H3N2 strains. Before vaccination, the majority of the children had protective antibody titers (HI titer, ≥40) against the H1N1 strain, which did not increase after vaccination but remained elevated 1 year after vaccination. This may be because H1N1 was a dominant circulating influenza A strain in 2009 and 2010. Hence, most of the children were primed, either by natural infection or pandemic vaccination [33]. As LAIV must replicate to elicit an immune response, the presence of preexisting antibodies or cross-reactive T cells in primed children could inhibit virus infection and replication, resulting in lower HI responses. In contrast, the H3N2 strain circulated to a much lesser degree in Norway; hence, most children were unprimed against this strain [33]. The B strain had limited circulation in the prior seasons, except during 2010–2011; it is therefore possible that most children were naive to this strain, consistent with the observation that LAIVs elicit stronger immune responses in unprimed children [34]. Apart from priming, differences in infectivity among the vaccine strains could also impact the subsequent immune response. We saw no difference in durability of the immune response after 1 or 2 doses of vaccine (Supplementary Figures 3 and 4).

Induction of long-term immunological memory is the ultimate goal of vaccination. In this study, levels of influenza virus–specific memory B cells increased after vaccination and were maintained for 1 year. Memory B cells can rapidly differentiate into antibody-secreting plasmablasts upon antigen reencounter. They may possess broad cross-reactivity and the ability to go through secondary affinity maturation to altered antigenic epitopes [35, 36]. The IgG^+ and IgA^+ memory B-cell responses in our study were strain dependent. When we divided the children according to a protective HI titer of 40, we observed that, in influenza virus–primed children, the levels of memory B cells were not boosted upon vaccination (data not shown). The lack of a boosting response after vaccination in B cells in primed individuals has previously been described in adults [37, 38]. In contrast to the IgG^+ and IgA^+ memory B-cell responses, the IgM^+ memory B-cell response increased significantly against all 3 influenza virus strains. Recent research suggests that isotype-switched, affinity-matured memory B cells dominate the antibody-secreting cell response on antigen recall, while the majority of IgM^+ memory B cells contain less somatic hypermutations and dominate the formation of new germinal centers [38, 39]. Furthermore, it has been suggested that IgM^+ memory B cells live longer than their isotype-switched counterparts [39]. Thus, the influenza virus–specific IgM^+ memory B cells may contain a population with the potential to respond to novel antigenic variants (eg, drifted influenza viruses). The observed strain variations may indicate a biological threshold for memory B-cell responses in children with preexisting memory B cells due to previous influenza virus exposure.

LAIV mimics natural infection and activates the innate immune system, as well as both humoral and T-cell responses, which play a key role in cross-reactive anti–influenza virus responses [10, 21]. T cells depend on major histocompatibility complex presentation of viral antigens and, hence, cannot prevent infection per se. Human studies have shown naturally acquired $CD4^+$ and $CD8^+$ T cells to be important in limiting disease and may provide heterosubtypic immunity, which may influence the influenza A (H1N1 and H3N2) response [40, 41]. T cells respond to conserved epitopes, which is why the response to influenza A viruses is interlinked. Importantly, cross-reactive T cells elicited by LAIV have the potential to protect against drifted strains and shifted pandemic strains. This has been demonstrated in animal models [42, 43]. LAIV-induced cross-reactive antibodies have been found in humans, but it has yet to be determined whether LAIV induces cross-protective T cells in humans.

Using a direct ELISpot assay, we determined the IFN-γ T-cell response following LAIV immunization. This ELISpot assay is more sensitive than serum antibody responses in determining the influenza virus–specific memory immune response with an arbitrary number of 100 SFC/10^6 PBMCs suggested as a protective level against clinical influenza after LAIV in a trial of >2000 children [15]. Interestingly, 5 subjects in our study who, on the basis of HI assays, did not seroconvert to H1N1 had a significant increase in their IFN-γ response after 2 vaccine

doses, which may provide clinical protection. In fact, HI titers are known to underestimate the protective effect achieved by the LAIV in children, and clinical efficacy studies on LAIV have shown high levels of protection against laboratory-confirmed influenza despite low HI titers [7, 44, 45]. Studies in adults have found that LAIV elicits higher $CD4^+$ T-cell responses than TIV to the variant region of hemagglutinin, suggesting that antigenically distinct mutants that escape antibody responses may still be recognized by T cells [21].

Recent studies suggest that $CD4^+$ T cells that simultaneously secrete IFN-γ, IL-2, or TNF-α (multifunctional T cells) are functionally superior than single cytokine producers at inducing anti–influenza virus immunity [46]. In this study, we have shown that LAIV induces a significant increase in both single-cytokine and multifunctional Th1 responses in children. The magnitude of the Th1 cytokine responses induced after LAIV receipt was lower than observed in adult subjects after intramuscular vaccination with candidate pandemic vaccines [25, 47]. Differences in the route of administration (intramuscular or intranasal) and formulation with the adjuvant in immunologically naive subjects could partly explain the superiority of the parenteral vaccine at inducing a Th1 response in peripheral blood. However, in children, LAIV has been shown to be a better inducer of T-cell responses than TIV [10, 48, 49]. This may explain, at least in part, the higher efficacy of LAIV, compared with TIV, in children during head-to-head clinical trials [29]. Nonetheless, vaccine-induced long-lived memory $CD4^+$ T cells may provide broader protection and should be a goal of novel vaccines [18]. With respect to cytotoxic T cells, we did not detect an increase in antigen-specific $CD8^+$ T-cell responses at any time point after LAIV receipt. This is most likely due to the use of split virus proteins for in vitro PBMC stimulation, resulting in inefficient antigen cross-presentation to stimulate a $CD8^+$ T-cell response.

Here, we have demonstrated that LAIV elicits elevated and sustained humoral and T-cell responses in young children at least 1 year after vaccination and that there is great interstrain variation in responses. This was recently addressed by the Advisory Committee on Immunization Practices, which noted that the LAIV gave less protection than TIV against the H1N1 strain alone [6]. This study provides support to public health officials in determining the benefit of their childhood vaccine programs when considering safety and obtaining long-lasting immune responses toward influenza virus.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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II

The Journal of Infectious Diseases
MAJOR ARTICLE

Live Attenuated Influenza Vaccine in Children Induces B-Cell Responses in Tonsils

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Background. Tonsils play a key role in eliciting immune responses against respiratory pathogens. Little is known about how tonsils contribute to the local immune response after intranasal vaccination. Here, we uniquely report the mucosal humoral responses in tonsils and saliva after intranasal live attenuated influenza vaccine (LAIV) vaccination in children.

Methods. Blood, saliva, and tonsils samples were collected from 39 children before and after LAIV vaccination and from 16 age-matched, nonvaccinated controls. Serum antibody responses were determined by a hemagglutination inhibition (HI) assay. The salivary immunoglobulin A (IgA) level was measured by an enzyme-linked immunosorbent assay. Antibody-secreting cell (ASC) and memory B-cell (MBC) responses were enumerated in tonsils and blood.

Results. Significant increases were observed in levels of serum antibodies and salivary IgA to influenza A(H3N2) and influenza B virus strains as early as 14 days after vaccination but not to influenza A(H1N1). Influenza virus–specific salivary IgA levels correlated with serum HI responses, making this a new possible indicator of vaccine immunogenicity in children. LAIV augmented influenza virus–specific B-cell responses in tonsils and blood. Tonsillar MBC responses correlated with systemic MBC and serological responses. Naive children showed significant increases in MBC counts after LAIV vaccination.

Conclusions. This is the first study to demonstrate that LAIV elicits humoral B-cell responses in tonsils of young children. Furthermore, salivary IgA analysis represents an easy method for measuring immunogenicity after vaccination.

Keywords. pediatric; influenza; LAIV; lymphoid tissue; tonsils; mucosa; saliva immune response; humoral; memory B cell; antibody-secreting cell; longevity.

Influenza continues to be an important infectious disease, with annual epidemics claiming up to half a million lives and causing a significant economic burden [1]. Annual seasonal immunization with inactivated trivalent influenza vaccine (TIV) is the most widely used and cost-effective measure for limiting the impact of influenza. An alternative vaccination strategy is to use live attenuated influenza vaccine (LAIV), which was licensed in Europe for children (2–17 years old) in 2012 [2]. LAIV is genetically stable and attenuated to have limited replication in the upper respiratory tract. Meta-analysis of LAIV efficacy studies have demonstrated up to 80% efficacy to matched strains in children <6 years old and 40% efficacy in adults [3–5]. However, the immunological mechanisms and correlates of protection of LAIV are not yet clearly understood.

Serum antibody levels are known to underestimate the protection achieved by LAIV [6]. Other immunological mechanisms are thought to be involved in conferring protection after intranasal immunization, and mucosal responses warrant further investigation. Tonsils are local lymph nodes serving the upper respiratory tract and are a collection of mucosa-associated lymphoid tissues. They consist of a pharyngeal (adenoid) and lingual tonsil and 2 tubal and palatine tonsils (referred to as tonsils). Tonsils play a key role in eliciting mucosal immune responses against respiratory pathogens [7], but their role in eliciting immune response against antigens delivered by intranasal vaccination is not widely reported.

Delivery of LAIV via the intranasal route is perhaps the most efficient way of boosting mucosal immunity at the site of viral entry and induces a weaker systemic response as compared to that of TIV [8]. The tonsil's location at the site of entry into the upper respiratory tract suggests a major role in anti-influenza immunity. The tonsillar epithelium is composed of deep crypts to maximize the surface area exposed to antigens, with Langerhans and M cells transporting luminal antigens into the tonsillar

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tissue [9, 10]. Evidence suggests that tonsils have functional T cells and can provide B cells for mucosal effector sites, including upper airway mucosa and lacrimal and salivary glands [11, 12].

In this unique study, we have vaccinated young children with LAIV at specific time points prior to elective tonsillectomy. We aimed to characterize the early local immune responses after LAIV vaccination, using the blood, saliva, and tonsils obtained from these children. We have previously reported that the systemic B- and T-cell responses persisted for 1 year after LAIV vaccination in some children [13]. Here we show that the LAIV induces early salivary antibody and B-cell responses in the tonsils, which may play a significant role in mediating protection against influenza.

MATERIALS AND METHODS

Study Design

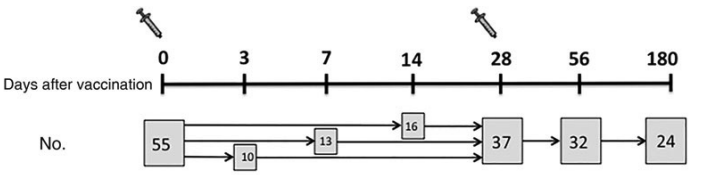
Fifty-five healthy children (3–17 years old) scheduled for tonsillectomy were recruited from outpatients at the ear, nose, and throat (ENT) clinic at Haukeland University Hospital (Figure 1). Thirty-nine children were vaccinated with trivalent LAIV (Fluenz, Astra Zeneca, United Kingdom) during the influenza season from October 2012 to February 2013. The study had ethical and regulatory approval (clinical trials registration NCT01866540). Exclusion criteria have been published previously [13].

To study the early immunological responses after LAIV, we chose the earliest time point that was considered safe by the anesthesiologist (day 3), in addition to 1 and 2 weeks after vaccination. The sampling days were based around the distribution of antibody-secreting cells (ASCs) in peripheral blood, the levels of which peak around day 7 after vaccination, but they have been observed as early as day 2 after TIV vaccination [14–16]. The children were randomized into 3 subgroups, depending on scheduled tonsillectomy date: 3–5 days (n = 10), 7 days (range, 6–9 days; n = 13), and 14 days (range, 11–20 days; n = 16) after vaccination. The number of children was obtained from asking eligible children set up for elective tonsillectomy during the vaccination period. A nonvaccinated control group consisted of 16 age-matched children as a prevaccination comparator for the tonsillar responses in the vaccinated children; the controls were recruited in parallel to the study subjects. Samples were collected at a single time point during the operation (Table 1). Blood and saliva specimens were only used to show the suitability of the controls, as prevaccination (day 0) blood and saliva samples were collected from the vaccinated children for comparison with samples obtained at subsequent time points up to 180 days after vaccination.

Vaccine

LAIV was administered intranasally as a 0.1-mL spray dose into each nostril. LAIV contained 10^7 fluorescent focus units of A/California/7/2009(H1N1)pdm09, A/Victoria/361/2011(H3N2), and B/Wisconsin/1/2010. Children <10 years of age (n = 28) were given 2 doses of LAIV as per the manufacturer's recommendations.

None of the children had earlier received LAIV, as it was not licensed in Norway in 2012–2013. Most children were born after



Vaccination	39#				28[a]		
Controls	16*						
Samples collected:							
Serum	38+16*	10	13	16	37	32	24
Saliva	37+15*	10	13	14	37	32	21
Tonsils[b]	16*	10	13	16			

Figure 1. Study design and sample collection. Children scheduled for tonsillectomy were recruited from outpatients at the ear, nose, and throat clinic at Haukeland University Hospital during the influenza season of October 2012–February 2013. Control children were recruited in parallel throughout the study from the same patient population (ie, if the parents were willing for their child to join the study but did not wish for their child to receive the live attenuated influenza vaccine). Asterisks denote nonvaccinated controls. One control did not provide a sufficient saliva sample owing to dry mouth prior to operation. The hash indicates that 1 vaccinated child provided samples on the day of tonsillectomy but no sample on day 0. [a]Only children aged <10 years old required 2 doses of LAIV. Two children aged <10 years did not receive a second dose, 1 child was sick on the day of the second vaccination, and another child withdrew from the study due to postoperative discomfort. [b]The patients had both of their tonsils removed in 1 operation, and therefore tonsils were only sampled at a single time point. Nonvaccinated controls were used as a prevaccination (day 0) comparator for tonsillar samples. Tonsils were collected from vaccinated children at 3–5 days, 7 days (range, 6–9 days), or 14 days (range, 11–20 days) after vaccination. Serum and saliva samples were collected at multiple time points from each vaccinated subject and at only a single time point, at the time of tonsillectomy, from the nonvaccinated controls. The exclusion criteria and study details for this clinical trial have been published earlier [13].

Table 1. Demographic and Clinical Characteristics of the Study Children

Characteristic	All Subjects, Value	Vaccinated Subjects				Control Subjects, Value
		Overall	Day 3–5	Day 6–9	Day 14	
Subjects	55 (100)	39[a] (100)	9[a]	13	16	16 (100)
Sex						
Male	28 (51)	20 (51)	6	7	6	8 (50)
Female	27 (49)	19 (49)	3	6	10	8 (50)
Age, y						
3–4	31 (56)	23 (59)	6	8	8	8 (50)
5–9	15 (27)	8 (20.5)	2	3	3	7 (44)
10–17	9 (16)	8 (20.5)	1	2	5	1 (6)
Weight, kg, median (range)	19 (9–81)	19 (13–81)	18.5 (14–81)	19.3 (13–60)	19.4 (13–61)	19 (9–64)
Height, cm, median (range)	107 (88–177)	107 (88–177)	104.2 (99–176)	108.4 (95–171)	105.9 (88–172)	100 (91–164)[b]
A(H1N1)pdm09[c] vaccination	26 (47)	21 (54)	3	7	4	6 (38)[d]
Reason for tonsillectomy[e]						
Only recurrent tonsillitis	11	8	3	2	2	3
Only hypertrophy[f]	20	13	5	4	4	7
Both reasons	22	16	1	6[g]	9[g]	6

Data are no. (%) of children, unless otherwise indicated. Thirty-nine subjects received 1 dose of live attenuated influenza vaccine, and 28 children received 2 doses. Ten subjects did not receive the second dose owing to age (8 subjects were >10 years old), illness on the day of the second dose (1 subject), and postoperative discomfort and later withdrawal from study (1 subject).

[a] One child had the operation delayed, therefore no samples were collected at the day of tonsillectomy, but the rest of the time points were collected.

[b] Data for 4 subjects are missing.

[c] Vaccination with 2009 pandemic influenza A(H1N1) vaccine (Pandemrix) in 2009.

[d] Data for 5 subjects are missing.

[e] Data for 2 subjects on reason for tonsillectomy are missing.

[f] Defined as hypertrophy-related problems such as sleep apnea/snoring, speech impairment, and recurrent ear infections.

[g] Data for a subject is missing.

the pandemic, and the only influenza vaccine the older children had received was the monovalent, adjuvanted pandemic influenza A(H1N1) vaccine in 2009 (6 controls and 21 vaccinees).

Samples

Peripheral blood samples (8 mL) were collected at day 0 and after vaccination, using CPT tubes (BD), and peripheral blood mononuclear cells (PBMCs) and plasma were separated [17]. Plasma samples were stored at −80°C. Immediately following tonsillectomy, whole tonsils were collected to isolate the tonsillar mononuclear cells (TMCs) by Lymphoprep (Stemcell tech, United Kingdom). Saliva samples were absorbed from the lower buccal mucosa for 2 minutes, using a swab (Salimetrics). The swabs were placed in a tube and kept on ice until centrifuged (at $600 \times g$ for 10 minutes at 4°C) before storage at −80°C.

Serological Assays

Hemagglutination Inhibition (HI) Assay

Plasma samples from each subject were tested at the same time, in duplicate. In the HI assay, 8 hemagglutinin units of the homologous influenza A(H1N1) and influenza A(H3N2) virus strains or ether-treated influenza B virus vaccine strains (50 µL/well) were used, and 0.7% turkey red blood cells, with receptor destroying enzyme–treated serum at a starting dilution of 1:10 [18]. The influenza virus antigens were either provided by the WHO Influenza Reagent Resources or were grown in eggs in our laboratory.

B-Cell Assays

The influenza virus–specific immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin M (IgM) ASC [19] and memory B-cell (MBC) [20] responses were determined by an enzyme-linked immunospot (ELISPOT) assay using fresh lymphocytes from blood and tonsils. Results are presented as influenza virus–specific ASCs or MBCs per 1×10^6 PBMCs/TMCs.

Salivary IgA

The concentration of influenza virus–specific IgA antibodies in the saliva was measured in ELISA plates coated with 2 µg/mL of split influenza virus antigens (A(H1N1), A(H3N2), or B strains) as previously described [14].

Statistical Analysis

Statistical analysis was performed using GraphPad Prism, version 6, for Mac OS X. Differences between prevaccination and postvaccination ASC and MBC responses were analyzed by matched-paired signed rank *t* test, (Wilcoxon), and the *P* value was adjusted accordingly (by the Bonferroni method). The comparison of HI and saliva IgA responses over time were evaluated by analysis of variance, (nonparametric, Kruskal–Wallis) with the Dunn multiple comparisons test. Correlation analysis was performed by nonparametric Spearman correlation. A P value of $< .05$ was considered statistically significant.

RESULTS

Study Subjects

Fifty-five healthy children were enrolled in the study during the influenza season from October 2012–January 2013. Of these, 39 were vaccinated with LAIV, and 16 were nonvaccinated controls. The vast majority (32 of 39) were ethnic Norwegian caucasian individuals. Among the vaccinated children, there were 20 boys and 19 girls, with a median age of 4 years (range, 3–17 years). The children were vaccinated at 3 days (range, 3–5 days; n = 10), 7 days (range, 6–9 days; n = 13), or 14 days (range, 11–20 days; n = 16) prior to tonsillectomy, to allow evaluation of early tonsillar B-cell responses after LAIV vaccination. The demographic characteristics and vaccination history were similar in the 3 subgroups and controls (Table 1). Sequential blood samples were collected before vaccination, on the day of tonsillectomy, and 28, 56 and 180 days after vaccination (Figure 1) [13]. The median sampling time point was close to the target sampling day. For comparison of differences in kinetics in blood and tonsils, the early time points (days 3, 7, and 14) were used, while the later time points were used to study the duration of the systemic and salivary responses after LAIV vaccination. For the comparison of background prevaccination tonsillar responses and the responses in vaccinated children, 16 matched, nonvaccinated controls were used.

Among the 39 vaccinated children, 21 (54%) had received the inactivated, monovalent influenza A(H1N1) pandemic vaccine in 2009. Two vaccinees (5%) were born to mothers who had been immunized with the pandemic vaccine during pregnancy. Apart from 1 child, none had earlier received seasonal TIV or LAIV, as routine influenza vaccination of children is not recommended in Norway.

Serological Responses

An HI titer of ≥40 is considered protective against seasonal influenza [21]. No significant changes were observed in the postvaccination response against influenza A(H1N1) virus, with 45%–82% having titers of ≥40 after LAIV vaccination (Figure 2*A*). Significant increases in influenza B and influenza A (H3N2) virus antibody responses were observed from 14 and 28 days after vaccination, respectively, and maintained until day 180 (Figure 2*B* and 2*C*). Overall, the percentage of subjects with HI titers of ≥40 against influenza A(H3N2) virus increased from 49% at day 0 (geometric mean titer [GMT], 36) to 94% in the group that underwent tonsillectomy 14 days after vaccination (GMT, 137; Figure 2*B*) and was maintained until 180 days. The majority (89%) of children had no detectable antibodies to the influenza B virus strain before vaccination. As early as 14 days after vaccination, 69% had protective antibody titers, increasing to 76% and 84% at days 28 and 56 ($P = .0001$; Figure 2*C*). The nonvaccinated controls had similar antibody titers to the prevaccination (day 0) titers, justifying their use as a day 0 comparator for tonsillar responses (Figure 2; controls/day 0). When studying the individual responses, no increase in titers was observed on day 3, but increases were observed in 2 children on day 7 (influenza A(H3N2) and B virus strains), and in 10 children at day 14 (71%) for the influenza B virus strain (Supplementary Figure 2). A boost after the second dose was observed in 1 child (for influenza A(H1N1) virus), 7 children (for influenza A(H3N2) virus), and 11 children (for the influenza B virus strain), with the strongest responses in the unprimed children. There were no significant differences in responses over time in the 3 groups, except at the time of tonsillectomy (Supplementary Figure 2).

IgA Response in Saliva

Figure 3*A*–3*C* shows the influenza virus–specific IgA response in saliva after LAIV vaccination. Significant increases ($P < .001$) in saliva IgA response were detected against influenza B virus and influenza A(H3N2) virus strains from 0 to 14 days after vaccination and also at days 56 and 180 for the influenza B virus strain. The IgA response was maintained to day 180

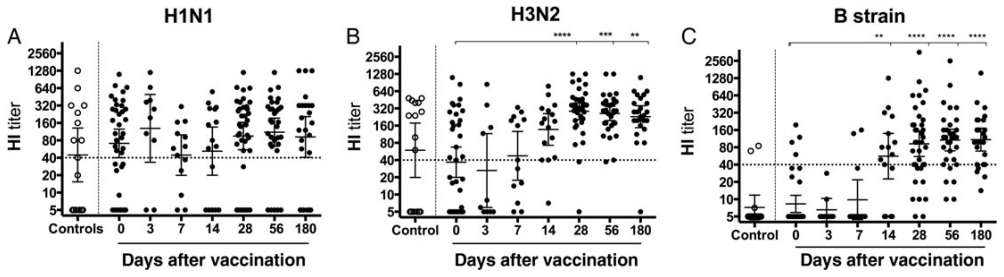


Figure 2. Serological response after live attenuated influenza vaccine (LAIV) vaccination. Plasma was collected from nonvaccinated controls (open circles) and LAIV recipients (closed circles), and the serological antibody response was investigated by a hemagglutination inhibition (HI) assay. The data indicate influenza A(H1N1) virus–specific (*A*), influenza A(H3N2) virus–specific (*B*), and influenza B virus–specific titers (*C*). Each symbol represents an individual subject, and the horizontal lines represent the geometric mean titers ± 95% confidence intervals. The dotted lines represents an HI titer of 40, considered indicative of a protective level. Statistical significance between prevaccination and postvaccination responses was measured by the nonparametric Kruskal–Wallis multiple comparisons test. $^{**}P \leq .01$, $^{***}P \leq .001$, and $^{****}P \leq .0001$.

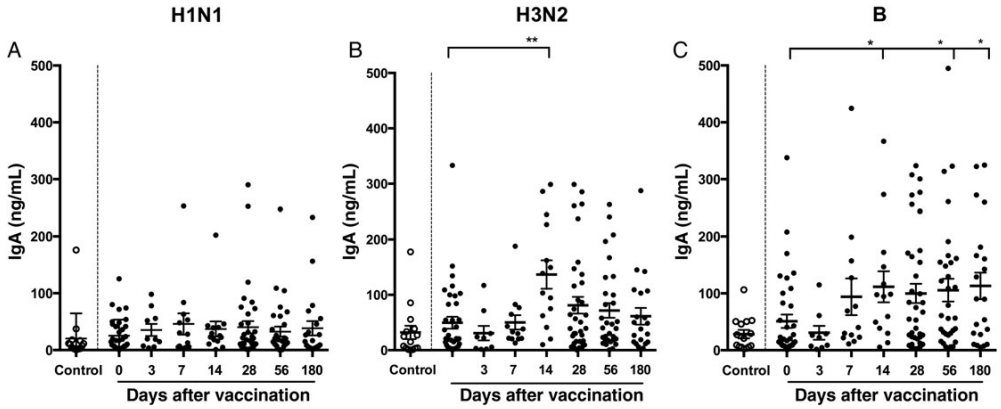


Figure 3. The immunoglobulin A (IgA) response in saliva after live attenuated influenza vaccine (LAIV) vaccination. Saliva samples were collected from nonvaccinated controls (open circles) and LAIV recipients (closed circles) on the day of tonsillectomy (day 3, 7, or 14) and 28–180 days after vaccination. The IgA antibody levels in saliva were determined by enzyme-linked immunosorbent assay for each strain *A* (H1N1), *B* (H3N2), and *C* (B strain). Each symbol represents the IgA response of 1 subject, with means and standard errors of the mean indicated. Statistical significance between prevaccination and postvaccination responses was measured by the nonparametric Kruskal–Wallis multiple comparisons test. $^{*}P < .05$ and $^{**}P \leq .01$.

above prevaccination levels for the influenza A(H3N2) and B virus strains. However, no significant increase in IgA responses was observed against the influenza A(H1N1) virus strain at any time point after vaccination. Furthermore, there was a significant positive correlation between the postvaccination (day 3–14), salivary IgA titers and the serum HI responses for all 3 strains ($r = 0.37–0.48$; $P < .05$). The controls had titers that matched the prevaccination titers of the vaccinated children.

ASC Responses in Tonsils and Blood

As tonsils could only be collected at a single time point, nonvaccinated control children were included to show background prevaccination tonsillar B-cell responses (Figure 1 and Table 1). Serological and salivary IgA titers (Figures 2 and 3) observed in controls were similar to the prevaccination (day 0) samples from the vaccinated children, making them suitable for comparison in the ASC and MBC assays. Influenza virus–specific ASC responses in blood and tonsils were analyzed by ELISPOT to a mixture of the 3 vaccine strain antigens (influenza A(H1N1), A(H3N2), and B virus strains; Figure 4*A*–4*F*). The antigen-specific ASC response in TMCs was dominated by IgM and increased significantly 7 days after vaccination (Figure 4*C*). There were low numbers of IgG and IgA ASCs detected in the tonsils, with the highest responses on day 14 after vaccination, compared with control responses (Figure 4*A* and 4*B*).

Figure 4*D*–4*F* shows the influenza virus–specific ASC response in PBMCs, with very low numbers detected before and 3 days after vaccination but with significant increases in IgG and IgA ASCs on days 7 and 14 (Figures 4*D* and 4*E*). IgM also increased although not significantly. At day 28, the IgA and IgM frequencies were similar to prevaccination levels (mean, 3 and 4 ASCs/1×10^6 PBMCs, respectively). We found a significant positive correlation between influenza virus–specific IgG ASC frequencies detected in tonsils and blood after LAIV vaccination ($r = 0.51$; $P = .007$), suggesting that peripheral ASCs reflect the local tonsillar response.

MBC Responses in Tonsils and Blood

Influenza virus–specific MBC responses were detected by ELISPOT in blood and tonsils. No significant increases were observed in short-term MBC responses in peripheral blood or tonsils up to day 14 (Supplementary Figure 1). In general, much higher frequencies of IgG and IgM MBCs were detected, compared with IgA, in both tonsils and blood.

We observed a significant positive correlation between the IgG MBC responses in the TMCs and PBMCs to the 3 LAIV strains ($r = 0.82–0.59$; $P < .05$). A significant positive correlation was also detected between the IgG MBC responses in the TMCs and the HI responses at the corresponding time points to the influenza A(H1N1) virus ($r = 0.68$; $P = .0004$) and influenza A(H3N2) virus ($r = 0.47$; $P = .0189$) strains but not to the influenza B virus strain.

To see whether previous infection (priming status) of the subjects influenced their short-term MBC response after LAIV vaccination, we stratified each individual on the basis of their prevaccination serological titer as primed (HI titer of ≥40) or naive (unprimed; HI titer of <40; Figure 5). The primed subjects had significantly higher IgG MBC frequencies than the naive subjects against influenza A(H1N1) virus in both tonsils (mean, 469 and 51 MBCs/10^6 TMCs, respectively) and blood (mean, 1100 and 130 MBCs/10^6 PBMCs, respectively) and against the influenza A(H3N2) virus strain in tonsils (mean,

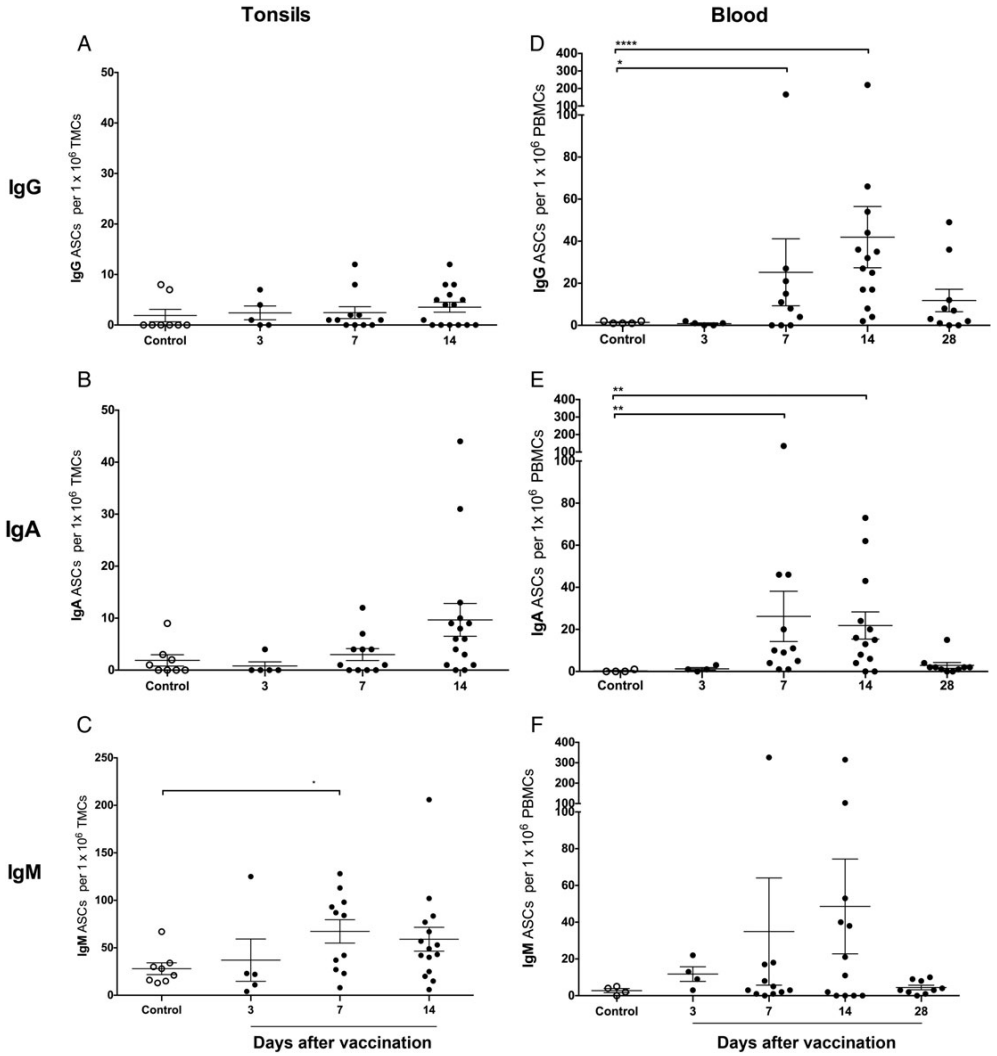


Figure 4. Antibody-secreting cell (ASC) response in tonsils and peripheral blood after live attenuated influenza vaccine (LAIV) vaccination. Children were vaccinated with 2012–2013 seasonal LAIV, and the immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin M (IgM) ASC responses in tonsillar mononuclear cells (TMCs) and peripheral blood mononuclear cells (PBMCs) were measured by enzyme-linked immunospot assay. The influenza virus–specific IgG (*A*), IgA (*B*), and IgM (*C*) ASC responses against a combination of the 3 viruses (influenza A(H1N1), A(H3N2), and B viruses) were determined in TMCs isolated from nonvaccinated controls (open circles) and LAIV recipients (closed circles) 3, 7, and 14 days after vaccination. The IgG (*D*), IgA (*E*), and IgM (*F*) ASC responses against a combination of 3 influenza viruses (influenza A(H1N1), A (H3N2), and B viruses) were measured in PBMCs isolated before vaccination (day 0) and at 3, 7, 14, and 28 days after vaccination. *A–F*, Each symbol represents influenza virus–specific ASCs per 1×10^6 cells with mean ± standard error of the mean indicated. Statistical differences between vaccinated and nonvaccinated subjects were determined by the nonparametric Kruskal–Wallis multiple comparisons test. $^*P < .05$, $^{**}P \leq .01$, and $^{****}P \leq .0001$.

710 and 182 MBCs/10^6 TMCs, respectively). Low frequencies of MBCs were observed for the influenza B virus strain in the unprimed children, and generally a higher response was observed in the primed child (only 1 of the 4 primed children had results for the B strain). No significant differences in influenza virus–specific IgA and IgM MBC responses were observed between primed and naive individuals (data not shown).

We have earlier shown that LAIV significantly increases MBC responses in these children, which persist for up to 6–12 months [13]. When we analyzed these long-term IgG

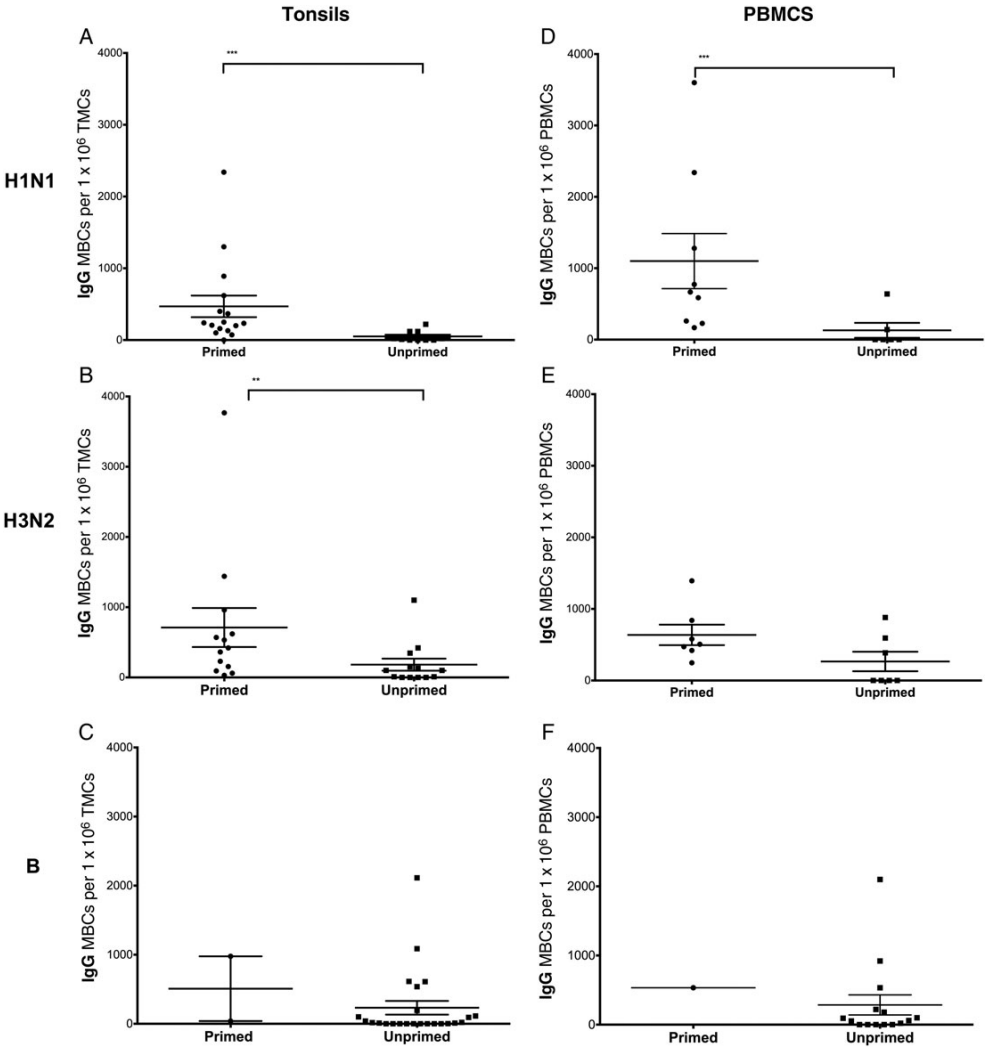


Figure 5. The short-term effect of priming on memory B-cell (MBC) response in tonsils and blood after live attenuated influenza vaccine (LAIV) vaccination. The children were classified as "primed" if they had a hemagglutination inhibition (HI) antibody titer of ≥40 and as "naive" if the HI titer was <40 prior to vaccination. The figure shows the influenza A(H1N1) virus–specific (*A*), influenza A(H3N2) virus–specific (*B*), and influenza B virus–specific (*C*) immunoglobulin G (IgG) MBC response in tonsillar mononuclear cells (TMCs). The IgG MBC results for the 3 strains in peripheral blood mononuclear cells (PBMCs) are shown in panels *D*–*F* from primed and naive subjects. Each symbol represents the MBC response of 1 subject, and the horizontal lines represent the means ± standard errors of the mean indicated. Statistical differences between the primed and naive groups were measured by the Mann–Whitney nonparametric test. $^{**}P \leq .01$ and $^{***}P \leq .001$.

MBC responses (up to day 180) according to the priming status, we found a significant increase in MBCs in the unprimed children after LAIV vaccination and not in the primed children (Figure 6*A*–6*C*). This indicates that the increase in the MBC response after LAIV vaccination was largely influenced by the priming status of the child.

DISCUSSION

Tonsils represent both an induction and maintenance site for mucosal immune responses in the nasopharynx against respiratory pathogens (ie, influenza virus) encountered through natural infection [22]. However, limited data are available on the tonsillar role and contribution to the local immune response

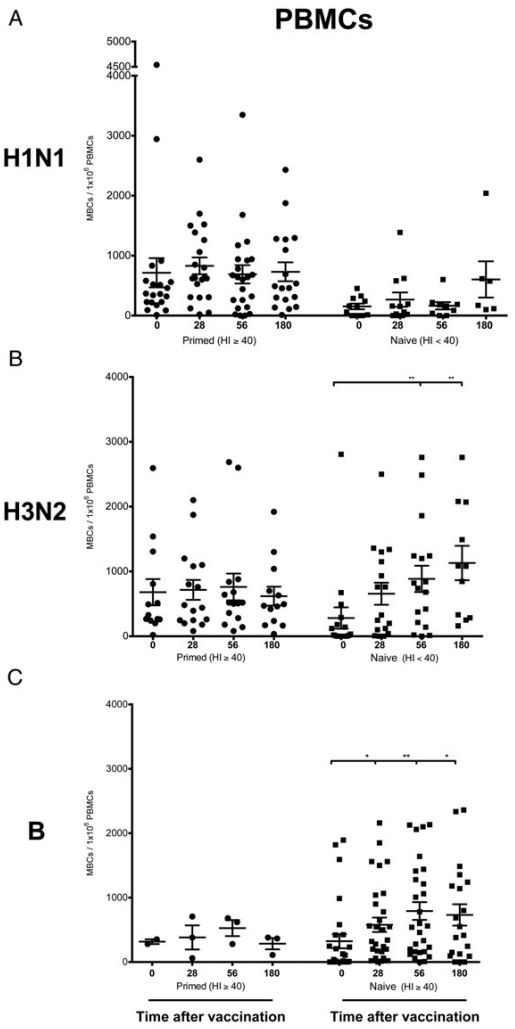


Figure 6. The long-term effect of priming on memory B cells (MBCs) in blood after live attenuated influenza vaccine (LAIV) vaccination. The children were classified as "primed" if they had a hemagglutination inhibition (HI) antibody titer of ≥40 and as "naive" if the HI titer was <40 prior to vaccination. The immunoglobulin G (IgG) MBC results for the 3 strains were measured in peripheral blood mononuclear cells (PBMCs) isolated before vaccination (day 0) and 28, 56, and 180 days after vaccination (*A*–*C*). Each symbol represents influenza virus–specific MBCs per 1×10^6 cells with mean ± standard error of the mean indicated. Statistical differences between the different time points and day 0 were determined by the nonparametric Kruskal–Wallis multiple comparisons test. $^*P < .05$, $^{**}P \leq .01$, and $^{***}P \leq .001$, respectively.

after intranasal vaccination. In this unique study, we were able to collect tonsils, saliva, and blood samples from young children (median age, 4 years) who were intranasally vaccinated with a LAIV prior to elective tonsillectomy. These pediatric samples enabled us to assess the local lymphoid and saliva responses, as well as the systemic immune responses.

The immune responses elicited by LAIV are multifaceted, similar to those after natural infection. The induction of adequate immune responses to LAIV is dependent on local replication of the virus, and hence preexisting mucosal antibodies may reduce viral replication. Protection after LAIV is thought to be associated with induction of mucosal antibodies, but challenges in sampling and assaying these antibodies have hampered development of mucosal antibodies as a correlate of protection [2]. IgA is the predominant secreted antibody at mucosal surfaces [23, 24]. We detected elevated IgA levels in saliva 14 days after vaccination, and the response persisted in some subjects for 180 days, which is similar in duration to the nasal IgA response observed after LAIV vaccination in children [8, 25]. This indicates that the mucosal immune response is well developed in young children and that the LAIV provides local protection in the nasal and oral cavities. Importantly, a significant positive association was observed between the influenza virus–specific salivary IgA and serum antibody responses. This implies that salivary IgA levels could be a possible noninvasive biomarker to predict the immunogenicity of LAIV and could be particularly useful when assessing the effectiveness of the vaccine in children.

The LAIV enhanced the systemic antibody responses toward the influenza A(H3N2) and B virus strains in most subjects, but no boost was observed against the influenza A(H1N1) virus strain. A lack of measurable effectiveness against the influenza A(H1N1) virus strain in the 2013 LAIV has been reported in the United States, and the LAIV is no longer the sole recommended vaccine for children [26–28]. The LAIV influenza A(H1N1) virus strain had reduced viral fitness due to temperature instability, and the vaccine manufacturer has developed a new influenza A(H1N1) virus strain to overcome these problems [29]. However, the preexisting antibodies to influenza A(H1N1) virus may have also contributed to the low effectiveness, since most children had previously been infected or vaccinated with this strain during the 2009 pandemic.

The humoral immune response was further characterized by analyzing the ASC responses in the tonsils and blood. Our earlier work in adults has shown that after intramuscular TIV vaccination, antigen-specific ASC responses appear transiently in the blood. These ASCs peak 1 week after vaccination, corresponding to the peak plasmablast ($CD19^+CD20^-CD27^{high}CD38^{high}$) response [14, 19]. The kinetics of the circulating ASC response was slower after LAIV vaccination as compared to TIV vaccination, with the highest response observed 2 weeks after vaccination. The extended ASC response after intranasal immunization could be due to longer persistence of the vaccine viruses. Data from respiratory syncytial virus–infected subjects show that ASCs are produced for as long as the virus is shed [30]. An extended ASC response was also detected in the tonsils; however, we cannot

rule out a peak later than our last sampling point (day 14). In the tonsils, the predominant influenza virus–specific responses were unswitched IgM ASCs, which indicates activation of naive B-cell responses to novel epitopes on the viral surface glycoproteins. Relatively low IgA responses were observed in tonsils after LAIV vaccination, in agreement with our previous observation in children after TIV vaccination [15]. Although the number of influenza virus–specific IgG and IgA ASCs remained relatively low in tonsils, compared with blood, it still represents a significant immunological response, as $>10^9$ cells were isolated from some tonsils.

Recall antibody responses produced by MBCs are crucial for influenza vaccine–induced protective immunity. We observed influenza virus–specific MBCs in both tonsils and blood before vaccination in most children. The presence of tonsillar MBCs could be a significant factor in providing long-term protection at the site of initial virus infection in the upper respiratory tract, as murine studies have shown that long-lived antibody responses against viruses originate from existing MBCs [31]. The importance of mucosal MBCs in protective immunity is highlighted by the fact that at baseline, primed subjects had significantly higher influenza virus–specific IgG MBCs in their tonsils than unprimed subjects. The MBCs measured at day 0 or in the controls reflected prior infection history. The observation of high levels of influenza A(H1N1) virus– and A(H3N2) virus–specific MBCs in some of the control children in both tonsils and blood (Supplementary Figure 1) is probably a result of earlier infection by the circulating influenza A(H1N1) or A(H3N2) virus strains, not previous vaccination, as influenza vaccination is only recommended for children with high-risk conditions in Norway. We observed interstrain differences in the immune response after LAIV vaccination, with the influenza B virus strain inducing the highest antibody responses both locally (saliva and tonsils) and systemically (blood). Most of the children were naïve to the influenza B virus strain; nonetheless, the majority reached protective HI titers 14 days after vaccination, indicating a rapid induction of protective antibody. Although individual variance will affect the kinetics of the immune response, the results from our study may represent different types of immune responses: an experienced population (with the majority previously exposed to influenza A(H1N1) virus) and a naïve population (with the majority without prior influenza A(H3N2) and B virus strain exposure).

Importantly, there was a significant positive correlation between influenza virus–specific tonsillar IgG MBC responses and serum HI titers, which is consistent with findings that IgG MBCs are more likely to become plasmablasts that appear in the circulation, capable of producing influenza virus–specific antibody responses [32–34]. Although the early influenza virus–specific MBC responses were not boosted by vaccination within the 2-week period (Supplementary Figure 1), the MBC responses increased in blood by 28–56 days after vaccination, lasting up to 12 months [13]. Importantly, our results show that the increased MBC responses after LAIV occur mainly in unprimed (naïve) children (Figure 6).

MBC responses are rapid, producing more high-affinity antibodies than naïve B cells. The primed children had higher MBC levels, which were not boosted further after LAIV vaccination, possibly because a threshold level was reached (Figure 6), which has been observed by Sasaki et al [16]. LAIV may however help maintenance and maturation of the MBC response, producing antibodies with a broader repertoire to influenza virus [35]. The finding that primed children had a greater IgG MBC response in both tonsils and blood toward the 2 influenza A virus strains (Figure 5) could therefore suggest that a more mature immune response is achieved in these children. This is supported by earlier studies showing that serum IgG levels induced after influenza virus infection or vaccination are correlated with resistance to infection [26], with higher antibody levels with increased avidity. The lack of correlation between influenza B virus strain serological titers and IgG MBCs could indicate that the influenza B virus strain kinetics are different or that the response arises at a later time point, as the vast majority were naïve to this strain. Only the naïve children showed a significantly increased long-term MBC response in blood beyond the time points we sampled the tonsils (Figure 6). The weaker influenza A(H1N1) virus strain [29] did not boost the MBC response, but the influenza A(H3N2) and B virus strains elicited boosted MBCs responses in naïve children, and the majority seroconverted. Our findings support European recommendations of LAIV vaccination by children only and can explain the lower effectiveness of LAIV found in adults as compared to children. Our study is limited by the small number of subjects and because we asked the parents about influenza illnes in their children but did not test the children during the trial.

Future studies may elucidate the ability of LAIVs to induce cross-reactive antibodies and cellular immunity. In the present study, we are the first to show that vaccination with a mucosal influenza vaccine enhances antibody and B-cell immune responses in palatine tonsils. Better understanding of the tonsillar immune responses will aid the development of vaccination strategies aimed at enhancing local immunity against influenza.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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III

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RESEARCH ARTICLE

Immune Responses in Acute and Convalescent Patients with Mild, Moderate and Severe Disease during the 2009 Influenza Pandemic in Norway

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Abstract

Increased understanding of immune responses influencing clinical severity during pandemic influenza infection is important for improved treatment and vaccine development. In this study we recruited 46 adult patients during the 2009 influenza pandemic and characterized humoral and cellular immune responses. Those included were either acute hospitalized or convalescent patients with different disease severities (mild, moderate or severe). In general, protective antibody responses increased with enhanced disease severity. In the acute patients, we found higher levels of TNF-α single-producing $CD4^+$T-cells in the severely ill as compared to patients with moderate disease. Stimulation of peripheral blood mononuclear cells (PBMC) from a subset of acute patients with peptide T-cell epitopes showed significantly lower frequencies of influenza specific $CD8^+$ compared with $CD4^+$ IFN-γ T-cells in acute patients. Both T-cell subsets were predominantly directed against the envelope antigens (HA and NA). However, in the convalescent patients we found high levels of both $CD4^+$ and $CD8^+$ T-cells directed against conserved core antigens (NP, PA, PB, and M). The results indicate that the antigen targets recognized by the T-cell subsets may vary according to the phase of infection. The apparent low levels of cross-reactive $CD8^+$ T-

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cells recognizing internal antigens in acute hospitalized patients suggest an important role for this T-cell subset in protective immunity against influenza.

Introduction

During the 2009 influenza pandemic, young and otherwise healthy people experienced severe illness and mortality [1–4]. During the main wave of the pandemic in Norway, 1300 people were hospitalized, 200 patients received intensive care treatment, and 29 patients died [5]. Nevertheless, in hindsight, this pandemic was regarded as mild [6]. Post-pandemic studies have described the clinical picture, the risk factors associated with disease outcome, and effects of vaccines and antiviral medication [1,3,7–12]. Specific viral mutations and several host factors and underlying conditions, such as obesity and pregnancy, were identified and associated with increased disease severity [13–17]. People older than 65 years old experienced less severe infection, probably due to pre-existing cross-reactive immunity generated by previous H1N1 infections [18].

Seasonal vaccination or infection induces strain-specific neutralizing antibodies directed towards the viral surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). HA-specific antibodies measured by the hemagglutination inhibition assay (HI) are defined as the primary correlate of protection against influenza in man (HI titers $\geq$40) [19]. However, strain-specific antibodies do not provide cross-protection against new epidemic or pandemic viruses [20]. Hence, due to the lack of protective antibodies, the novel A(H1N1)pdm09 virus spread rapidly worldwide.

In contrast to antibodies, T-cells may mediate cross-protective immunity between strains due to recognition of epitopes from the conserved core antigens of the virus, which have a high degree of homology, e.g. (nucleoprotein (NP), the polymerases (PB1, PB2 and PA) and matrix (M) proteins. T-cells play important roles in coordinating and regulating the immune response against influenza [21]. $CD4^+$ T-cells help B-cells in producing neutralizing antibodies and secrete cytokines, which direct the activity of $CD8^+$ T-cells. $CD8^+$ T-cells contribute to protection by killing virus-infected host cells, and are essential for viral clearance. Infection with seasonal influenza A H1N1 virus induces memory T-cells that cross-react with the pandemic strain [22–25]. In a recent study from the UK, the presence of NP-specific T-cells prior to exposure was associated with significantly less symptomatic, PCR-positive seasonal and pandemic influenza disease [25]. More specifically, pre-existence of $CD8^+$ T-cells against conserved viral core epitopes correlated inversely with symptomatic illness in antibody naïve adults during the 2009 pandemic [26]. However in a human, high dose challenge model of seasonal influenza A virus, pre-existing influenza-specific $CD4^+$ T-cells, rather than $CD8^+$ T-cells, correlated with protection against mild disease [27]. In the early phase of A(H1N1)pdm09 virus infection, high levels of peripheral $CD4^+$ T-cells may correlate with disease severity [28], and different immune memory profiles develop depending on the severity of pandemic infection [29].

In the absence of strain specific antibodies, cross-reactive T-cells are considered important, as cellular immune responses may limit disease severity and death when infection is already established [21]. Current knowledge of human T-cell responses after natural infection with influenza remains limited. Due to the sudden nature of pandemics, with a stretched healthcare system primarily focused on treatment, there is limited immunological data from hospitalized patients with different disease severities [30]. Here, we describe and compare the immune

responses in acute and convalescent patients with different pandemic disease severities, with the hypothesis that the severely ill patients would have less cross-protective T-cell immunity. Although our study has limitations in sample sets and study design imposed by the pandemic, our results suggest that both the antigen targets and the T-cell subsets involved in recognition vary according to the phase of infection. This study increases our understanding of the immune responses associated with severe disease and hospitalization and may guide future treatment and development of improved influenza vaccines.

Material and Methods

Study design

We conducted a prospective observational study in 46 adult patients (>15 years old) during the main wave of influenza A(H1N1)pdm pandemic in October/November 2009 in Norway. Two groups of patients were recruited from two Norwegian university hospitals (Haukeland (HUS) and Akershus (AHUS) university hospital). Nasopharyngeal swabs were collected from patients at inclusion in the study, for confirmatory viral diagnosis by real-time RT PCR. Acute, patients (n = 27) hospitalized >24 hours at HUS provided one blood sample (peripheral blood mononuclear cells (PBMC) and serum) in the acute phase of disease (Fig 1). Convalescent patients (n = 19) were diagnosed initially at AHUS and blood samples were collected in the convalescent phase at 3 and 32 weeks post-infection at a designated outpatient clinic (Fig 1). Patient data were grouped according to disease severity into mild (no hospitalization), moderate (hospitalization $\leq$ 2 days) and severe illness (hospitalization > 2 days, often with lung infiltrations and oxygen requirement) [12] (S1 Table). All patients met the modified clinical case definition for A(H1N1)pdm09 disease, described previously [12]. Nineteen convalescent patients with influenza like illness (ILI) symptoms were recruited from hospitalized and outpatients, with mild, moderate or severe disease, after RT-PCR confirmation of A(H1N1)pdm09.

The Regional Ethical Committees approved the study (Regional Ethics Committee South-East Norway 2009/1853 and Regional Ethics Committee Western Norway 2009/2295). Written informed consent was obtained upon inclusion.

Isolation of PBMC and sera

PBMC were separated immediately by density gradient centrifugation of cell preparation tubes (CPT) or from ETDA tubes through Lymphoprep™ (StemCell technologies) according to the manufacturer's instructions and stored in liquid nitrogen in 90% foetal calf serum (FCS)/10% DMSO or 25% FCS/10% DMSO. Serum samples were aliquoted and stored at -20°C.

Hemagglutination inhibition test

Sera were pre-treated with receptor destroying enzyme (RDE) (Seiken, Japan) [31]. HI antibodies were detected in sera with 0.7% turkey red blood cells and 8 HA units of influenza A/California/07/09(H1N1) virus. HI titers are expressed as the reciprocal of the last serum dilution inhibiting hemagglutination. Negative titers were assigned a value of 5 for calculation purposes.

Microneutalization assay

Microneutralization (MN) was carried out as described in the WHO protocol [32] with minor modifications. Inactivated sera were pre-incubated with A/California/07/09 like virus. Cells were fixed in methanol containing 0.6% H_2O_2 for 20 min. Detection of influenza infected cells was done by ELISA using monoclonal anti-influenza A nucleoprotein primary antibody diluted

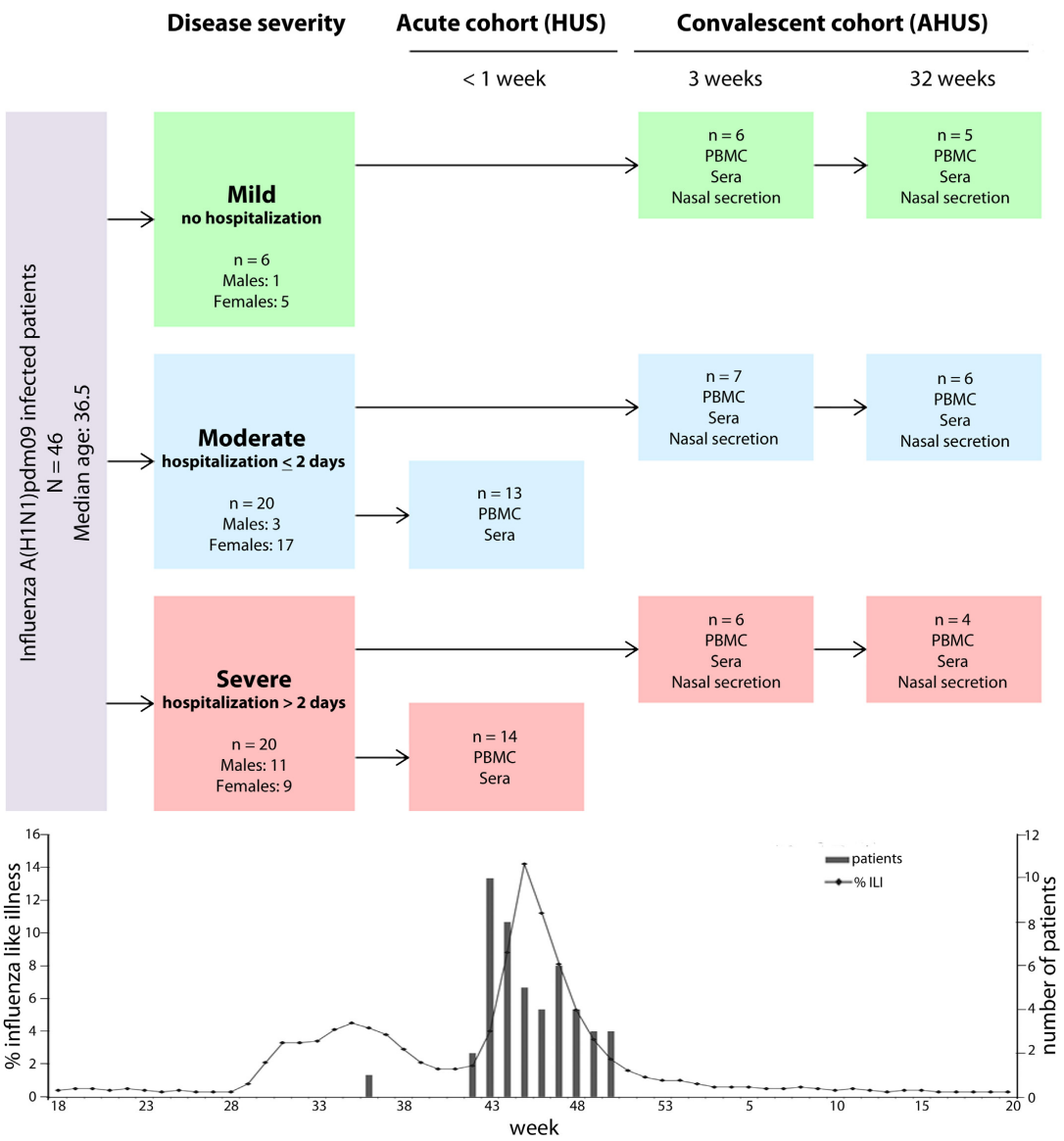

Fig 1. Study design. The inclusion of patients, time and types of samples collected in this study are shown in this flow-chart. Patients were recruited from two Norwegian university hospitals; Haukeland (HUH) or Akershus (AHUS). Nasopharyngeal swabs for influenza A(H1N1)pdm viral RT-PCR diagnosis were collected from patients at the time of inclusion for routine diagnosis. Subsequently sera and peripheral blood mononuclear cells (PBMC) were collected from hospitalized patients at HUH in the acute phase of disease. Sera and PBMC were collected at 3 and 32 weeks post disease onset from patients at AHUS (hospitalized and out-patients). Sample types available from individual patients are described in S1 Table. Sufficient numbers of PBMC were not available for all analyses. Patients were grouped according to disease severity as mild (no hospitalization), moderate (hospitalization $\leq$ 2 days) and severe (>2 days hospitalization) [12]. The trend-line in the graph below shows people with influenza like illness in Norway from end of April 2009 to May 2010. The first wave was due to other respiratory viruses than pandemic H1N1. In the same graph the number and time of inclusion of patients in this study are shown as columns.

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PBS, 5% skimmed milk powder and 0.1% Tween 20 and Horseradish peroxidase-labeled rabbit anti-mouse IgG secondary antibody diluted PBS, 5% skimmed milk powder, 0.1% Tween 20 and 1% bovine serum albumin. Liquid TMB substrate was used and the reaction was stopped after 18 minutes using 0.5M HCl. The absorbance was measured at 450 and 620nm and the 620nm readings were subtracted from the 450nm readings.

IgG ELISA

ELISA was used to measure influenza specific IgG in serum [33]. Ninety-six-well plates (maxisorb, Nunc) were coated with 4μg/ml of inactivated virus solution (100μl/well) and allowed to bind for ≥2 days at 4°C [34]. Specific antibody concentrations (arbitrary units) in unknown samples were determined based on defined pools of human sera.

Intracellular cytokine staining

Frozen PBMC were thawed and rested overnight in RPMI 1640 medium containing L-glutamine, 0.1 mM non-essential amino acids, 10 mM Hepes pH 7.4, 1 mM sodium pyruvate, 100 IU⁄ml penicillin, 100 μg⁄ml streptomycin, 0.25 μg⁄ml fungizone, and 10% FCS. Lymphocytes were stimulated for 16 hours with A/California/07/09 split virus antigen (2.5μg/ml HA, kindly provided by GSK, Belgium) and anti-CD28 (1μg/ml) anti-CD49d (1μg/ml) antibodies (Pharmingen, USA), Brefeldin A (1μg/ml) and Monensin (0.7μg/ml) (BD Biosciences, USA) [35]. For each patient sample, cytokine levels in non-stimulated cells (cells incubated in lymphocyte medium containing anti-CD28 (1μg/ml) anti-CD49d (1μg/ml) antibodies, Brefeldin A (1μg/ml) and Monensin (0.7μg/ml)) were subtracted from cytokine levels observed in corresponding influenza-stimulated cells, in order to determine the influenza-specific responses. HA-specific cells were stained for CD3, CD4, IFN-γ, IL-2 and TNF-α and analysed by a BD LSRFortessa flow cytometer (acquiring $\geq 3x10^5$cells). Representative gating strategy is illustrated in S1 Fig.

IFN-gamma ELISpot assay

IFN-γ responses were measured in pre-coated 96-well plates (Mabtech AB, Sweden) using 200,000 PBMC in AIM-V medium (Gibco) [31]. Positive (anti-CD3 or ConA) and negative controls (DMSO/AIM-V), A/California/07/09 whole virus, split virus antigen and peptide libraries (final concentration of 2μg/ml in DMSO/AIM-V) were added. Peptide assays were conducted for a subset of patients with available PBMCs (n = 16, 11 acute and 5 convalescent patients). The plates were read using a CTL S 6 Ultra V Immunospot analyzer (Cellular Technology Limited, OH, USA), and the results were plotted using GraphPad Prims 5 software (GraphPad Software, Inc., USA).

Peptide libraries

Synthetic peptide sets have been compiled in order to assess T-cell responses against different influenza antigens (internal or envelope) targeted by different T-cell subsets (CD4 or CD8) [36]. Experimentally verified T-cell epitopes were identified based on querying the Immune Epitope Database [37]. Universal epitopes were selected from influenza strains circulating between 1934–2009 according to prevalence, conservancy, and HLA super type coverage [27]. This approach circumvents the need for individual HLA typing. Peptides unique for A (H1N1)pdm09 have also been identified [36]. The peptide lengths were 8–11 amino acids for MHC class I and ≥ 13 amino acids for MHC class II. The peptides (31 MHC class I and 33 MHC class II) were chemically synthesized by Fmoc chemistry and HPLC purified (Mimotopes, Australia). The peptides were pooled into 7 distinct sets according to strain specificity

(universal or pandemic), T-cell subset (CD4/CD8 T-cells), and antigen source (internal or external) [36].

Statistics

Statistical analyses were performed in GraphPad Prism, version 5 for Mac and Prism 6 for Windows (GraphPad Software, USA). For all statistical tests, a *P*-value of ≤ 0.05 was considered significant. For comparisons of intracellular cytokine production we used Student's t-test. All other data were analyzed using non-parametric methods; Mann-Whitney test, Kruskal-Wallis or Wilcoxon test (paired group). Correlations were calculated using Spearman's correlation coefficient.

Results

Study patients enrolled from two major University hospitals

In total 46 patients were enrolled in two separate groups from two university hospitals in Norway during the 2009 pandemic (Fig 1). The patients had a median age of 36.5 years old (age range 19–93), and a female/male ratio of 31/15 (S1 Table). Laboratory samples, epidemiological and demographic data were collected from the two groups of patients: either from acute hospitalized (n = 27, sampled once), or convalescent patients (n = 19, sampled twice, at weeks 3 and 32 post infection). Twenty patients had moderate disease (hospitalized $\leq$ 2 days), and 20 patients had severe influenza disease (hospitalized >2 days). Six convalescent patients had mild influenza disease (no hospitalization) without any underlying disease conditions. Forty percent of the moderately and severely ill patients had comorbidities: 11 had pulmonary or coronary diseases, six patients had other underlying diseases (autoimmunity, malignancy), and five were pregnant, reflecting the diversity of patients hospitalised during the pandemic in Norway. Of these, seven patients were vaccinated with the Pandemrix vaccine (GSK) (S1 Table).

Serological responses

Severely ill patients had higher antibody responses. To study the humoral immune response, sera were collected from all patients (one time point for the acute patients and two time points for 15 paired convalescent samples) and tested for A(H1N1)pdm09-specific antibodies with HI, MN and IgG ELISA assay (Fig 2A,2B, and 2C). No data on pre-infection antibody titers were available, as patients were recruited upon clinical presentation with confirmed A(H1N1)pdm09 disease. HI titers $\geq$40 were present in sera from 11/27 of acute and 17/19 of convalescent patients at three weeks post infection (Fig 2A). Seven patients in the acute group (3 moderately and 4 severely ill) received pandemic vaccination, but only 4 of these patients had HI titers $\geq$40 (2 moderate and 2 severe). In the convalescent patient group, 2 patients (severely ill) were vaccinated and both had titers $\geq$ 40 (S1 Table). We did not find differences in HI or MN responses in patients with or without comorbidities.

The convalescent patients recovering from severe disease had significantly higher HI titers (all subject $\geq$40), MN titers and IgG antibody levels than patients with mild disease at 3 weeks post post-infection (Fig 2A–2C). Similarly, in the acute patients there was a trend that the severely ill patients had higher antibody responses than the moderately ill (Fig 2A–2C). In order to study the functionality of the antibody response, MN assay was performed (Fig 2B). The MN data correlated with the HI results (both patient groups; $r = 0.9$, $p<0.0001$). For the convalescent patients the kinetics of the antibody response was investigated in the paired subjects, and the antibody levels correlated significantly with severe and moderate disease (HI-titer: $r = 0.786$, $p = 0.0001$ and IgG: $r = 0.677$, $p = 0.0014$). The HI and IgG antibody levels

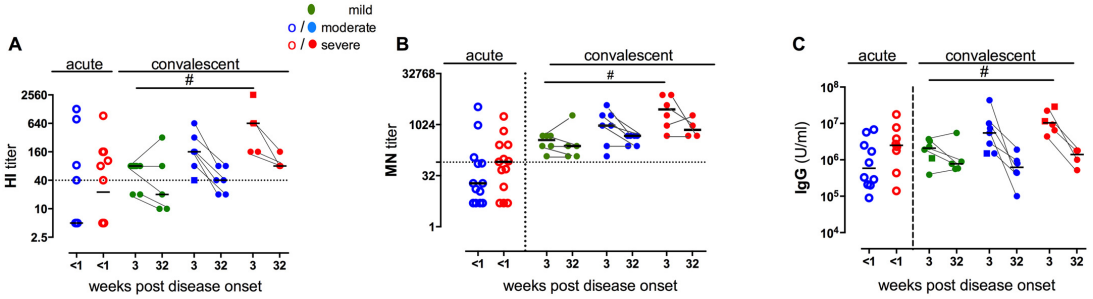

Fig 2. Humoral responses against A(H1N1)pdm09 virus. Humoral responses against A(H1N1)pdm09 virus in acute (one time point) and convalescent patients (3 and 32 weeks following disease onset) plotted according to the disease severity. A) HI titers in acute (n = 27) and convalescent patients (n = 19 and 15). The dotted line represents an HI titer of 40. B) Microneutralization (MN) titers in acute (n = 27) and convalescent patients (n = 19 and 15). The dotted line represents an MN titer of 80. C) Serum IgG concentration in acute (n = 25) and convalescent patients (n = 19 and 15). ○ = acute samples, ● = convalescent samples □ = single samples in the convalescent group. Disease severity is defined as mild (out-patients), moderate (hospitalized ≤ 2 days) or severe (hospitalized > 2 days). #p≤ 0.05 (Mann Whitney test (HI and IgG) unpaired t-test (MN)).

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declined significantly between 3 and 32 weeks post infection in the convalescent patients, but remained above the protective HI-threshold in most patients (67%), with severely ill patients showing higher antibody responses compared to patients with mild disease. Fig 2A).

Cellular responses

High CD4+ T-cell TNF-α response in acute severely ill patients. To study the functionality of T-cell responses, PBMC from acute subjects (n = 24) were stimulated with split virus antigen (predominantly containing surface antigens) and analyzed for intracellular cytokines by flow cytometry. The percentages of $CD4^+$ T-cells secreting HA-specific single (Fig 3A and 3B) or multiple cytokines (Fig 3C and 3D) are shown. High unspecific background levels of TNF-α were observed in moderately and severely ill patients, with the highest levels in the severely ill patients (range 0.041–4.12% of $CD4^+$ T-cells, data not shown). Severely ill patients (n = 12) had significantly higher numbers of influenza specific T-cells secreting TNF-α than moderately ill patients (n = 12) (Fig 3A and 3B). When comparing the severely ill with moderately ill patients we also observed a non-significant trend with higher levels of IL-2 (mean of 0.12% and 0.04%, respectively) and lower levels of IFN-γ (mean of 0.01% and 0.09% of, respectively) positive $CD4^+$ T-cells. The percentage of $CD4^+$ T-cells simultaneously expressing two (IL-2/TNF-α) or three (IFN-γ/IL-2/TNF-α) cytokines was higher in the severely ill than in the moderately ill group, although the difference was not significant (Fig 3C and 3D).

Acute patients have a high envelope specific-$CD4^+$ and low $CD8^+$ T-cell response. To further dissect the T-cell responses, PBMC from a subset of acute patients (n = 11) were stimulated with different peptide pools of influenza A specific epitopes in the IFN-γ Elispot assay [36] (Fig 4A). We measured $CD4^+$ or $CD8^+$ responses induced by epitopes from either universal (envelope or internal) or pandemic specific antigens [36]. We observed significantly higher frequencies of cells within the $CD4^+$ compared with $CD8^+$ compartment (5-fold) (Fig 4A). The number of $CD4^+$ T-cells recognizing epitopes from the viral envelope (HA and NA; CD4e peptides) was significantly higher than $CD4^+$ T-cells recognizing the conserved core antigens (PA, PB, M, NP, NS2 and NS1; CD4i peptides) (Fig 4A). Overall, the acute patients were characterized by low $CD8^+$ T-cell responses. No significant differences in T-cell responses were found

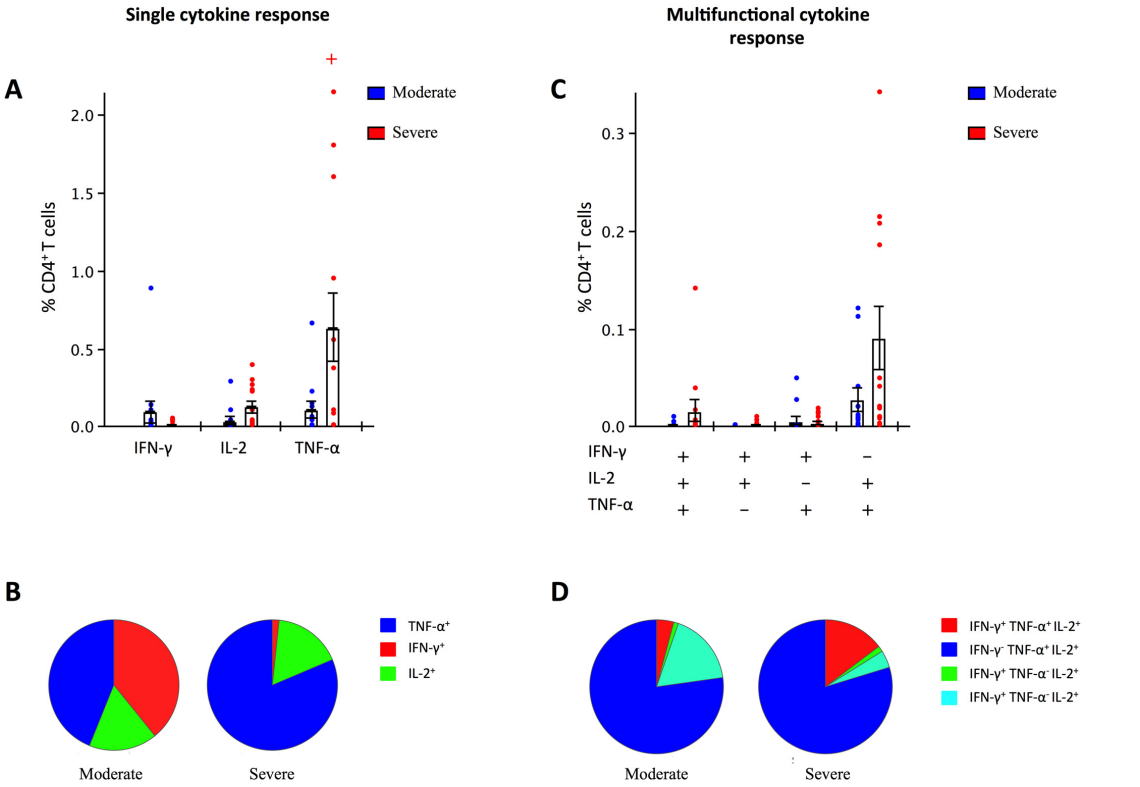

Fig 3. CD4+ T-cell cytokine responses in moderately and severely ill acute patients. PBMC from acute patients (n = 24) were stained for intracellular cytokines and the percentage of CD4+ T-cells secreting either single (A and B) or multiple (C and D) cytokines were measured by flow cytometry. Each symbol represents the response of one individual with bars depicting the mean and SEM percentage of CD4+ T-cells. +p<0.05 (Student's t test). Gating strategy is shown in S1 Fig. Disease severity is defined as moderate (hospitalized ≤ 2 days) or severe (hospitalized > 2 days).

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between moderately and severely ill patients (Fig 4A). When PBMC from the acute patients were stimulated with CD4+ and CD8+ T-cell epitopes unique to the pandemic strain, the CD4+ T-cell responses were also predominantly directed against the HA and NA antigens (pCD4e) of the pandemic strain.

CD4+ and CD8+ T-cell responses were directed against internal epitopes in the convalescent patients. In a subset of the convalescent patients paired samples from two time points (3 and 32 weeks) were available (n = 5). In contrast to acute phase patients, similar total CD4+ and CD8+ T-cell responses were observed at 3 and 32 weeks post-infection (Fig 4C). Influenza specific CD4+ and CD8+ T-cell responses were higher in the convalescent patients (week 3) as compared with the acute patients (3- or 4-fold, respectively). Moreover, higher frequencies of both CD4+ and CD8+ T-cells recognized internal antigens compared with envelope antigens at both time points (Fig 4C) (p = 0.063 at week 3 for both CD4+ and CD8+ T-cells). A clear decline in frequencies of both IFN-γ producing CD4+ and CD8+ T-cells was observed over time (Fig 4C). Stimulation with live virus showed similar T-cell kinetics as obtained by peptide pools (data not shown). No clear trends linking the T-cell responses to clinical severity could

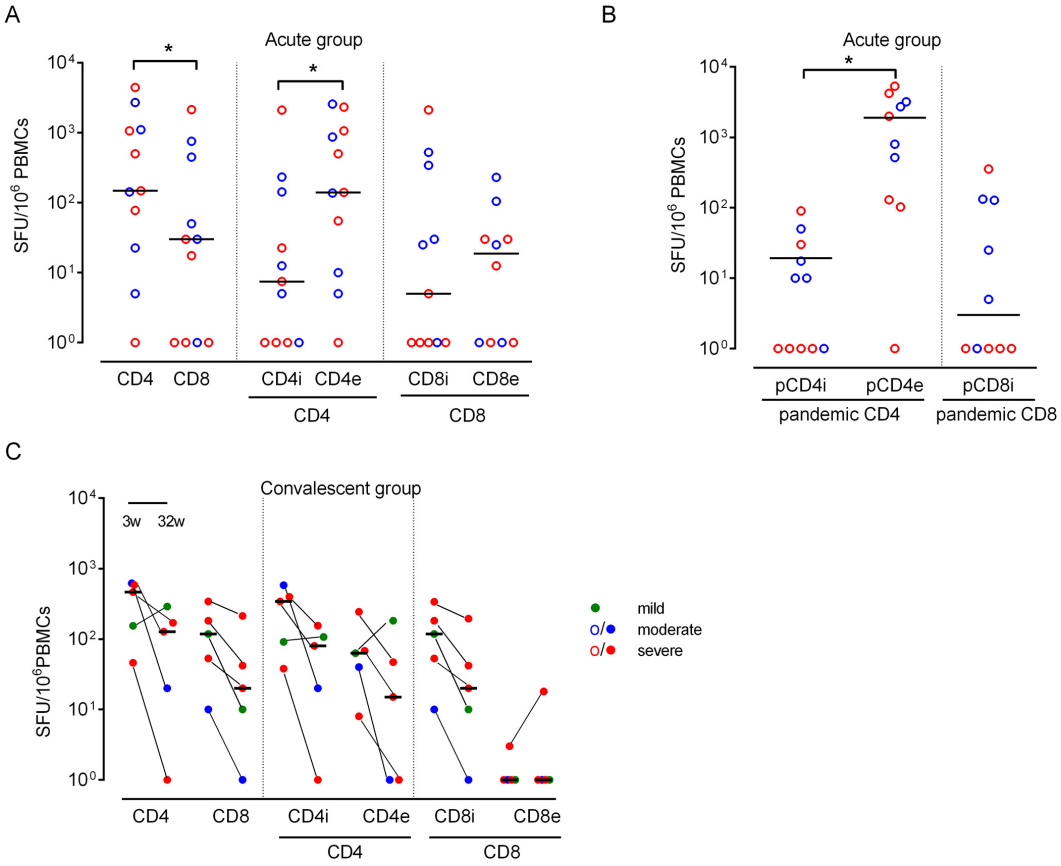

Fig 4. T-cell responses measured by IFN-γ Elispot assay after stimulation with universal or A(H1N1)pdm09 specific peptides. A) T-cell responses against universal influenza epitopes in a subset of acute patients (n = 11). B) T-cell responses against A(H1N1)pdm09 specific epitopes in a subset of acute patients (n = 11). C) T-cell responses against universal influenza epitopes in a subset of convalescent patients (n = 5) with paired samples for 3 and 32 weeks. ○ = acute samples, ● = convalescent samples. Disease severity is defined as mild (out-patients), moderate (hospitalized ≤ 2 days) or severe (hospitalized > 2 days). The bars are plotted as median with range. The T-cell responses were directed against epitopes from internal (i = NP, M1, PA, PB and NS) or external (e = HA and NA) influenza antigens. CD4 = CD4i + CD4e and CD8 = CD8i + CD8e. *p≤ 0.05 (Wilcoxon matched-pair signed-rank test). PBMC were also shown to respond to stimulation with live influenza virus (data not shown).

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be observed for the convalescent patients, possibly due to the low number of patients in each disease group.

Discussion

Identifying risk factors and immune correlates of protection against human influenza is crucial for future pandemic preparedness. There is currently limited data on immunological responses in hospitalized patients due to the difficulties of collecting samples in the midst of a pandemic. The emergence of the 2009 influenza pandemic represented a unique opportunity to relate clinical outcomes to influenza specific immune responses. Here, we have characterized both

antibody and T-cell responses in two study groups of either acute or convalescent patients with different disease severities (mild, moderate and severe) in Norway. We were particularly interested in studying T-cell responses in severely as compared with moderately ill patients. Limitations in the possibilities to obtain an ideal sample set, including base line and follow up samples from the same patients have to be taken into consideration when interpreting the results. Nevertheless, the patients in our study reflect the pandemic situation in Norway with respect to age, comorbidities and vaccination status, resulting in different pre-existing immunity status.

Patients were included upon infection and therefore we unfortunately do not have data on pre-infection antibody levels in our patients. However, Norwegian seroprevalence data showed low frequencies of pre-existing protective antibodies (1,7%) in the population prior to the 2009 pandemic as well as in health care workers [38,39]. Therefore the antibodies measured in our patients are probably induced by infection with the pandemic virus. Only four of the seven vaccinated patients in the acute group had high antibody levels, which may have been influenced by the timing of pandemic vaccination in Norway since the pandemic wave, and vaccination occurred almost simultaneously [40].

Antibody responses were influenced by disease severity with the highest levels of antibodies found in patients with severe disease. Although the antibody levels declined 8 months after infection, protective antibody levels remained present in patients with severe disease. This is in agreement with a recent study showing a correlation between the severity of influenza disease and antibody levels [41]. Another study from the UK showed high antibody titers for a minimum of 15 months after natural infection with A(H1N1)pdm09 [31]. It has previously been shown that severely ill patients had elevated levels of viral replication in the lower respiratory tract or extended viral shedding, which might lead to high antibody levels [42]. No measurable differences in upper respiratory tract viral loads were found between the different illness severities in our patients when comparing PCR data at the time of inclusion (Ct values in S1 Table). Two patients with mild disease did not achieve protective HI antibody levels, confirming previous findings that not all infected individuals show HI seroconversion [43].

Pandemic influenza may occasionally cause viremia in severely ill or immunocompromised patients [44–46]. Four of the 27 hospitalized patients in the acute group with moderate or severe disease had viremia, with systemic viral dissemination. Viral factors, in particular a point mutation in HA, have been associated with severe disease [16,17]. No such mutation was detected in the viruses sequenced from our PCR positive patients (S2 Table). However, *de novo* mutations may occur and a low frequency of mutant virus in a high background of 222D virus may not be detected in the upper respiratory tract. Several patients with clinical pandemic influenza were PCR negative, possibly with lower respiratory tract infection not detected by nasopharyngeal swabs. Therefore we cannot exclude that this mutation may have been present in the lower respiratory tract of these patients [44,47,48].

T-cells are central in coordinating the immune response, and both the magnitude and the quality of T-cell responses are critical for the control of viral infections [42,49]. The high unspecific TNF-α background levels observed in the severely ill patients in this study, suggests an overall elevated and possibly dysregulated immune activation in these individuals. Moreover, the high frequency of single TNF-α $CD4^+$ T-cells in the severely ill patients suggest exhaustion or an altered immune response in these patients, affirmed by low single IFN-γ $CD4^+$ T-cells and low frequencies of multifunctional cells [50]. Protective responses to respiratory viruses are typically biased towards a Th1 response, producing high levels of IFN-γ, but also IL-2 and TNF-α, promoting cytolytic activity and viral clearance [51,52]. The multifunctional $CD4^+$ T-cells are regarded as functionally superior to single-cytokine producers [53]. The low levels of multifunctional $CD4^+$ T-cells in the acute, severely ill patients could possibly

be linked to the severity of their disease. However, future studies with higher numbers of participants are needed to confirm this. Although present at low frequencies, the multifunctional HA specific $CD4^+$ T-cells were dominated by IFN-γ^-IL2$^+$TNF-α cells. This is in agreement with previous findings that after A(H1N1)pdm09 exposure, a primed IFN-γ^-IL2$^+$TNF-α^+ non-polarized precursor T-cell population (Thpp) has been observed [54], representing a recently induced memory response to influenza [55]. This small multifunctional $CD4^+$ T-cell population has high proliferative potential and may be important for protection against future infection [54]. The general elevated T-cell responses in the convalescent group may reflect recall expansion of this subpopulation.

After natural A(H1N1)pdm09 infection $CD4^+$ T-cells recognize both unique and conserved HA epitopes [56]. HA specific naïve T-cells undergo significant expansion, whereas memory T-cells directed towards conserved epitopes have a more restricted expansion [56]. This may be reflected in the striking dominance of T-cell responses against external envelope antigens seen in the acute patients as opposed to conserved internal antigens in the convalescent patients.

Recent studies suggest that different immune memory profiles may develop depending on the severity of A(H1N1)pdm09 infection [28,29]. In agreement with this, we found significant differences in T-cell responses between and within our patient groups. Acute patients showed higher $CD4^+$ than $CD8^+$ T-cell responses compared to the healthy Norwegian population (5-fold /1.5-fold respectively) [36]. The convalescent patients showed higher levels of both $CD4^+$ and $CD8^+$ T-cells, compared to acute patients and healthy individuals. However, no significant differences were found between disease severities.

Although pre-existing cross-reactive $CD8^+$ T-cells have been correlated with reduced severity of symptoms during natural influenza infection [26], it is not clear whether these circulating cells protect against disease or whether they potentially reflect local pulmonary T-cells that mediate viral clearance [57]. The low levels of peripheral $CD8^+$ T-cells may be linked to disease severity. However, the low $CD8^+$ levels may be due homing of this T-cell subset to the infected lung tissue, and hence absence in the blood. In support of this, Zhao and coworkers found that high levels of peripheral $CD4^+$ T-cells against internal viral proteins in the early phase of infection, rather than low levels of $CD8^+$ cells T-cells, correlated with disease severity during the 2009 pandemic [28]. The higher levels of both $CD4^+$ and $CD8^+$ T-cells in the convalescent patient group could indicate subsequent proliferation in the blood. The phenotype of T-cells protecting against influenza infection remains to be defined. While our T-cell data provide preliminary evidence for the importance of T-cells in protection from severe disease, future studies should focus on the functionality of these T-cells (killing and degranulation markers) to dissect how T-cells influence disease severity.

Excessive immune responses have been assumed to play a role in the pathogenesis of influenza virus disease, this assumption has been challenged by the findings that severe disease is characterized by inadequate, rather than excessive, adaptive immune responses and robust viral replication [57]. Our data suggests that both the phenotype of T-cells and the influenza epitopes they target vary according to the phase of infection. However, additional studies, following larger cohorts of well-characterized influenza infected individuals will be necessary to define the relationship between T-cell subpopulations and disease severity or phase of infection.

Despite the limitations of this study, the apparently low levels of $CD8^+$ T-cell responses in patients hospitalized during the acute phase, suggests an important role of these T-cells in protective immunity against influenza. Moreover, the observation that both $CD4^+$ and $CD8^+$ T-cell responses are directed against epitopes from conserved internal antigens in the convalescent phase of infection may guide universal influenza vaccine development. Our results

support the idea that the clinical severity of pandemic infection is influenced by the host´s immune response and not only the characteristics of the novel virus.

Supporting Information

S1 Fig. Representative gating strategy for unstimulated and influenza-specific intracellular cytokine secretion analysis by flow cytometry. Frozen PBMC form a severely ill acute patient were thawed and rested overnight and A) incubated for 16 hours (5%CO_2, 37°C) in lymphocyte medium containing anti-CD28 (1μg/ml) antibodies, Brefeldin A(1μg/ml) and Monensin (0.7μg/ml) or B) stimulated for 16 hours with 2.5μg/ml HA of A/California/07/09 split vius vaccine and anti-CD28 (1μg/ml) anti-CD49d (1μg/ml) antibodies, Brefeldin A(1μg/ml) and Monensin (0.7μg/ml). The basal cytokine levels in non-stimulated cells were subtracted from the cytokine levels observed in the influenza stimulated cells. Cells were stained with fluorochrome conjugated antibodies against CD3, CD4, IFN-γ, IL-2 and TNF-α and acquired by a BD LSRFortessa flow cytometer (acquiring $\geq 3x10^5$cells per sample) and data analyzed by FloJo software (Version 8.8.7).
(TIFF)

S1 Methods and Results. .
(DOCX)

S1 Table. Demographics of the patients included in the study. [1]Disease severity is defined as mild (out-patients), moderate (hospitalized $\leq$ 2 days) or severe (hospitalized $>$ 2 days).
[2]Time from onset of clinical symptoms [12].
[3]PBMCs: + samples included in analyses, (+) samples excluded from analyses,—samples never received for analyses
[4]HI titer only.
[5]For convalescent patients, the HI titers are given as: titer at 3 weeks (titer at 32 weeks), e.g. 160 (20).
(DOCX)

S2 Table. Overview of the mutations found in the HA gene.
(DOCX)

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Author Contributions

Conceived and designed the experiments: KGIM RJC SM JEB CMJ. Performed the experiments: KGIM RJC AGH ÅJL SM FO GT CMJ JEB. Analyzed the data: KGIM RJC AGH ÅJL SM FO GT CMJ. Contributed reagents/materials/analysis tools: KGIM RJC AGH ÅJL SM FO GT CMJ BP JEB. Wrote the paper: KGIM RJC AGH SM FO GT CMJ ÅJL.

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Appendix papers I-IV

Effectiveness of neuraminidase inhibitors in reducing mortality in patients admitted to hospital with influenza A H1N1pdm09 virus infection: a meta-analysis of individual participant data

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Summary

Background Neuraminidase inhibitors were widely used during the 2009–10 influenza A H1N1 pandemic, but evidence for their effectiveness in reducing mortality is uncertain. We did a meta-analysis of individual participant data to investigate the association between use of neuraminidase inhibitors and mortality in patients admitted to hospital with pandemic influenza A H1N1pdm09 virus infection.

Methods We assembled data for patients (all ages) admitted to hospital worldwide with laboratory confirmed or clinically diagnosed pandemic influenza A H1N1pdm09 virus infection. We identified potential data contributors from an earlier systematic review of reported studies addressing the same research question. In our systematic review, eligible studies were done between March 1, 2009 (Mexico), or April 1, 2009 (rest of the world), until the WHO declaration of the end of the pandemic (Aug 10, 2010); however, we continued to receive data up to March 14, 2011, from ongoing studies. We did a meta-analysis of individual participant data to assess the association between neuraminidase inhibitor treatment and mortality (primary outcome), adjusting for both treatment propensity and potential confounders, using generalised linear mixed modelling. We assessed the association with time to treatment using time-dependent Cox regression shared frailty modelling.

Findings We included data for 29 234 patients from 78 studies of patients admitted to hospital between Jan 2, 2009, and March 14, 2011. Compared with no treatment, neuraminidase inhibitor treatment (irrespective of timing) was associated with a reduction in mortality risk (adjusted odds ratio [OR] 0·81; 95% CI 0·70–0·93; p=0·0024). Compared with later treatment, early treatment (within 2 days of symptom onset) was associated with a reduction in mortality risk (adjusted OR 0·48; 95% CI 0·41–0·56; p<0·0001). Early treatment versus no treatment was also associated with a reduction in mortality (adjusted OR 0·50; 95% CI 0·37–0·67; p<0·0001). These associations with reduced mortality risk were less pronounced and not significant in children. There was an increase in the mortality hazard rate with each day's delay in initiation of treatment up to day 5 as compared with treatment initiated within 2 days of symptom onset (adjusted hazard ratio [HR 1·23] [95% CI 1·18–1·28]; p<0·0001 for the increasing HR with each day's delay).

Interpretation We advocate early instigation of neuraminidase inhibitor treatment in adults admitted to hospital with suspected or proven influenza infection.

Funding F Hoffmann-La Roche.

Introduction

The neuraminidase inhibitors, oral oseltamivir and inhaled zanamivir, were the predominant medical countermeasure available from emergence of the influenza A H1N1pdm09 virus in early 2009, until the first release of monovalent H1N1 vaccines in October, 2009. Prescribing data from seven countries (Australia, Canada, France, Germany, Japan, UK, USA) suggest at least 18·3 million individuals received oseltamivir between May 1, 2009, and Dec 31, 2009.[1] Country-specific policies for use of neuraminidase inhibitors during the 2009–10 pandemic varied from no use, to targeted use in at-risk patients (most countries), to treatment of all patients with clinical illness (UK). Most use of neuraminidase inhibitors worldwide was in the form of oseltamivir—eg, 97·5% of neuraminidase inhibitors used in the USA.[2]

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There is little prepandemic evidence pertaining to the effectiveness of neuraminidase inhibitors in reducing mortality in patients admitted to hospital with influenza; most evidence comes from observational studies of treatment of seasonal influenza, often in highly specific groups of patients.[3–9] Thus, in 2009–10, neuraminidase inhibitors were used on the basis of rational deduction that they would reduce mortality due to influenza A H1N1pdm09 virus infection rather than on strong pre-existing evidence, although data from treatment of human influenza A H5N1 cases suggested this reduction in mortality might be possible.[10,11] Japanese clinicians used neuraminidase inhibitors widely to treat all people presenting with clinical influenza in 2009–10 and recorded the lowest pandemic mortality rate of any developed country.[12–14] Although a similar treat-all policy existed in the UK in 2009, uptake of neuraminidase inhibitors in patients admitted to hospital with influenza A H1N1pdm09 was low.[15]

Two systematic reviews and meta-analyses have examined the effectiveness of neuraminidase inhibitors in reducing mortality due to influenza. Both suggest substantial reductions in mortality by two-thirds to three-quarters compared with no treatment.[16,17] However, limitations are apparent, such as the heterogeneity of studies included and inadequate adjustment for potential confounding. Importantly, neither was able to adjust for the likelihood of a patient receiving antiviral treatment (propensity)—a crucial consideration when antiviral drugs might have been prioritised towards the sickest patients—and neither was able to use a pooled analysis approach with individual participant data.[18]

Methods

Study design and identification of datasets

The Post-pandemic Review of anti-Influenza Drug Effectiveness (PRIDE) research consortium was set up in October, 2011, and is coordinated by the Health Protection and Influenza Research Group at the University of Nottingham, Nottingham, UK. The aim of the collaboration is to do individual participant data meta-analyses of the effectiveness of antiviral use on outcomes of public health importance during the 2009–10 influenza pandemic. Members of the PRIDE research consortium are listed in appendix pp 1–6.

The initial identification of potential data contributors was done on the basis of a systematic search of 11 databases (date of last search April 19, 2012) for observational studies (case series, case-control, and cohort studies) and randomised controlled trials done between March 1, 2009 (Mexico), or April 1, 2009 (rest of the world), until the WHO declaration of the end of the pandemic (Aug 10, 2010), assessing the association between neuraminidase inhibitor treatment and clinical outcomes (mortality, influenza-related pneumonia, admission to critical care, length of stay in hospital and admission to hospital). We searched Ovid Medline (reports from 1996 onwards) and Embase (1980 onwards) using a comprehensive search strategy. We also searched CINAHL, CAB Abstracts, ISI Web of Science, PubMed, UK PubMed Central, Scopus, WHO regional indexes, LILAC, and J-STAGE databases using Boolean logic and core search terms relating to pandemic influenza (including influenza A virus OR H1N1 subtype OR swine origin influenza AH1N1 virus) AND exposure of interest—ie, antiviral drugs (including neuraminidase inhibitors OR oseltamivir OR zanamivir OR peramivir) AND clinical outcome measures (including pneumonia, or critical care/intensive care, or mortality). We identified further studies from reference lists of relevant articles and through contact with subject area experts (via JSN-V-T). All search results were limited to human beings with no language restrictions. Our detailed search strategy is reported elsewhere.[17]

On the basis of this search, we contacted 401 potential data contributors, identified during the conduct of our previously reported systematic review;[17] these potential contributors included several corresponding authors from different papers but potentially related to the same source dataset, as an all-inclusive approach. We recruited additional centres through our network of global collaborators, publicity at conferences attended, and by word of mouth. Centres fulfilling the minimum dataset requirement (appendix pp 7–8) were eligible for inclusion. We requested data for both laboratory confirmed and clinically diagnosed pandemic influenza A H1N1pdm09 cases, but allowed centres to provide individual patient data extending to March 14, 2011 (third pandemic wave cases). Clinically diagnosed cases that could not be confirmed by virology were diagnosed on the basis of clinical signs and symptoms that, in the opinion of the attending physician, were judged to be representative of influenza-like illness, in the absence of any other more likely diagnosis. We deliberately accepted diagnoses made on clinical judgment rather than specifying a set of clinical criteria, since case definitions of influenza-like illness vary within and between countries. This study was granted exemption from full ethical review by the University of Nottingham Medical School Research Ethics Committee, provided that each contributing centre held its own institutional review board approval for data collection and sharing.

Data standardisation, exposures, outcomes, and covariates

A common data dictionary was developed and individual datasets standardised according to these definitions (appendix pp 9–15) before pooling for analysis.

The primary outcome variable was mortality, defined as death occurring during admission to hospital or individual study follow-up period for the generalised linear mixed regression models and as death occurring within 30 days of illness onset in the Cox regression models. Use of neuraminidase inhibitors (exposure) was defined and compared as follows: neuraminidase inhibitor treatment (irrespective of timing) versus none; early neuraminidase inhibitor treatment (starting treatment ≤2 days after

symptom onset) versus later (initiation >2 days after symptom onset); early neuraminidase inhibitor treatment versus none; and later neuraminidase inhibitor treatment versus none. Additionally, we created a continuous exposure variable, representing time (in days) between symptom onset and treatment initiation (0 meaning treatment commenced on day of symptom onset). Covariates in the final multivariable models were "inpatient treatment with oral or intravenous antibiotics" and "inpatient treatment with systemic corticosteroids" prescribed during the admission to hospital for influenza along with treatment propensity scores. We were unable to adjust for dose or duration of such treatments because of the scarce availability of these data across the individual datasets.

Propensity scoring

We calculated propensity scores for the likelihood of neuraminidase inhibitor treatment for each patient within individual datasets using multivariable logistic regression for binary treatment variables and generalised propensity score estimation for the continuous time to treatment variable as described by Hirano and Imbens.[19] For each separate study dataset we calculated propensity scores (likelihood of treatment) for each of the four main exposure measures: neuraminidase inhibitor at any time (yes or no), early versus late neuraminidase inhibitor, early versus no neuraminidase inhibitor, and later (>2 days) versus no neuraminidase inhibitor. Covariates were then included as follows, irrespective of significance: age, sex, comorbidity (yes or no), a proxy indicator of severe disease (yes or no), which were, in order of preference, severe respiratory distress; shortness of breath; unweighted symptom score; or, if none of these indicators were available, we used one of the following measures of severity: AVPU (alert, voice, pain, unresponsive) mental status examination score, Glasgow Coma Scale score, Sequential Organ Failure Assessment score, or CURB-65 (confusion, urea, respiratory rate, blood pressure, age ≥65 years) pneumonia severity scores, if these were available, entered as a continuous variable. We added the following variables when available to create an extended model, using a parsimonious approach that retained only significant covariates in the final model: obesity, smoking, pregnancy, asthma, chronic obstructive pulmonary disease, lung disease, heart disease, immunosuppression, neurological disease, renal disease, and diabetes. We rejected variables with more than 25% missing data. Some variables used for the propensity score calculation, such as comorbidity (binary) and illness severity at presentation (binary), were derived at individual study level only and were not appropriate for inclusion in the pooled dataset analysis because of the heterogeneity in definition of these variables between studies.

The appropriateness of the propensity derivation models was assessed graphically by comparing the distribution of estimated propensity scores across treatment groups for each individual dataset.[20] Propensity scores were then categorised into quintiles for each individual dataset.

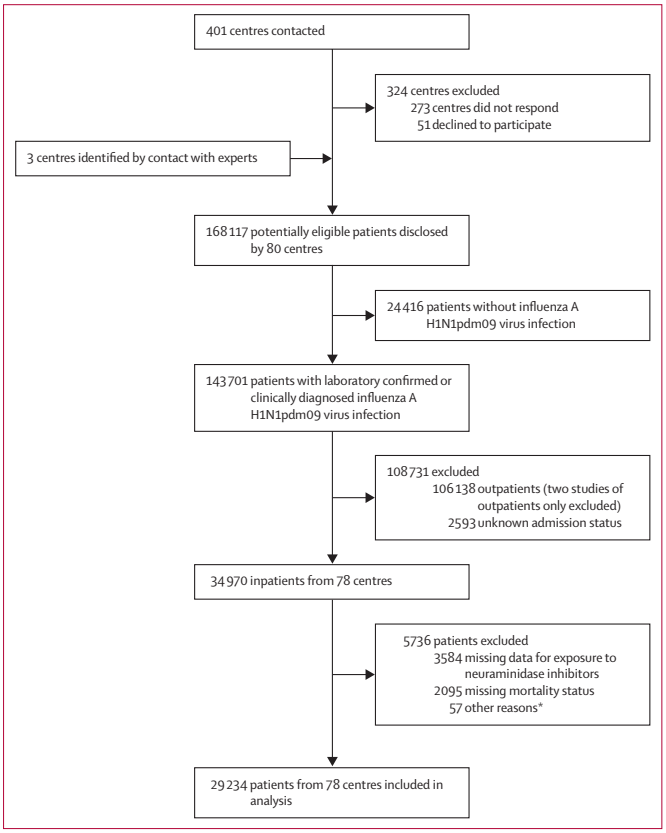

Figure 1: **Study flow diagram**
*47 overlapping data; one onset of illness before March 1, 2009 (Mexico); nine missing data for key variables.

Statistical analysis

We used a generalised linear mixed model to account for clustering of effects by study using the *xtmelogit* command in Stata (version 12). We included "study" as a random intercept to account for differences in baseline crude mortality rate at each site. We adjusted the model for treatment propensity, inpatient antibiotics, and systemic corticosteroids. We included missing data in covariates as a separate dummy category. The overall analysis included patients of all ages with laboratory or clinically diagnosed influenza A H1N1pdm09. We did prespecified stratified analyses for adults and children (<16 years), pregnant women (irrespective of age), laboratory confirmed influenza A H1N1pdm09 cases, and patients admitted to critical care units. Additionally, for a subset of our sample for whom exact onset and treatment initiation times were

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available, we investigated the association between time to initiation of antiviral treatment and mortality within 30 days of illness onset using a time-dependent Cox regression shared frailty model (to account for clustering by study) adjusted for propensity score and inpatient treatment with antibiotics or systemic corticosteroids. Antiviral treatment was modelled as a time-dependent covariate to overcome immortal time bias (ie, survivor bias). Results from the generalised linear mixed model are expressed as relative risks of mortality using odds ratios (ORs) and hazard ratios (HRs) for the Cox regression analysis with 95% CIs. We used Stata (version 12) for all analyses.

The protocol[21] for this study was registered with the PROSPERO register of systematic reviews, number CRD42011001273.

Role of the funding source

The funder of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The funder has not and will never have access to the data. Each collaborator had access to the raw data from his or her centre. SGM, SV, PRM, JL-B, and JSN-V-T had access to the pooled dataset. The corresponding author (JSN-V-T) had full access to all the data in the study and the final responsibility for the decision to submit for publication.

Results

We received replies from 128 (32%) of 401 centres contacted; of these 77 (60%) confirmed willingness to participate and the remainder declined (36 [28%] had no data; three [2%] agreed initially but later withdrew because of lack of capacity for data extraction, institutional review board restrictions preventing sharing of individual participant data, or failure to obtain government approval for data sharing; 12 [9%] had agreed in principle, but were unable to share data within project timescales). No data were requested from nor provided by pharmaceutical companies. After exclusion of duplicate responses (same

	All patients	Deceased	Survived
Number of patients*	29 234 (100%)	2784 (10%)	26 450 (90%)
Number of male cases (n=29 226)†	14 431 (49%)	1433 (51%)	12 998 (49%)
Age in years (n=29 034)†	26 (11–44)	40 (26–54)	25 (10–42)
Adults (≥16 years)	19 816 (68%)	2450 (88%)	17 366 (66%)
Children (<16 years)	9218 (32%)	325 (12%)	8893 (34%)
Obese‡ (n=22 527)†	2607 (9%)	517 (19%)	2090 (8%)
Smoking (n=19 066)†	2406 (8%)	285 (10%)	2121 (8%)
Pregnant women§	2166/9513 (23%)	177/951 (19%)	1989/8562 (23%)
WHO region (n=29 234)†			
African	41 (<1%)	14 (1%)	27 (<1%)
Americas	14 186 (49%)	1477 (53%)	12 709 (48%)
Eastern Mediterranean	5262 (18%)	518 (19%)	4744 (18%)
European	7272 (25%)	680 (24%)	6592 (25%)
South-East Asia	210 (1%)	14 (1%)	196 (1%)
Western Pacific	2263 (8%)	81 (3%)	2182 (8%)
Influenza A H1N1pdm09 diagnosis (n=29 234)†			
Laboratory confirmed	25 001 (86%)	2486 (89%)	22 515 (85%)
Clinically diagnosed	4233 (14%)	298 (11%)	3935 (15%)
Comorbidities†¶			
Any comorbidity (n=28 672)	11 011 (38%)	1471 (53%)	9540 (36%)
Asthma (n=20 518)	2820 (10%)	134 (5%)	2686 (10%)
COPD (n=17 081)	1012 (3%)	171 (6%)	841 (3%)
Other chronic lung disease (n=17 853)	2479 (8%)	272 (10%)	2207 (8%)
Heart disease (n=18 419)	1624 (6%)	317 (11%)	1307 (5%)
Renal disease (n=19 860)	710 (2%)	151 (5%)	559 (2%)
Liver disease (n=12 264)	295 (1%)	81 (3%)	214 (1%)
Cerebrovascular disease (n=9803)	304 (1%)	34 (1%)	270 (1%)
Neurological disease (n=13 598)	1013 (3%)	136 (5%)	877 (3%)
Diabetes (n=24 764)	2087 (7%)	418 (15%)	1669 (6%)
Immunosuppression (n=25 268)	1803 (6%)	346 (12%)	1457 (6%)
Pandemic H1N1 vaccination (n=4382)†‖	347/15 349 (2%)	27/1604 (2%)	320/13 745 (2%)
Time from symptom onset to hospital admission in days (n=23 769)†	2 (1–5)	4 (2–6)	2 (1–4)

(Table 1 continues on next page)

	All patients	Deceased	Survived
(Continued from previous page)			
Antiviral agents used			
No NAI treatment	10 431 (36%)	959 (34%)	9472 (36%)
Any NAI	18 803 (64%)	1825 (66%)	16 978 (64%)
Oral oseltamivir**	17 309/18 803 (92%)	1675/1825 (92%)	15 634/16 978 (92%)
Intravenous or inhaled zanamivir**	435/18 803 (2%)	52/1825 (3%)	383/16 978 (2%)
Intravenous peramivir**	49/18 803 (<1%)	28/1825 (2%)	21/16 978 (<1%)
NAI (regimen unknown)**	1251/18 803 (7%)	140/1825 (8%)	1111/16 978 (7%)
NAI and non-NAI**	94/18 803 (<1%)	18/1825 (1%)	76/16 978 (<1%)
NAI combination therapy**	238/18 803 (1%)	69/1825 (4%)	169/16 978 (1%)
Early NAI (≤2 days of symptom onset) (n=13 254)†**	5995/18 803 (32%)	358/1825 (20%)	5637/16 978 (33%)
Later NAI (>2 days after symptom onset) (n=13 254)†**	7259/18 803 (39%)	942/1825 (52%)	6317/16 978 (37%)
Time from symptom onset to antiviral treatment in days (n=12 284)†	3 (1–5)	4 (2–7)	3 (1–5)
Other inhospital treatment†			
Antibiotics (n=20 362)	13 230 (45%)	1096 (39%)	12 134 (46%)
Corticosteroids (n=9982)	2745 (9%)	453 (16%)	2292 (9%)
Hospital length of stay in days (n=22 366)†	5 (2–9)	7 (2–15)	5 (2–8)
Other patient outcomes†			
Influenza-related pneumonia†† (n=16 551)	7225 (25%)	1035 (37%)	6190 (23%)
Admission to critical care (n=24 435)	6848 (23%)	1957 (70%)	4891 (18%)

Data are n (%) or median (IQR). COPD=chronic obstructive pulmonary disease. NAI=neuraminidase inhibitor. *All percentages have been calculated using these denominators unless otherwise specified. †Missing data; n shows number of cases with data. ‡Reported as clinically obese or using WHO definition for obesity (BMI ≥30 kg/m² in adults aged ≥20 years). §Proportions were calculated as a percentage of pregnant patients among female patients of reproductive age (13–54 years); the broader age range was selected in preference to the WHO definition (15–44 years) after consultation with data contributors to reflect the actual fertility experience of the sample. ¶For definition of comorbidity, see appendix pp 9–11. ||Denominators for pandemic vaccine based on patients admitted after Oct 1, 2009 (when vaccine potentially available). **Percentages calculated as a proportion of the sample receiving NAI therapy. ††Clinically or radiologically diagnosed pneumonia.

Table 1: **Characteristics of pooled dataset of patients admitted to hospital with influenza A H1N1pdm09 virus infection included in mortality analysis**

source dataset), and addition of three further datasets provided through informal contact with domain experts, 80 research groups from 38 countries in six WHO regions contributed anonymised data for 168 117 patients, of whom 24 416 had laboratory results indicative of non-influenza A H1N1 disease. Among the remaining 143 701 laboratory confirmed or clinically diagnosed (without standard study-wide case definition) influenza A H1N1pdm09 cases, 106 138 were outpatients and 2593 had missing information for hospital admission. The remaining 34 970 inpatients were eligible for inclusion (figure 1).

Of the 34 970 inpatients eligible for inclusion, 2095 (6%) had missing information for mortality status, and 3584 (10%) for exposure to neuraminidase inhibitors; 57 (<1%) were unsuitable for inclusion for other reasons (figure 1). Ultimately, we included 29 234 records from 78 studies (two studies provided only outpatient data and were excluded from analysis) of patients admitted to hospital between Jan 2, 2009, and March 14, 2011: 25 001 (86%) laboratory confirmed; 9218 (32%) children; and 1600 (5%) aged 65 years or older. Appendix p 16 show the incidence of cases by month. Full characteristics of the pooled dataset are listed in table 1 with absolute risks of mortality for various exposure categories and subgroups summarised in appendix p 16. Baseline characteristics of each constituent dataset are presented in appendix pp 17–21.

Patients without neuraminidase inhibitor treatment data and therefore excluded from analysis were more likely to be older, to have presented to hospital later, less likely to have a laboratory confirmed diagnosis, and more likely to be treated with antibiotics than were patients included in the analysis (appendix pp 26–27). However, they were less likely to be smokers, obese, or to have an underlying comorbidity. Additionally, their hospital stay was shorter, and they were less likely to have severe outcomes (admission to critical care unit or death), or influenza-related pneumonia (appendix pp 26–27).

After adjustment for propensity score and corticosteroid and antibiotic treatment, the likelihood of mortality in patients treated with a neuraminidase inhibitor was 0·81 (95% CI 0·70–0·93), compared with no treatment (table 2). The OR did not change substantially when only laboratory confirmed cases were included (adjusted OR 0·82 [95% CI 0·70–0·95]). Similarly, we identified significant associations with a reduced mortality risk in adults, pregnant women, and critically ill adult patients (table 2). However, there was no significant association between neuraminidase inhibitor treatment and mortality in children aged 0–15 years (table 2). Post-hoc analyses restricted to

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	Crude analysis		Adjusted* analysis	
	OR (95% CI)	p value	OR (95% CI)	p value
Laboratory confirmed or clinically diagnosed, all ages; n=29 234	0·92 (0·81–1·05)	0·21	0·81 (0·70–0·93)	0·0024
Laboratory confirmed cases, all ages; n=25 001	0·94 (0·81–1·09)	0·42	0·82 (0·70 to 0·95)	0·0104
Adults (≥16 years); n=19 816	0·82 (0·70–0·95)	0·0071	0·75 (0·64–0·87)	0·0002
Children (<16 years); n=9218	1·02 (0·73–1·42)	0·90	0·82 (0·58–1·17)	0·28
Pregnant women; n=2166	0·47 (0·24–0·90)	0·0228	0·46 (0·23–0·89)	0·0215
Critical care patients				
Adults (≥16 years); n=5103	0·74 (0·57–0·95)	0·0187	0·72 (0·56–0·94)	0·0155
Children (<16 years); n=1725	0·84 (0·52–1·37)	0·49	0·70 (0·42–1·16)	0·17

OR=odds ratio. *Adjusted for treatment propensity (by quintile), corticosteroid use, and antibiotic use.

Table 2: **Neuraminidase inhibitor treatment (at any time) versus none**

	Crude analysis		Adjusted* analysis	
	OR (95% CI)	p value	OR (95% CI)	p value
Early treatment versus later treatment				
Laboratory confirmed or clinically diagnosed, all ages; n=13 254	0·36 (0·31–0·41)	<0·0001	0·48 (0·41–0·56)	<0·0001
Laboratory confirmed cases, all ages; n=12 992	0·36 (0·31–0·41)	<0·0001	0·48 (0·41–0·56)	<0·0001
Adults (≥16 years); n=9270	0·37 (0·32–0·44)	<0·0001	0·45 (0·38–0·54)	<0·0001
Children (<16 years); n=3899	0·53 (0·35–0·80)	0·0026	0·67 (0·44–1·03)	0·07
Pregnant women; n=917	0·20 (0·09–0·46)	0·0002	0·27 (0·11–0·63)	0·0026
Critical care patients				
Adults (≥16 years); n=3385	0·64 (0·51–0·79)	<0·0001	0·62 (0·49–0·77)	<0·0001
Children (<16 years); n=683	1·12 (0·63–1·99)	0·69	1·15 (0·64–2·06)	0·64
Early treatment versus none				
Laboratory confirmed or clinically diagnosed, all ages; n=16 425	0·54 (0·40–0·72)	<0·0001	0·50 (0·37–0·67)	<0·0001
Laboratory confirmed cases, all ages; n=13 200	0·53 (0·39–0·71)	<0·0001	0·48 (0·36–0·66)	<0·0001
Adults (≥16 years); n=10 607	0·39 (0·28–0·55)	<0·0001	0·38 (0·27–0·54)	<0·0001
Children (<16 years); n=5696	1·08 (0·61–1·93)	0·79	0·85 (0·47–1·53)	0·59
Pregnant women; n=1303	0·16 (0·04–0·64)	0·0099	0·16 (0·04–0·67)	0·0118
Critical care patients				
Adults (≥16 years); n=1608	0·30 (0·19–0·45)	<0·0001	0·31 (0·20–0·47)	<0·0001
Children (<16 years); n=572	0·88 (0·40–1·91)	0·74	0·76 (0·34–1·67)	0·49

OR=odds ratio. *Adjusted for treatment propensity (by quintile), corticosteroid use, and antibiotic use.

Table 3: **Early neuraminidase inhibitor treatment (≤2 days after onset) versus later (>2 days) or none**

	Crude analysis		Adjusted* analysis	
	OR (95% CI)	p value	OR (95% CI)	p value
Laboratory confirmed or clinically diagnosed, all ages; n=17 670	1·27 (1·00–1·61)	0·0497	1·20 (0·93–1·54)	0·15
Laboratory confirmed cases, all ages; n=14 409	1·25 (0·98–1·59)	0·07	1·17 (0·92–1·51)	0·21
Adults (≥16 years); n=12 269	1·01 (0·77–1·32)	0·94	1·01 (0·76–1·33)	0·96
Children (<16 years); n=5282	1·34 (0·78–2·31)	0·29	1·29 (0·75–2·21)	0·36
Pregnant women; n=1302	0·72 (0·26–2·01)	0·53	0·70 (0·24–2·06)	0·51
Critical care patients				
Adults (≥16 years); n=2977	0·61 (0·43–0·86)	0·0045	0·65 (0·46– 0·93)	0·0183
Children (<16 years); n=644	0·65 (0·32–1·36)	0·25	0·75 (0·35–1·57)	0·44

OR=odds ratio. *Adjusted for treatment propensity quintiles, corticosteroid use and antibiotic use.

Table 4: **Later neuraminidase inhibitor treatment (>2 days) versus none**

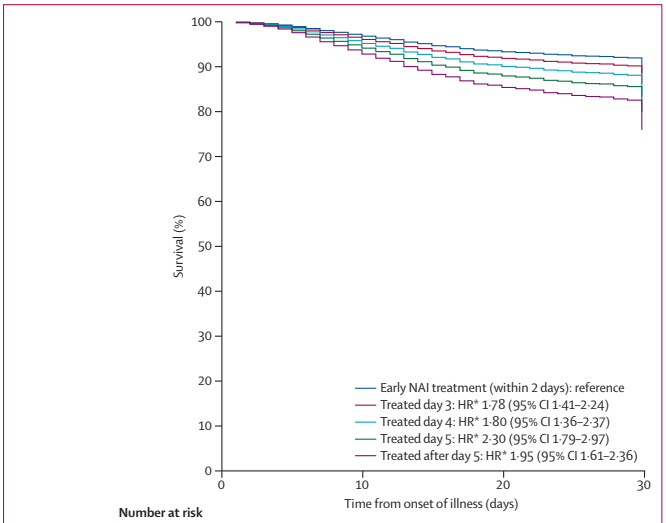
children up to 1 year of age and up to 5 years of age did not change this finding (appendix p 27).

Early neuraminidase inhibitor treatment compared with later treatment initiation was associated with an overall significant reduction in mortality risk (adjusted OR 0·48 [95% CI 0·41–0·56]; table 3). The ORs remained essentially unchanged when only laboratory confirmed cases were considered, but risk reduction was higher in pregnant women (table 3). Notably, there was again no significant association between early treatment and mortality in children after adjustment (table 3).

Neuraminidase inhibitor treatment within 2 days of symptom onset compared with none was also associated with a significant reduction in mortality in all patients (adjusted OR 0·50 [95% CI 0·37–0·67]; table 3), with significant risk reductions also noted among laboratory confirmed cases, adults, pregnant women, and adult patients admitted to critical care (table 3). However, there was no significant association with a lower mortality risk in children aged 0–15 years (table 3).

With regard to neuraminidase inhibitor treatment started more than 2 days after symptom onset compared with none, we identified no significant association with mortality in all patients (adjusted OR 1·20 [95% CI 0·93–1·54]), nor in laboratory confirmed cases, adults, pregnant women, or children (table 4). However, we noted an associated mortality risk reduction of about a third (adjusted OR 0·65 [95% CI 0·46–0·93]) in adult patients admitted to critical care.

Information about exact timing of neuraminidase inhibitor treatment from symptom onset was available for 65% (12284 of 18803) of those receiving such treatment. After taking into account clustering by study, propensity score quintiles, and inhospital treatment with antibiotics or systemic corticosteroids, when antiviral use was modelled as a time-dependent covariate to overcome potential immortal time bias (ie, survivor bias), neuraminidase inhibitor treatment was significantly associated with decreased hazard rate of mortality over a 30-day follow-up period (adjusted HR 0·51 [95% CI 0·45–0·58], p<0·0001) as compared with no antiviral treatment. When only treated cases were included, there was an increase in the hazard with each day's delay in initiation of treatment up to day 5 as compared with treatment initiated within 2 days of symptom onset (adjusted HR 1·23 [95% CI 1·18–1·28], p<0·0001 for the increasing HR with each day's delay). The unadjusted and adjusted survival curves comparing survival by time to treatment initiation are shown in figure 2 and appendix pp 28–29.



Number at risk

Early NAI treatment (within 2 days)	5192	2414	1939	1843
Treated with NAI day 3	1477	689	492	460
Treated with NAI day 4	1012	567	322	282
Treated with NAI day 5	812	541	272	241
Treated with NAI after day 5	2298	1981	1009	735

Figure 2: **Survival by time to treatment**
HR=hazard ratio. NAI=neuraminidase inhibitor. *Cox regression shared frailty model (adjusted for treatment propensity and in hospital steroid or antibiotic use).

Discussion

Our results show that neuraminidase inhibitor treatment was associated with reduced mortality in adult patients admitted to hospital with influenza A H1N1pdm09 virus infection. Neuraminidase inhibitor treatment of influenza A H1N1pdm09 at any stage of illness compared with none revealed an associated reduction in the likelihood of mortality (table 2). We identified an associated likelihood of lower mortality when comparing early versus later initiation of treatment and when comparing early treatment with none (table 3, panel). Although we included 4233 patients (14%) without laboratory confirmed influenza A H1N1pdm09, restriction to laboratory-confirmed cases produced near identical estimates, suggesting that the data are not confounded by misclassification bias attributable to other causes (tables 2, 3). Additionally, we noted much the same findings in adults, pregnant women, and adult patients needing admission to critical care. The finding regarding critical care suggests that neuraminidase inhibitors were associated with mortality reduction across the spectrum of severity in adult patients admitted to hospital with influenza A H1N1pdm09. These findings accord closely with previous studies[16,17] but have increased precision and reduced estimates of effectiveness consistent with more complete adjustment for confounders and treatment propensity. They are also consistent with ecological data.[23–25]

We were consistently unable to show any association of neuraminidase inhibitor treatment with mortality reduction in children. Possible explanations include lower case fatality proportion in paediatric patients (thus reduced statistical power),[26,27] higher influenza A H1N1pdm09 viral

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***Panel:* Research in context**

Systematic review

Two systematic reviews and meta-analyses have examined the effectiveness of neuraminidase inhibitors in reducing mortality due to influenza. Hsu and colleagues[16] considered reported observational data, mainly for seasonal influenza, and concluded that oral oseltamivir might reduce mortality (odds ratio [OR] 0·23 [95% CI 0·13–0·43]). In our own systematic review, we included only reported data from the 2009–10 influenza A H1N1 pandemic (all observational) and showed that early neuraminidase inhibitor treatment versus none reduced mortality by two thirds (OR 0·35 [95% CI 0·18–0·71]).[17] We applied search terms relating to pandemic influenza (including "Influenza A Virus" OR "H1N1 Subtype" OR "swine origin influenza AH1N1 virus"), AND exposure of interest—ie, antiviral drugs (including "neuraminidase inhibitors" OR "oseltamivir" OR "zanamivir" OR "peramivir") AND clinical outcome measures (including "pneumonia", "critical or intensive care", "mortality") to 11 databases (search range from Jan 1, 2009, to Aug 10, 2010; last search on April 19, 2012) without imposing language restrictions. Importantly, both studies acknowledged limitations such as the heterogeneity of studies included and inadequate adjustment for potential confounding. Moreover, neither was able to adjust for the likelihood of a patient receiving antiviral treatment (propensity)—a crucial consideration when antiviral drugs might have been prioritised towards the sickest patients.

Interpretation

By using a meta-analysis of individual patient data, which permits a uniform approach to potential confounding and adjustment for treatment propensity, and through the assembly of a very large international dataset, our study adds substantially to the evidence that neuraminidase inhibitors administered to adults admitted to hospital with influenza A H1N1pdm09 reduced mortality, especially when started promptly. Since placebo-controlled randomised controlled trials of neuraminidase inhibitors are not ethically feasible during a pandemic, the evidence we have assembled is likely to be the best that will be available. The US Centers for Disease Control and Prevention recommend neuraminidase inhibitor treatment as early as possible for any patient with confirmed or suspected influenza who is hospitalised; has severe, complicated or progressive illness; or is at higher risk for influenza complications.[22] Neuraminidase inhibitors are also widely prescribed in Japan, but elsewhere their use is far less common. Although a similar treat-all policy existed in the UK in 2009, uptake of neuraminidase inhibitors inpatients admitted to hospital with influenza A H1N1pdm09 virus was low.[35] We advocate early instigation of neuraminidase inhibitor treatment in adults admitted to hospital with suspected or proven influenza infection.

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See Online for appendix

load in children[28] than adults leading to reduced drug effectiveness, suboptimum dosing in very young children,[29] secondary bacterial infections (eg, meticillin-resistant *Staphylococcus aureus*), confounding by indication[30] (children might have been more likely to have had antivirals prescribed if they had more severe disease or if they failed to respond to other treatments), or a combination of these factors. Since it has been suggested that younger children might be admitted with milder disease compared with older children and adults (precautionary physician behaviour), that the pharmacokinetics and pharmacology of oseltamivir might be different in very young children,[29] and that influenza pathogenesis might differ by age,[30] we did post-hoc sensitivity analyses separately in children up to 1 year of age and up to 5 years of age, but our findings did not change (appendix p 27). However, we note that these results contrast with those of Louie and colleagues,[32] who recently showed a two-thirds reduction in mortality among children treated with neuraminidase inhibitors admitted to hospital with influenza (OR 0·36 [95% CI 0·16–0·83]).

The finding that no treatment was better than late treatment is probably explained by confounding due to illness severity at the point of treatment initiation (ie, confounding by indication). Untreated patients probably had milder disease and patients treated later in the course of their illness might have had delays in hospital admission, delays in diagnosis after admission, or delays in being considered for neuraminidase inhibitor treatment (treatment only started once their condition deteriorated), or combinations of these factors. We advocate early consideration of a diagnosis of influenza in patients admitted to hospital with respiratory infection during periods of known influenza activity, and early instigation of neuraminidase inhibitor treatment based on rapid laboratory confirmation or clinical suspicion.

Our analyses examining the effect of later treatment versus none are especially relevant to the continued clinical debate about the value of delayed therapy. Combining all subgroups of patients, we did not identify any protective association with treatment delayed more than 2 days after symptom onset (table 4). This finding could be explained by confounding by indication. However, we noted that in adult patients admitted to critical care, delayed treatment was associated with reduced likelihood of mortality compared with no treatment (table 4), suggesting that delayed therapy might still be worthwhile in severely ill patients; this finding is plausible since, within this subgroup, treated and untreated patients (who all needed admission to critical care) are likely to have been more balanced in terms of illness severity thereby overcoming confounding by this factor to some extent. Additionally, some patients admitted to critical care might have had prolonged influenza A H1N1pdm09 virus replication in the lower respiratory tract, which might benefit from later initiation of neuraminidase inhibitor treatment. To gain further understanding about overall timing-dependent benefit, we modelled time to start of antiviral treatment using a time-dependent Cox regression model, which showed a significant detrimental survival benefit associated with delay in treatment beyond 2 days after symptom onset ($p<0·0001$), albeit with overlapping 95% CIs when time to treatment was modelled as a categorical variable; the latter finding suggests that potential differences in treatment benefit between starting on day 3 after symptom onset through to more than 5 days after symptom onset cannot be further clarified through our data. This finding could seem to conflict with the findings in table 4 comparing later neuraminidase inhibitor treatment to no neuraminidase inhibitor treatment but is not surprising, because by comparing only treated patients in figure 2, we possibly eliminated some of the confounding due to indication, which allowed us to identify the potential survival benefits conferred by later treatment, albeit detrimental in proportion to treatment delay.

One of the strengths of this study is the very large number of patients from geographically diverse clinical centres and source populations. We made exhaustive efforts to identify suitable datasets from around the world, but nevertheless cannot comment on the extent to which bias might have been introduced by failing to include centres that did not respond (we cannot say if they had suitable data or not), or that declined to share data; in a worst case scenario, it is possible that less than 20% of potential sites contributed to this analysis. Furthermore, comparatively few cases were from the WHO African (0·1%) and South-East Asia (0·7%) regions, which might limit the extent to which our findings can be generalised.

A clear limitation of our study is that we were unable to adjust specifically for disease severity in our multilevel models because of the heterogeneity of severity measures used across individual datasets. However, we made every effort to include relevant data including severity measures, within each propensity score, but there is still likely to be some residual confounding, particularly due to illness severity at presentation. Likewise, we attempted to control for study-level biases, such as treatment policies, and health-care seeking behaviour, using multilevel models but there might be residual confounding. A further limitation of our dataset is that 10% of the patients had missing data for exposure to neuraminidase inhibitors and were excluded from the analysis; the characteristics of these patients are compared with those with data for neuraminidase inhibitor exposure in appendix pp 26–27; these patients were more likely to be older, to have presented to hospital later, less likely to have a laboratory confirmed diagnosis, and more likely to be treated with antibiotics.

The decision to adjust for treatment with antibiotics and corticosteroids was taken after consultation with clinical colleagues within the PRIDE study collaboration. This decision results from widespread clinical practice to treat patients admitted to hospital with respiratory illness with corticosteroids and antibiotics. There is particular uncertainty about the possible effect of corticosteroids on the course of severe influenza infection.[33,34] Therefore, it was necessary to separate out the possible effects of antivirals from these other commonly used treatments. We did not do specific analyses to establish the potential effect of antibiotic or corticosteroid use on mortality, but recognise that these factors both warrant further research. Although we were able to adjust for inpatient antibiotics and systemic corticosteroid use, we were unable to adjust for pandemic H1N1 vaccination since 35% (8284 of 23633) of our case series were admitted to hospital before the first availability of vaccine in October, 2009, and 71% (10 967 of 15 349) of data for vaccination status were missing among those admitted after that juncture; however, the available data suggest uptake was no higher than 8% during the study period.

This meta-analysis of individual patient data offers the most rigorous assessment of mortality benefits of neuraminidase inhibitor treatment during the 2009–10 pandemic that is likely to be possible using retrospective observational data. The greatest likelihood of reduced mortality seems to be attributable to treatment started within 2 days of symptom onset. These data offer evidence of the effectiveness of neuraminidase inhibitors during the 2009–10 pandemic and are superior to extrapolations from earlier data on seasonal influenza; they could retrospectively vindicate prepandemic neuraminidase inhibitor antiviral stockpiling decisions made by governments worldwide. Treatment guidance policies should increase emphasis on early empirical neuraminidase inhibitor treatment of adult patients admitted to hospital after presenting with proven or clinically suspected influenza A H1N1pdm09 virus infection. However, most adult patients with suspected or confirmed influenza are not admitted to hospital within 48 h of illness onset. Therefore, the implications of these findings, although based on patients admitted to hospital with influenza A H1N1pdm09, encourage early initiation of neuraminidase inhibitor treatment in outpatients who are appreciably unwell with suspected or confirmed influenza, or at increased risk of complications, including those with influenza A H3N2 or influenza B. Further studies are needed in children to confirm the adequacy of present dose regimens and duration of therapy in terms of clinical efficacy.

Contributors

JSN-V-T, PRM, SGM, SV, and JL-B conceived and designed the study. All authors, apart from SGM, SV, and JL-B, contributed to acquisition and local preparation of constituent datasets. SGM, SV, PRM, and JL-B contributed to dataset amalgamation and standardisation, design of statistical analyses, and data analysis. JSN-V-T, PRM, SGM, SV, and JL-B interpreted the data and wrote the paper. All authors contributed to critical examination of the paper for important intellectual content and approval of the final report. Each author acts as the guarantor of data from their individual study centre; JSN-V-T and PRM act as overall guarantors for the pooled analysis and the report.

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Declaration of interests

Between October, 2007, and September, 2010, JSN-V-T did ad-hoc paid consultancy and lecturing for several influenza vaccine manufacturers (Sanofi Pasteur MSD, Sanofi Pasteur, GlaxoSmithKline, Baxter AG, Solvay, Novartis) and manufacturers of neuraminidase inhibitors (F Hoffmann-La Roche: oseltamivir [Tamiflu] and GlaxoSmithKline: zanamivir [Relenza]). He is a former employee of SmithKline Beecham (now part of

GlaxoSmithKline), Roche Products (UK), and Sanofi Pasteur MSD, all before 2005. He has no outstanding interests related to shares, share options, or accrued pension rights in any of these companies. He is in receipt of current or recent research funding, related to influenza vaccination from GlaxoSmithKline and AstraZeneca and non-financial support (travel) from Baxter AG. His brother became an employee of GlaxoSmithKline in January, 2014. JL-B is Statistical Editor of the Cochrane Skin Group. PRM is the recipient of the unrestricted educational grant for research in the area of pandemic influenza from F Hoffman-La Roche, used to fund this work. She has also received travel grants from F Hoffman-La Roche and its subsidiaries to attend clinical seminars to present this work. RB has received financial support from CSL, Sanofi, GlaxoSmithKline, Novartis, Roche, and Wyeth to do research and present at scientific meetings. Any funding received is directed to an NCIRS research account at The Children's Hospital at Westmead, NSW, Australia, and is not personally accepted by RB. All other named authors declare that they have no competing interests.

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Original Article

Impact of neuraminidase inhibitors on influenza A(H1N1) pdm09-related pneumonia: an individual participant data meta-analysis

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Background The impact of neuraminidase inhibitors (NAIs) on influenza-related pneumonia (IRP) is not established. Our objective was to investigate the association between NAI treatment and IRP incidence and outcomes in patients hospitalised with A(H1N1) pdm09 virus infection.

Methods A worldwide meta-analysis of individual participant data from 20 634 hospitalised patients with laboratory-confirmed A (H1N1)pdm09 (n = 20 021) or clinically diagnosed (n = 613) 'pandemic influenza'. The primary outcome was radiologically confirmed IRP. Odds ratios (OR) were estimated using generalised linear mixed modelling, adjusting for NAI treatment propensity, antibiotics and corticosteroids.

Results Of 20 634 included participants, 5978 (29·0%) had IRP; conversely, 3349 (16·2%) had confirmed the absence of radiographic pneumonia (the comparator). Early NAI treatment (within 2 days of symptom onset) versus no NAI was not significantly associated with IRP [adj. OR 0·83 (95% CI 0·64–1·06; P = 0·136)]. Among the 5978 patients with IRP, early NAI treatment versus none did not impact on mortality [adj. OR = 0·72 (0·44–1·17; P = 0·180)] or likelihood of requiring ventilatory support [adj. OR = 1·17 (0·71–1·92; P = 0·537)], but early treatment versus later significantly reduced mortality [adj. OR = 0·70 (0·55–0·88; P = 0·003)] and likelihood of requiring ventilatory support [adj. OR = 0·68 (0·54–0·85; P = 0·001)].

Conclusions Early NAI treatment of patients hospitalised with A (H1N1)pdm09 virus infection versus no treatment did not reduce the likelihood of IRP. However, in patients who developed IRP, early NAI treatment versus later reduced the likelihood of mortality and needing ventilatory support.

Keywords Hospitalisation, individual participant data meta-analyses, influenza-related pneumonia, neuraminidase inhibitors.

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Introduction

Influenza-related pneumonia (IRP) was a common and severe complication during the 2009–2010 influenza pandemic.[1–5] Neuraminidase inhibitors (NAIs), primarily oseltamivir and zanamivir, were widely recommended for patients with suspected or confirmed influenza A(H1N1)pdm09 virus infection.[6,7] However, prior to the 2009–2010 pandemic, evidence of their effectiveness in seasonal influenza, while strong for modest symptom alleviation, was less robust for reductions in pneumonia incidence or improvements in pneumonia outcome.[8–10] The findings from meta-analyses have been inconsistent. One study based on observational data from 150 660 patients with mainly seasonal influenza suggested no statistically significant reduced likelihood of pneumonia.[9] Another used clinical trials data from 4452 community adult patients with uncomplicated seasonal influenza and concluded that oseltamivir significantly reduced 'self-reported, investigator-mediated, unverified pneumonia' by 45%, compared with placebo, but data on radiologically confirmed pneumonia were not available.[11]

A recent individual participant data (IPD) analysis of clinical trial data investigating the efficacy of oseltamivir when compared to placebo in patients with seasonal influenza reported a reduction in risk of pneumonia by 60%.[12] Individual observational studies during the 2009–2010 pandemic suggest a possible benefit of NAIs in reducing pneumonia incidence, but are limited by small sample sizes.[13–16] A meta-analysis of 2009–2010 pandemic data from patients hospitalised with influenza A(H1N1)pdm09 virus infection reported that early treatment with NAIs reduced the likelihood of IRP compared to late treatment by 65%.[17] But this work encountered high degrees of heterogeneity and inconsistent or incomplete adjustment for potential confounders.

We present a global meta-analysis based on IPD, controlling for potential confounders and treatment propensity. We investigate the association between NAI treatment and radiologically confirmed IRP in patients hospitalised with A(H1N1)pdm09 virus infection, and outcomes including admission to intensive care units (ICUs), ventilatory support, acute respiratory distress syndrome (ARDS) and mortality in patients with IRP.

Some of these results have been previously reported in the form of an abstract.[18]

Methodology

The PRIDE research consortium

Details of the Post-pandemic Review of anti-Influenza Drug Effectiveness (PRIDE) study have been published previously.[19] Briefly, participating research centres were identified during the conduct of a systematic review of published studies on the same topic.[17] Additional centres were recruited through this network of global collaborators, publicity at conferences and by word of mouth. Centres that fulfilled the minimum data set requirements (Table S2) were eligible for inclusion in the consortium. In total, 79 research groups from 38 countries and six World Health Organization (WHO) regions contributed data on 143 786 patients with laboratory- or clinically diagnosed influenza A(H1N1)pdm09 virus infection (Figure 1). No data were provided or funded for collection by pharmaceutical companies. The protocol was registered with the PROSPERO register of systematic reviews, number CRD42011001273.[20]

Data standardisation, exposure and outcome variables

Data were standardised using a common data dictionary[19] before pooling for analysis. For this analysis, the primary outcome was IRP defined as laboratory-confirmed or clinically diagnosed influenza A(H1N1)pdm09 virus infection plus pneumonia confirmed by chest radiography, occurring at any time after the onset of influenza-like illness. For radiographic evidence of pneumonia, we accepted:

1. A formal chest radiograph or computerised tomograph report documenting 'pneumonia'.
2. Data sets reporting pneumonia and chest radiograph as discrete variables, in which both items were marked positive or 'yes'.
3. Formal chest radiograph reports of one or more abnormalities consistent with pneumonia: pulmonary infiltrates, lobar consolidation, homogeneous segmental consolidation with or without cavitation, diffuse bilateral interstitial and/or interstitial–alveolar (mixed) infiltrates, segmental consolidation, lobar consolidation, rounded pneumonia, bronchopneumonia, interstitial pneumonia, pneumatoceles, acute pulmonary infiltrates, as previously validated by Bewick *et al.* and Franquet,[21,22] unless a formal radiograph report also stated 'no pneumonia'.
4. Chest radiograph report not provided, but specific mention in the clinical case notes that a radiograph had been formally reported as showing pneumonia.

The absence of IRP ('no IRP') was defined as laboratory-confirmed or clinically diagnosed influenza A(H1N1)pdm09 infection plus a radiographic report that did not identify abnormalities consistent with pneumonia, or which stated that pneumonia was 'not present' (irrespective of any specific features reported).

Comparative exposure to NAI treatment was defined as follows: early NAI treatment (≤2 days after symptom onset) versus no NAI treatment; early NAI treatment versus later NAI treatment (treatment commenced >2 days after symptom onset); later NAI treatment versus no NAI treatment; and NAI treatment (irrespective of timing) versus no NAI treatment.

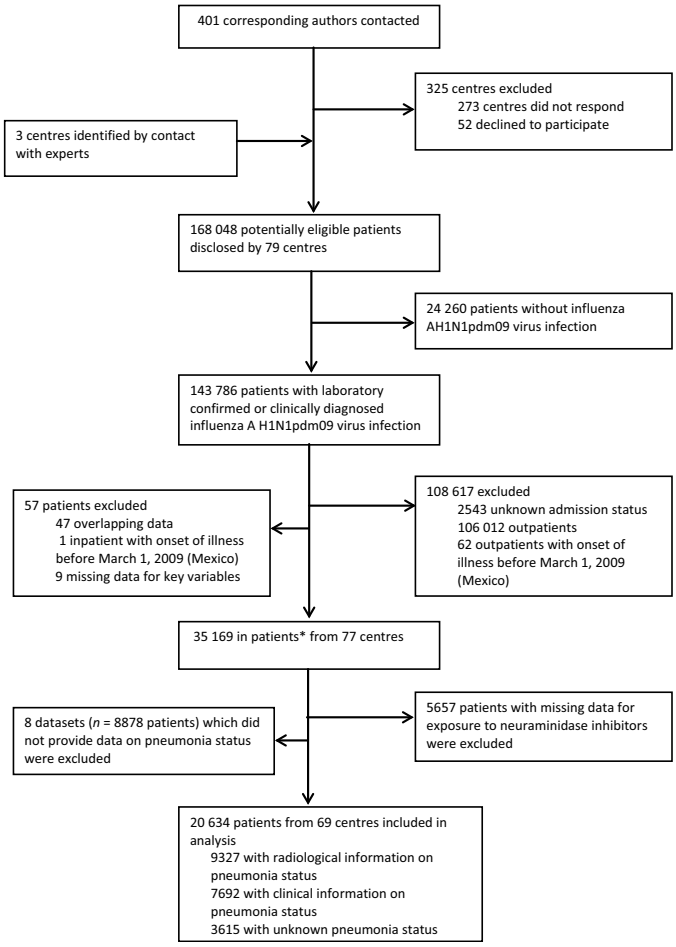

Figure 1. Study flow diagram. *Two hundred and sixty patients added since publication of Muthuri *et al.*[17] following clarification of inpatient status from data collaborator.

Propensity scoring

Propensity scores for the likelihood of NAI treatment were calculated for each patient within individual data sets using multivariable logistic regression for each of the three NAI exposure measures, using covariates as described by Muthuri *et al.*[19] (Table S3). Subsequently, propensity scores were categorised into quintiles for each individual data set.

Statistical analysis

To investigate the association between the use of NAI treatment and IRP, we compared patients with IRP against those with no IRP. We used generalised linear mixed modelling to conduct separate analyses for each NAI exposure comparison using the xtmelogit command in STATA (version 13; StataCorp LP, College Station, TX, USA). Individual studies were included in the model as a random intercept in order to account for differences in baseline outcome. Adjustment was performed for propensity of NAI treatment, antibiotics administered during hospitalisation and corticosteroids administered during hospitalisation. Missing data in the covariates were included as a separate dummy category to allow for comparisons across the crude and adjusted analyses. We excluded data sets in which all patients (n = 1352 from 14 data sets) were diagnosed with IRP. Stratified analyses were conducted for adults (≥16 years), children (<16 years; including <5- and 5- to 15-year subgroups), pregnant women, laboratory-confirmed A(H1N1)pdm09 cases and patients admitted to critical care units. We did not include patients with unknown pneumonia status (n = 3615 across 21 data sets) in this analysis.

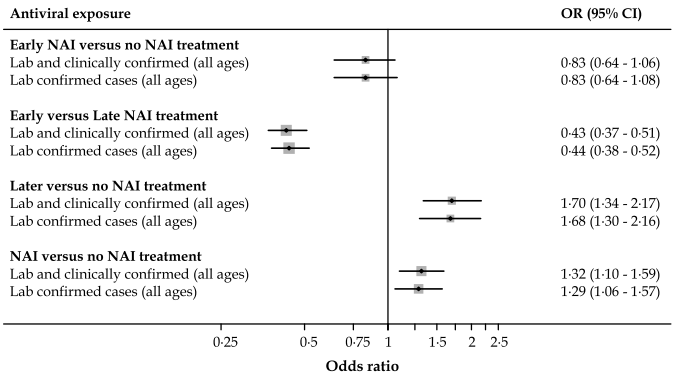

Figure 2. Summary of main findings for influenza-related pneumonia (IRP) in laboratory- and clinical diagnosed influenza patients, all ages.

In the subgroup of patients with IRP, we further examined the effect of NAI treatment on secondary clinical outcomes: admission to ICUs, ventilatory support, ARDS and mortality. At this juncture, we re-included the 14 data sets in which all patients were diagnosed with IRP.

Sensitivity analysis

In some clinical settings, chest radiography is not routinely performed for hospitalised patients with influenza unless a pulmonary complication is also suspected; therefore, reliance on radiographic abnormalities is likely to give a conservative estimate of pneumonia incidence. Accordingly, we also performed a sensitivity analysis, which considered a diagnosis of 'any pneumonia' by combining IRP with physician-diagnosed pneumonia (PDP), the latter defined as laboratory-confirmed or clinically diagnosed influenza A(H1N1) pdm09 plus a physician diagnosis of pneumonia, but where no chest radiograph report was available. For this analysis, patients categorised as 'no pneumonia' had laboratory-confirmed or clinically diagnosed influenza A(H1N1)pdm09 with no evidence of IRP on chest radiography; unknown pneumonia status; or, in the absence of a chest radiograph report, no documented clinical record of PDP, recognising that clinicians record positive findings in the case record, but not all negative findings.

Results are presented as unadjusted and adjusted odds ratios (OR) with 95% confidence intervals (95% CI), and two-sided *P*-values < 0·05 were considered statistically significant. Statistical analyses were conducted using STATA (version 13).

Results

Overall, data were obtained on 35 169 individuals hospitalised with A(H1N1)pdm09 virus infection (Figure 1). Of these, 29 512 (84%) patients were admitted from January 2009 through March 2011 (Figure S1) with information available on NAI treatment. A further eight data sets comprising 8878 hospitalised patients that did not provide data on pneumonia status were excluded from the analysis (Figure 1; Table S4).

Of the 20 634 patients included, 9327 (45%) had a positive or negative diagnosis of IRP confirmed by chest radiography, while 7692 (37%) did not have chest radiography, but had a positive or negative diagnosis of PDP documented. The remaining 3615 (18%) hospitalised patients had neither radiological nor clinical documentation of pneumonia status; they were included in the sensitivity analysis (only) as having 'no pneumonia'. The characteristics of hospitalised patients with and without pneumonia included in the pooled data set are shown in Table 1. Baseline characteristics of each constituent data set included in the analysis are presented in Table S5.

Overall, patients with IRP were more likely than patients with no IRP to be adult ($P < 0·001$), non-pregnant ($P < 0·001$), free of underlying medical conditions ($P = 0·038$), be from outside the WHO European region ($P < 0·001$) and have laboratory-confirmed influenza A (H1N1)pdm09 infection ($P < 0·001$). They were more likely to receive NAI treatment ($P < 0·001$), antibiotics ($P < 0·001$) and corticosteroids ($P < 0·001$), be admitted to critical care facilities ($P < 0·001$) and require ventilatory support (<0·001) or die ($P < 0·001$) (Table 1).

Association between NAI treatment and IRP

Overall, 63 data sets provided data on 9327 hospitalised patients with a positive or negative diagnosis of pneumonia confirmed by chest radiography. After the exclusion of 14 data sets in which all patients had IRP ($n = 1352$, Table S5), 7975 patients remained in the analysis.

Early NAI (≤2 days) versus no NAI treatment

Early NAI use compared with no NAI use was not significantly associated with IRP in our overall sample

[adjusted OR 0·83 (95% CI 0·64–1·06)], nor when we considered laboratory-confirmed cases, adults, pregnant women or children (Table 2 and Figure 2). However, point estimates for subgroups tended to suggest an OR below unity, except in ICU patients. When considering 'any pneumonia', we found a borderline significant reduced OR associated with early NAI use in all patients [adjusted OR 0·83 (95% CI 0·70–0·98)], with further borderline significant risk reductions also noted among laboratory-confirmed cases; these findings lost a statistical significance when further stratified by patient subgroups but the point estimates remained consistent (Table 2).

For this exposure, we also looked at the impact of corticosteroids on the association between NAI treatment and IRP. A test for interaction between NAI treatment and corticosteroids did not show any significant interaction (*P*-value: 0·275). Stratified analysis (by corticosteroid use) did not show any significant association between NAI use and IRP (Table S9).

Early NAI (≤2 days) versus later NAI (>2 days) treatment

Early NAI treatment compared with later was associated with significantly lower odds of IRP [adjusted OR, 0·43 (95% CI, 0·37–0·51)] (Table 2 and Figure 2). The odds ratios did not change substantially when only cases of laboratory-confirmed influenza were considered (Table 2). Similarly, statistically significant lower odds of IRP were observed in adults aged 16 years or older, children aged 0–15 years, pregnant women and among adult patients admitted to critical care. However, there was no statistically significant association with IRP among children admitted to critical care (Table 2). The pattern of these findings in terms of direction and significance was similar when considering 'any pneumonia' (Table 2).

Later NAI (>2 days) versus no NAI treatment

Neuraminidase inhibitor treatment beyond 2 days of symptom onset compared with no NAI was associated with statistically significant higher odds of IRP [adjusted OR, 1·70 (95% CI, 1·34–2·17)]. Similar statistically significant associations were observed among cases of laboratory-confirmed influenza, adults and critically ill children, but not among all children, pregnant women and critically ill adults. Likewise, with 'any pneumonia', the direction and statistical significance of these findings did not change (Table 2 and Figure 2).

NAI anytime versus no NAI treatment

After adjustment, the likelihood of IRP in patients treated with NAI (administered at any point after illness onset) was 1·32 (95% CI 1·10–1·59), compared with no NAI treatment (Table 2 and Figure 2). This OR did not change substantially when only patients with laboratory-confirmed A(H1N1) pdm09 were included [adjusted OR 1·29 (95% CI 1·06–1·57)]. Similarly, we observed significantly higher odds of IRP associated with NAI antiviral use in adults and borderline significantly increased odds of IRP in adults admitted to an ICU. However, there was no significant association between NAI treatment and IRP in children aged 0–15 years, pregnant women and critically ill children. The pattern of these findings was not changed by considering 'any pneumonia', except in children admitted to critical care where we observed statistically significant higher odds of IRP for patients treated with an NAI (at any time).

Post hoc analyses on non-ICU patients (all ages) are shown in Table S6; children's subgroups aged <5 years and 5–15 are shown in Tables S7 (all severities) and S8 (critically ill).

Impact of NAI treatment on clinical outcomes among patients with pneumonia

We performed a further analysis, restricted to patients with IRP (n = 5978) (Table 3), and a sensitivity analysis by including 'any pneumonia' patients (n = 7054). Data sets in which all patients had IRP (n = 1352 patients, 14 data sets) were re-added at this juncture.

In the IRP cohort, we did not observe any statistically significant associations with clinical outcomes when early NAI treatment was compared with no NAI treatment; but for 'any pneumonia', we observed that early NAI treatment versus no NAI was associated with an increased likelihood of admission to an ICU [adjusted OR, 1·81 (95% CI, 1·27–2·58); P = 0·001], but a reduced likelihood of mortality [adj. OR, 0·62 (95% CI, 0·40–0·96); P = 0·032].

In patients with IRP, early NAI treatment compared to later NAI was associated with significantly lower odds of ventilatory support [adjusted OR, 0·68 (95% CI, 0·54–0·85); P = 0·001] and mortality [adjusted OR, 0·70 (95% CI, 0·55–0·88); P = 0·003]. These effects were similar and remained statistically significant for 'any pneumonia'.

Later NAI treatment versus no NAI was significantly associated with increased likelihood of ICU admission and ventilatory support. The pattern of these findings in terms of direction and significance was unchanged when considering 'any pneumonia'. Likewise, patients with IRP who received NAI at any time versus no NAI treatment were more likely to be admitted to an ICU [adj. OR, 1·59 (95% CI, 1·21–2·09), P = 0·001] and receive ventilatory support [adj. OR, 1·67 (95% CI, 1·22–2·29), P = 0·001].

Discussion

The strengths of this study include having data on a large number of patients of all ages hospitalised with influenza A (H1N1)pdm09 virus infection (mainly laboratory confirmed) from different geographical regions worldwide. Given the practical and ethical constraints likely to be involved in conducting placebo-controlled trials during

Table 1. Characteristics of pooled data set of 20 634 patients admitted to hospital with influenza A(H1N1)pdm09 virus infection with and without pneumonia

Characteristics	Radiologically diagnosed pneumonia status		Radiologically or PDP status	
	IRP	No IRP	Any pneumonia*	No pneumonia**
Number of patients***	5978 (100·0)	3349 (100·0)	7054 (100·0)	13 580 (100·0)
Number of male cases	3266 (54·6)	1879 (56·0)	3811 (54·0)	6645 (48·9)
Age: median (IQR) in years	36 (17–52)	26 (14–46)	35 (14–51)	22 (8–38)
Adults (≥16 years)	4560 (76·3)	2436 (72·7)	5208 (73·8)	8482 (62·5)
Children (<16 years)	1411 (23·6)	912 (27·2)	1821 (25·8)	4966 (36·6)
Obese†	952 (15·9)	229 (6·8)	1072 (15·2)	744 (5·5)
Smoking	914 (15·3)	481 (14·4)	958 (13·6)	867 (6·4)
Pregnant women††	219 (13·1)	150 (16·0)	279/1967 (14·2)	1153/4397 (26·2)
WHO regions				
African region	28 (0·5)	1 (0·03)	31 (0·4)	10 (0·1)
Region of the Americas	2314 (38·7)	550 (16·4)	2703 (38·3)	4948 (36·4)
Eastern Mediterranean region	178 (3·0)	206 (6·2)	549 (7·8)	3086 (22·7)
European region	2635 (44·1)	2032 (60·7)	2932 (41·6)	4080 (30·0)
South-East Asia region	45 (0·8)	86 (2·6)	45 (0·6)	157 (1·2)
Western Pacific region	778 (13·0)	474 (14·2)	794 (11·3)	1299 (9·6)
A(H1N1)pdm09 diagnosis				
Laboratory confirmed	5755 (96·3)	3146 (93·9)	6827 (96·8)	13 194 (97·2)
Clinically diagnosed	223 (3·7)	203 (6·1)	227 (3·2)	386 (2·8)
Comorbidities†††				
Any comorbidity	3021 (50·5)	1795 (53·6)	3531 (50·1)	5449 (40·1)
Asthma	856 (14·3)	777 (22·7)	968 (13·7)	1430 (10·5)
COPD	432 (7·2)	249 (7·4)	454 (6·4)	345 (2·5)
Other chronic lung disease	492 (8·2)	525 (15·7)	648 (9·2)	1668 (12·3)
Heart disease	650 (10·9)	341 (10·2)	713 (10·1)	786 (5·8)
Renal disease	278 (4·7)	113 (3·4)	328 (4·7)	349 (2·6)
Liver disease	122 (2·0)	73 (2·2)	127 (1·8)	121 (0·9)
Cerebrovascular disease	121 (2·0)	122 (3·6)	133 (1·9)	170 (1·3)
Neurological disease	436 (7·3)	237 (7·1)	492 (7·0)	508 (3·7)
Diabetes	634 (10·6)	280 (8·4)	725 (10·3)	690 (5·1)
Immunosuppression	525 (8·8)	242 (7·2)	610 (8·7)	852 (6·3)
H1N1pdm09 vaccination‡	121/2917 (4·2)	48/1701 (2·8)	163/3738 (4·4)	176/6237 (2·8)
Time from symptom onset to hospital admission, days, median (IQR)	4 (2–6)	2 (1–4)	3 (2–6)	2 (1–4)
Time from symptom onset to antiviral treatment, days, median (IQR)	4 (2–7)	2 (1–4)	4 (2–7)	2 (1–4)
Antiviral agents used				
No NAI treatment	582 (9·7)	540 (16·1)	724 (10·3)	4336 (31·9)
Any NAI	5396 (90·3)	2809 (83·9)	6330 (89·7)	9244 (68·1)
Oral oseltamivir‡‡	5356 (99·3)	2782 (99·0)	6263 (98·9)	9068 (98·1)
Intravenous/inhaled zanamivir‡‡	134 (2·5)	40 (1·4)	155 (2·5)	158 (1·7)
Intravenous peramivir‡‡	42 (0·8)	5 (0·2)	42 (0·7)	7 (0·1)
NAI (regimen unknown)‡‡	1 (0·02)	5 (0·2)	17 (0·3)	82 (0·9)
NAI and non-NAI‡‡	75 (1·4)	15 (0·5)	76 (1·2)	18 (0·2)
NAI combination therapy‡‡	134 (2·5)	23 (0·8)	144 (2·3)	71 (0·8)
Early NAI (≤2 days of symptom onset)‡‡	1067 (19·8)	1057 (37·6)	1353 (21·4)	3459 (37·4)
Later NAI (>2 days after symptom onset)‡‡	2843 (52·7)	998 (35·5)	3362 (53·1)	3221 (34·8)
Other in-hospital treatment				
Antibiotics	3604 (60·3)	1731 (51·7)	4265 (60·5)	5521 (40·7)
Corticosteroids	1658 (27·7)	626 (18·7)	1709 (24·2)	1024 (7·5)
Hospital length of stay, days, median (IQR)	9 (5–17)	5 (3–7)	8 (4–17)	4 (2–7)
Other patient outcomes				
Acute respiratory distress syndrome	265 (4·4)	10 (0·3)	341 (4·8)	43 (0·3)

Table 1. (*Continued*)

Characteristics	Radiologically diagnosed pneumonia status		Radiologically or PDP status	
	IRP	No IRP	Any pneumonia*	No pneumonia**
Ventilation support	2372 (39·7)	450 (13·4)	2619 (37·1)	1059 (7·8)
Admission to critical care	3335 (55·8)	764 (22·8)	3859 (54·7)	1989 (14·7)
Mortality	903 (15·1)	90 (2·7)	1014 (14·4)	496 (3·7)

*Any pneumonia includes influenza-related pneumonia (IRP) (n = 5978) and physician-diagnosed pneumonia (PDP) (n = 1076).
**No pneumonia includes no IRP (n = 3349), no PDP (n = 6616) and unknown pneumonia status (n = 3615).
***All percentages have been calculated using these denominators unless otherwise specified.
†Reported as clinically obese or using WHO definition for obesity (BMI ≥30 kg/m^2 in adults aged ≥20 years).
††Proportions were calculated as a percentage of pregnant patients among female patients of reproductive age (13–54 years); the broader age range was selected in preference to the WHO definition (15–44 years) after consultation with data contributors to reflect the actual fertility experience of the sample.
†††For definition of comorbidity, see Table S3.
‡Denominators for pandemic vaccine based on patients admitted after 1 October 2009 (when vaccine potentially became available).
‡‡Percentages calculated as a proportion of the total patients in that category who received neuraminidase inhibitor (NAI) therapy.

pandemic periods, the use of large-scale pooled observational data offers the best chance of producing meaningful results on the effect of NAIs on severe outcomes such as pneumonia.

Our definition of IRP, which required radiographic evidence of pneumonia, represents a conservative estimate of all cases of pneumonia as radiography was not routinely performed for every patient in all participating centres. We therefore also performed separate analyses, which included patients with PDP. Some patients with PDP would not have had pneumonia (false positives), and thus, we expect that the true effect estimates of the association of NAI with pneumonia and clinical outcomes probably fall somewhere between the values obtained in the analyses for IRP and 'any pneumonia'.

However, there are inevitable limitations, based on the use of retrospective observational data. Because we found an increase in IRP in several comparisons where we might have expected NAIs to have a protective effect, this suggests that our propensity scoring was not able to fully adjust for the tendency to use NAIs in more severe disease. We were unable to fully adjust for severity of illness within each propensity score because the different severity measures used across individual data sets were disparate. Furthermore, we included a broad spectrum of pneumonia severity and the available data did not permit stratification according to pneumonia severity (e.g. using CURB65 or the Pneumonia Severity Index).

NAI treatment and occurrence of pneumonia

Our findings that early initiation of NAI treatment (≤48 hours after illness onset) compared with later was associated with a significant reduction in IRP and 'any pneumonia' corroborate those previously reported from observational data on hospitalised influenza patients.[9,17,19] These trends were consistently observed across multiple subgroups: laboratory-confirmed influenza, adults, children, pregnant women and adults requiring critical care (but not children). For early treatment versus none, highly consistent, protective point estimates were also generated for most comparisons in adults and children, but failed to reach a statistical significance for IRP [possibly due to type II errors (sample size) although they reached borderline significance for 'any pneumonia' (all cases)]. As such, the results are somewhat incongruent with our previous work, which showed a 50% reduction in mortality associated with early treatment versus none.[18] It is possibly a combination of residual confounding and misclassification of pneumonia that has led to our current results, and it remains plausible that these weak signals still suggest a reduction in the occurrence of IRP.

Our other findings that NAI treatment at any time versus no NAI, and later NAI treatment compared with no NAI, universally increased the risks of IRP, contrast sharply with previous observational data on hospitalised influenza patients which found that NAI treatment (irrespective of timing) and later antiviral therapy (initiated >48 hours after illness onset) may improve a range of clinical outcomes.[19,23–28] Essentially similar observations were made for 'any pneumonia'.

Thus, in terms of the occurrence of pneumonia, our data suggest differential effects depending on the timing and use of NAIs; apparent harm associated with any or later NAI use versus no NAI; but potential benefit from early NAI use versus late NAI use or none. Based upon what is known

Table 2. Association between NAI treatment and pneumonia

Subgroups	Influenza-related pneumonia (IRP)		Any pneumonia†	
	Crude OR (95% CI)	Adjusted†† OR (95% CI)	Crude OR (95% CI)	Adjusted†† OR (95% CI)
Early NAI (≤2 days) versus no NAI treatment				
Laboratory and clinically confirmed (all ages) (n_1 = 2605; n_2 = 6710)	0·97 (0·77–1·23)	0·83 (0·64–1·06)	1·02 (0·87–1·19)	0·83 (0·70–0·98)*
Laboratory-confirmed cases (all ages) (n_1 = 2462; n_2 = 6541)	0·97 (0·76–1·24)	0·83 (0·64–1·08)	1·02 (0·87–1·19)	0·84 (0·70–0·99)*
Adults (≥16 years) (n_1 = 1934; n_2 = 3897)	0·90 (0·68–1·17)	0·80 (0·60–1·06)	1·00 (0·82–1·23)	0·82 (0·66–1·02)
Children (<16 years) (n_1 = 670; n_2 = 2765)	1·04 (0·61–1·77)	0·76 (0·42–1·36)	0·89 (0·69–1·14)	0·78 (0·59–1·03)
Pregnant (13–54 years) (n_1 = 130; n_2 = 424)	0·88 (0·27–2·93)	0·96 (0·29–3·20)	0·94 (0·41–2·18)	0·67 (0·26–1·76)
Intensive care unit (ICU) patients (all ages)				
Adults (≥16 years) (n_1 = 583; n_2 = 1015)	1·19 (0·67–2·13)	1·09 (0·59–2·02)	1·13 (0·76–1·67)	1·04 (0·69–1·56)
Children (<16 years) (n_1 = 197; n_2 = 447)	1·51 (0·58–3·97)	1·33 (0·46–3·78)	1·75 (0·99–3·12)	1·44 (0·79–2·62)
Early NAI (≤2 days) versus later NAI (>2 days)				
Laboratory and clinically confirmed (all ages) (n_1 = 5058; n_2 = 10 925)	0·34 (0·30–0·39)***	0·43 (0·37–0·51)***	0·40 (0·37–0·45)***	0·51 (0·46–0·57)***
Laboratory-confirmed cases (all ages) (n_1 = 4834; n_2 = 10 667)	0·35 (0·30–0·40)***	0·44 (0·38–0·52)***	0·41 (0·37–0·45)***	0·52 (0·47–0·58)***
Adults (≥16 years) (n_1 = 4189; n_2 = 7549)	0·34 (0·29–0·39)***	0·43 (0·36–0·51)***	0·41 (0·36–0·46)***	0·51 (0·45–0·58)***
Children (<16 years) (n_1 = 864; n_2 = 3295)	0·43 (0·29–0·62)***	0·47 (0·32–0·71)***	0·43 (0·35–0·53)***	0·53 (0·43–0·66)***
Pregnant (13–54 years) (n_1 = 256; n_2 = 649)	0·26 (0·13–0·53)***	0·32 (0·13–0·75)**	0·27 (0·17–0·44)***	0·34 (0·20–0·58)***
ICU patients (all ages)				
Adults (≥16 years) (n_1 = 1846; n_2 = 2850)	0·38 (0·29–0·51)***	0·47 (0·34–0·63)***	0·55 (0·45–0·68)***	0·62 (0·50–0·77)***
Children (<16 years) (n_1 = 251; n_2 = 655)	0·46 (0·22–0·94)*	0·45 (0·20–1·01)	0·61 (0·42–0·89)**	0·71 (0·47–1·05)
Later (>2 days) versus no NAI treatment				
Laboratory and clinically confirmed (all ages) (n_1 = 3991; n_2 = 8251)	2·53 (2·02–3·16)***	1·70 (1·34–2·17)***	2·41 (2·09–2·79)***	1·57 (1·34–1·84)***
Laboratory-confirmed cases (all ages) (n_1 = 3822; n_2 = 8048)	2·51 (1·98–3·16)***	1·68 (1·30–2·16)***	2·38 (2·06–2·76)***	1·55 (1·32–1·82)***
Adults (≥16 years) (n_1 = 3263; n_2 = 5572)	2·29 (1·78–2·95)***	1·64 (1·25–2·16)***	2·30 (1·91–2·77)***	1·58 (1·29–1·92)***
Children (<16 years) (n_1 = 724; n_2 = 2598)	2·26 (1·28–3·99)**	1·68 (0·89–3·16)	1·99 (1·55–2·57)***	1·42 (1·08–1·87)**
Pregnant (13–54 years) (n_1 = 186; n_2 = 383)	2·21 (0·76–6·45)	1·60 (0·40–6·49)	2·86 (1·30–6·25)**	1·58 (0·61–4·09)
ICU patients				
Adults (≥16 years) (n_1 = 1511; n_2 = 2249)	2·35 (1·31–4·23)**	1·55 (0·83–2·89)	1·68 (1·15–2·46)**	1·47 (1·00–2·17)*
Children (<16 years) (n_1 = 236; n_2 = 518)	5·84 (1·50–22·75)*	4·25 (1·07–16·88)*	3·50 (1·90–6·46)***	2·63 (1·39–4·96)**
NAI anytime versus no NAI treatment				
Laboratory and clinically confirmed (all ages) (n_1 = 7975; n_2 = 20 164)	1·57 (1·32–1·86)***	1·32 (1·10–1·59)**	1·62 (1·45–1·81)***	1·22 (1·08–1·38)**
Laboratory-confirmed cases (all ages) (n_1 = 7620; n_2 = 19 553)	1·55 (1·29–1·86)***	1·29 (1·06–1·57)*	1·58 (1·41–1·78)***	1·19 (1·05–1·35)**
Adults (≥16 years) (n_1 = 5964; n_2 = 13 247)	1·53 (1·24–1·91)***	1·30 (1·03–1·63)*	1·63 (1·40–1·89)***	1·24 (1·06–1·46)**
Children (<16 years) (n_1 = 2005; n_2 = 6760)	1·38 (1·00–1·90)*	1·30 (0·92–1·82)	1·41 (1·18–1·69)***	1·18 (0·97–1·43)
Pregnant (13–54 years) (n_1 = 348; n_2 = 1430)	1·48 (0·58–3·74)	1·03 (0·32–3·29)	1·74 (0·93–3·23)	1·08 (0·52–2·22)
ICU patients (all ages)				
Adults (≥16 years) (n_1 = 2721; n_2 = 4071)	2·02 (1·30–3·14)**	1·57 (1·00–2·48)*	1·58 (1·14–2·18)**	1·38 (1·00–1·92)*
Children (<16 years) (n_1 = 970; n_2 = 1579)	1·45 (0·89–2·38)	1·39 (0·85–2·29)	1·76 (1·22–2·53)**	1·59 (1·10–2·30)*

n_1 = total number of patients included in IRP analysis; n_2 = total number of patients included in 'any pneumonia' analysis.
*P < 0·05, **P < 0·01, ***P < 0·001.
†Influenza-related pneumonia and physician-diagnosed pneumonia.
††Adjusted for treatment propensity quintiles, corticosteroid use and antibiotic use.

about the mechanism of action of NAIs,[29,30] it is theoretically possible that treatment might be ineffective [tending to produce an odds ratio (OR) close to 1] but rather implausible that it would be genuinely harmful, producing an OR > 1 as we measured. Instead, we surmise that NAIs were often prescribed after the development of pneumonia or

Table 3. Association between neuraminidase inhibitor (NAI) treatment and clinical outcomes among patients with pneumonia

Clinical outcomes/exposures studied	Influenza-related pneumonia (IRP)		Any pneumonia†	
	Crude OR (95% CI)	Adjusted†† OR (95% CI)	Crude OR (95% CI)	Adjusted†† OR (95% CI)
Admission to an intensive care unit				
Early versus no NAI (n_1 = 1480; n_2 = 1855)	1·51 (1·01–2·25)*	1·44 (0·94–2·18)	2·02 (1·44–2·83)***	1·81 (1·27–2·58)**
Early versus later NAI (n_1 = 3905; n_2 = 4709)	1·15 (0·94–1·39)	0·89 (0·71–1·11)	1·09 (0·92–1·29)	0·95 (0·79–1·14)
Later versus no NAI (n_1 = 3255; n_2 = 3864)	2·59 (1·85–3·61)***	2·43 (1·71–3·45)***	2·91 (2·16–3·91)***	2·66 (1·95–3·62)***
NAI versus no NAI (n_1 = 5962; n_2 = 6976)	1·69 (1·30–2·19)***	1·59 (1·21–2·09)**	1·96 (1·55–2·50)***	1·78 (1·38–2·28)***
Ventilation support				
Early versus no NAI (n_1 = 1131; n_2 = 1287)	1·12 (0·70–1·79)	1·17 (0·71–1·92)	1·24 (0·82–1·87)	1·13 (0·73–1·75)
Early versus later NAI (n_1 = 3084; n_2 = 3459)	0·69 (0·56–0·86)**	0·68 (0·54–0·85)**	0·74 (0·60–0·90)**	0·75 (0·61–0·93)**
Later versus no NAI (n_1 = 2489; n_2 = 2760)	2·31 (1·50–3·55)***	2·48 (1·57–3·92)***	2·18 (1·48–3·21)***	2·21 (1·47–3·32)***
NAI versus no NAI (n_1 = 4739; n_2 = 5182)	1·70 (1·25–2·30)**	1·67 (1·22–2·29)**	1·69 (1·27–2·25)***	1·59 (1·19–2·13)**
Acute respiratory distress syndrome				
Early versus no NAI (n_1 = 454; n_2 = 546)	1·14 (0·32–4·07)	1·98 (0·46–8·54)	2·26 (0·76–6·67)	2·98 (0·77–11·60)
Early versus later NAI (n_1 = 1234; n_2 = 1434)	0·54 (0·33–0·90)*	0·65 (0·38–1·11)	0·55 (0·37–0·83)**	0·61 (0·40–0·94)*
Later versus no NAI (n_1 = 1032; n_2 = 1178)	2·34 (0·98–5·55)	2·23 (0·90–5·54)	3·42 (1·50–7·82)**	3·21 (1·36–7·58)**
NAI versus no NAI (n_1 = 1549; n_2 = 1836)	1·99 (0·84–4·70)	2·13 (0·87–5·21)	3·06 (1·35–6·94)**	3·14 (1·37–7·29)**
Mortality				
Early versus no NAI (n_1 = 1490; n_2 = 1866)	0·61 (0·38–0·96)*	0·72 (0·44–1·17)	0·59 (0·39–0·89)*	0·62 (0·40–0·96)*
Early versus later NAI (n_1 = 3906; n_2 = 4711)	0·84 (0·67–1·04)	0·70 (0·55–0·88)**	0·77 (0·63–0·95)*	0·69 (0·56–0·86)**
Later versus no NAI (n_1 = 3266; n_2 = 3875)	1·05 (0·73–1·52)	1·18 (0·81–1·74)	1·06 (0·76–1·49)	1·13 (0·80–1·61)
NAI versus no NAI (n_1 = 5974; n_2 = 7050)	0·88 (0·66–1·18)	0·90 (0·67–1·22)	0·89 (0·69–1·17)	0·89 (0·67–1·17)

n_1 = total number of patients included in IRP analysis; n_2 = total number of patients included in any pneumonia analysis.
*P < 0·05, **P < 0·01, ***P < 0·001.
†Influenza-related pneumonia and physician-diagnosed pneumonia.
††Adjusted for treatment propensity quintiles, corticosteroid use and antibiotic use.

clinical deterioration; furthermore, patients with IRP were admitted to hospital a median of 4 days from symptom onset, compared to 2 days for those with no pneumonia. A process of reverse causation is more likely to be responsible for the elevated risk of IRP associated with any or late NAI treatment versus none. Indeed, from our data set, we were able to record the timing of initiation of NAI treatment in relation to illness onset, but we lacked the ability to record the timing of treatment in relation to the development of pneumonia, which precluded us conducting a survival analysis. With regard to the severity of illness at the time of initiating NAI therapy, one functional measure would have been to consider site of NAI treatment initiation (outpatient, emergency department, hospital ward, ICU); unfortunately, we were not able to do this because overall there were too many missing data.

NAI treatment and clinical outcomes in pneumonia

Our other main finding relates to the effect of NAI treatment on clinical outcomes in patients with IRP. Our data reveal that patients with IRP, who were treated early with an NAI versus later, experienced a roughly one-third lower likelihood of dying or requiring ventilatory support. A mortality reduction of similar magnitude was noted when comparing early NAI versus no NAI, which was statistically significant for the analysis of 'any pneumonia', but not for IRP. Although we advise caution in the interpretation of these subgroup analyses, essentially the same finding has been made about ventilatory support in a very large cohort of children hospitalised with seasonal and pandemic influenza.[31]

We also found that among patients with 'any pneumonia', those who received NAIs were more likely to be managed in an ICU or require ventilatory support compared to those not treated with NAIs, regardless of the timing of treatment. Confounding by indication is an important consideration in relation to these data; that is, patients with severe pneumonia or ARDS who were escalated to ICU-based care would be more likely to be preferentially treated with NAIs compared to those not requiring ICU; indeed, in the PRIDE data set overall (n = 29 259), we noted that 82% of ICU patients received an NAI compared with 61% in non-ICU patients (P < 0·001). The alternative explanation that NAI treatment results in clinical deterioration with resultant increased requirements for ICU admission or ventilatory support, but no increase in mortality is unlikely and our results should

not be used to justify the avoidance of early empirical use of NAIs for patients who are severely unwell with suspected influenza.

Technical limitations

Insufficient data on influenza vaccination limited our ability to assess its potential effect on the clinical course of influenza A(H1N1)pdm09 virus infection, albeit that 9890 of 20 634 patients (48·5%) were admitted prior to November 2009 and could not have benefitted from H1N1pdm09 vaccine as it would not have been available by this point.

There were wide variations across included study centres in terms of individual study period, healthcare systems, clinical practice, treatment policies and resource availability. Although we attempted to control for these study-level biases using generalised linear mixed models, residual confounding is possible. Likewise, we cannot completely eliminate misclassification of exposure, covariate or outcome variables. Notwithstanding, we attempted to account for misclassification bias by conservatively restricting our main analysis to IRP based on chest radiograph reports. But we were unable to discriminate between viral pneumonia, bacterial pneumonia and concurrent viral and bacterial pneumonia, nor differentiate between community- and hospital-acquired pneumonia.

Despite requesting a minimum set of data variables (Table S2), the nature of the surveillance data sets provided, which were set up for monitoring during a public health emergency, meant that there were missing data on some variables of interest (e.g. admission diagnosis, comorbidities, interval from the onset of symptoms to NAI treatment, severity of disease at presentation, influenza vaccination, concomitant therapies, complications, information on follow-up).

Finally, this study does not reflect the full spectrum of disease caused by influenza A(H1N1)pdm09 virus infection in the community as it only examined hospitalised patients.

Implications and conclusions

Early NAI treatment probably reduces the likelihood of IRP. We observed highly consistent protective point estimates for early initiation of NAI treatment versus late and early treatment versus no NAI, but only the former was statistically significant; therefore, the evidence is strongest for an effect of early versus later NAI treatment. Overall, NAI treatment compared with no NAI treatment was associated with an increased likelihood of IRP; we surmise this because NAIs are sometimes started later in response to the development of pneumonia.

In patients with IRP, early NAI treatment versus later reduced the need for ventilatory support and subsequent mortality. Because randomised controlled trials of NAI treatment versus no NAI or placebo, or early NAI treatment versus late, are unlikely to be ethically or practically feasible, further evidence is needed from well-designed, prospective cohort studies in which disease severity and the dates of symptom onset, hospital admission, NAI treatment initiation and pneumonia onset are all accurately and consistently described.

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Conflict of interest

Puja R Myles and Jo Leonardi–Bee are recipients of grants from F. Hoffmann-La Roche, during the conduct of the study. Wei Shen Lim reports unrestricted investigator-initiated research funding from Pfizer to his department for research into pneumonia, a research grant from the National Institute for Health Research and consultancy fees paid to his department from the Wellcome Trust, outside the submitted work. Roland Bingisser has received consultancy fees from Philips and Alere. Jordi Carratala reports grants from Instituto de Salud Carlos III, personal fees from F. Hoffmann-La Roche, outside the submitted work. Allison McGeer reports grants from Hoffman LaRoche, during the conduct of the study, grants from GlaxoSmithKline, grants from Crucell (Johnson & Johnson), other from Cepheid, grants from Merck, grants from Sanofi-Pasteur, outside the submitted work. Barbara Rath reports grants from Hofmann LA Roche Inc. to her institution (Charité Universitätsmedizin Berlin), outside the submitted work. Dat Tran reports

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Author contributions

JSN-V-T, PRM, WSL, JL-B, SGM and SV conceived and designed the study. All authors, apart from SGM, SV, JL-B and WSL, contributed to the acquisition and local preparation of constituent data sets. SGM, SV, PRM and JL-B contributed to data set amalgamation and standardisation, design of statistical analyses and data analysis. JSN-V-T, PRM, JL-B, WSL, SGM and SV interpreted the data and wrote the paper. All authors contributed to critical examination of the paper for important intellectual content and approval of the final report. Each author acts as the guarantor of data from their individual study centre; JSN-V-T and PRM act as overall guarantors for the pooled analysis and the report.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Date of admission of 29 512 patients hospitalized with A(H1N1)pdm09 infection (by month).

Table S1. PRIDE study Investigators.

Table S2. Minimum dataset requirement.

Table S3. Standardised dataset – data dictionary with definitions used in this analysis.

Table S4. Comparison of hospitalised patients included in analysis compared with excluded patients.

Table S5. Characteristics of individual studies contributing to the current analysis.

Table S6. Sensitivity analysis excluding all ICU patients.

Table S7. Association between NAI treatment and pneumonia (all children).

Table S8. Association between NAI treatment and pneumonia (critically ill children).

Table S9. Stratified analysis based on steroid use.

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Research Article Open Access

Reduced Hospital Stay in Influenza Patients after Mass Vaccination during the 2009 Influenza Pandemic in Norway

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Abstract

Background: Norway had a preorder of pandemic vaccine, when the pandemic influenza A (H1N1)pdm09 in 2009 was declared. Mass vaccination occurred 1-3 weeks prior to the peak of the pandemic. Emergency plans were in place, but the predicted severe numbers of hospitalizations did not occur.

Objective: To study the epidemiology and clinical presentation of adult patients hospitalized with Influenza A(H1N1)pdm09, and to evaluate the impact of vaccination on the course of the pandemic at a tertiary hospital.

Methods: The low dose oil-in-water adjuvanted vaccine was used to vaccinate healthcare workers (HCWs) and at risk patients groups, and vaccination rates were recorded for the community and the hospital. Demographic and clinical information was obtained for 129 patients (>15 years), hospitalized with influenza A (H1N1)pdm09 between August 2009-January 2010. A confirmed case of influenza A (H1N1)pdm09 was defined as meeting a clinical case definition and/or laboratory confirmed disease (rt-PCR or serology). Hospital stay of more than 2 days was defined as a sign of severe illness.

Results: 1/3 of at risk patients in the community and >90% of frontline HCWs at the hospital were vaccinated. The median length of hospital stay of infected patients was significantly reduced 7 days after the onset of mass vaccination (p=0.029). There was a predominance of female and moderately obese (BMI 25-30) patients. Infiltration on chest X-ray upon admission was significantly associated with a hospital stay of >2 days (p=0.001).

Conclusion: Mass vaccination of frontline HCWs at the hospital and at risk patients in the community contributed to the observed significant reduction in hospital stay of patients infected with influenza. Almost no absenteeism enabled staff confidence and the ability for quick and safe patient turnover. This study highlights the importance of early influenza vaccination, to protect the high-risk patients and the integrity of the healthcare system.

Keywords: Influenza A(H1N1)pdm09; Pandemic, Mass vaccination; Healthcare worker; Hospital, Risk patients, Community, Epidemiology

Introduction

The emergence of a new influenza pandemic had long been anticipated since the 1968 Hong Kong Influenza H3N2 pandemic. The focus of pandemic preparedness had in later years been on the zoonosis caused by the highly pathogenic avian influenza virus (H5N1), and on increased surveillance of man and poultry, particularly in South-East Asia. Hence the world was taken somewhat by surprise when Mexico in April 2009 alerted the world to a novel influenza A virus of swine origin, subsequently referred to as Influenza A(H1N1)pdm09, heralding the advent of the first influenza pandemic for 4 decades [1,2].

Influenza vaccination remains the most effective prophylactic measure to prevent infection and limit viral spread in the general population. With limited vaccine-manufacturing capacity, a clear global and national prioritized vaccination strategy was developed. The WHO Strategic Advisory Group of Experts (SAGE) on Immunization recommended two high priority groups for vaccination: frontline health care workers (HCW) and high risk populations, including pregnant women, individuals with BMI >40 kg/m^2, and people <65 years with asthma/ chronic obstructive pulmonary disease (COPD), diabetes, chronic heart, - kidney, - hepatic or neurologic disease or immunocompromised individuals. Vaccination of HCWs was recommended to ensure the integrity of the health care system, reduce absenteeism and prevent spread of the virus in the hospital [3-6].

Norway, with a population of 4.9 million people [7], was fortunate to be well informed of the on-going pandemic [8,9-13] Preparedness plans were in place. Due to a pre order, the pandemic vaccine became rapidly available 1-3 weeks prior to the peak pandemic activity. Norway was one of the European countries with the highest vaccination coverage (45%). Haukeland University Hospital (HUH) provides specialized healthcare for approximately 260 000 inhabitants in Bergen, and is a tertiary hospital for Western-Norway, serving a population of 1 027 000 [7,14]. Vaccination among HCWs was voluntary, and willingness to become vaccinated increased with serious patient reports, and the death of a patient with no known risk factor at our hospital on October 18th (week 42). Immunization of frontline HCWs and the population at risk in Bergen commenced on October 21st 2009 (week 43), coinciding

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with a rapid increase in local hospital admissions due to Influenza A(H1N1)pdm09, which peaked three weeks later (week 46).

In this report, we studied adult patients (>15 years old) admitted to Haukeland University Hospital with confirmed Influenza A(H1N1) pdm09 disease from August 2009 to January 2010. Our aim was to describe the epidemiological characteristics, clinical features, treatment and outcome of these patients. The study period coincided with mass vaccination of both frontline HCWs at the hospital and patient groups at risk in the community, providing a unique situation to study the impact of the vaccine on the course of the pandemic. We hypothesize that early vaccination reduced the burden of the pandemic and protected the integrity of our healthcare system. The continued presence and lack of absenteeism among employees enabled confident and efficient patient care and increased handling capacity. In addition, immunization reduced the need for hospitalization in patients in the community at increased risk of severe influenza disease, thus reducing the burden of mass hospitalization.

Material and Methods

Study participants

Adult patients admitted with influenza-like illness (ILI) and hospitalized for more than 24 hours at the Medical and Thoracic Departments, HUH, were eligible to be included in the study. Of the 233 eligible patients, 104 were excluded due to lack of traceability, death from non-related illness, failing to provide informed consent, or not meeting the case definition.

A clinical case definition for Influenza A(H1N1)pdm09 disease (modified CDC case definition) included: temperature >38°C and one of the following criteria; I: ILI symptoms (two of the following symptoms): dry cough, sore throat, runny nose, muscle pain, joint pain, headache, malaise, dyspnea, vomiting/diarrhea; II: Pneumonia; III: Organ failure or collapse. Laboratory confirmed Influenza was defined as a positive (H1N1)pdm09 rt-PCR and/or positive serological antibody titers. All patients provided written, informed consent before inclusion and The Regional Ethical Committee of Western-Norway approved the study.

Study design

Vaccine: The arrival of the oil-in-water adjuvanated Influenza A (H1N1)pdm09 vaccine (Pandemrix® GlaxoSmithKline, Belgium) to Norway coincided with the increase in Influenza A(H1N1)pdm09 infection rate. Prioritized groups for vaccination were frontline HCWs at the hospital and patients in risk groups in the community. The Centre of Infection Control, HUH organized vaccination of HCWs, and the distribution of vaccine doses was initially strictly limited to frontline HCWs. Mass vaccination of the at risk population and general population was organized by the local health authorities in Bergen municipality. The city's soccer stadium and an elderly center were chosen as venues. Volunteers, retired doctors and nurses performed the vaccination with assistance from the Civil defense. The National Vaccine Register supplied data on the number of vaccinated individuals.

Subjects: Hospitalized patients were asked to provide demographic and clinical information, in addition to access to their medical charts and permission to store blood samples in a bio bank. The data were collected prospectively for each patient, including body-mass index (BMI), smoking and alcohol habits, possible Influenza A(H1N1)pdm09 exposure, influenza vaccination status, co-morbidities, pregnancy, symptoms at onset and upon admission, CRB-65 score at presentation (clinical score predicting mortality in community-acquired pneumonia (scale 0-4)), laboratory and radiological findings including Influenza A(H1N1)pdm09 rt-PCR and influenza A serology, antiviral and antibiotic treatment, supportive treatment, disease complications and outcome. Acute organ failures were specifically defined [14-16]. Data were collected through interviews with cases, and/or their parent/guardian or HCW. Physicians and two study nurses performed medical chart abstractions. Each patient was allocated a unique identification number and the data were plotted into SPSS Data entry (version 4.0).

Laboratory methods

Laboratory confirmation of Influenza A(H1N1)pdm09 was performed using swabs taken from the nasopharynx, tonsils, or lower respiratory tract (sterile brush from endotracheal tube or broncho-alveolar lavage) and collected into virus transport medium. All samples were tested at the Department of Microbiology, HUH, using rt-PCR according to CDC (Centre for Disease Control & Prevention) protocol on a light cycler 2.0 (Roche) [17-19]. Serum samples were collected from some patients and assayed in the complement fixation test (CFT) for influenza A or by haemagglutination inhibition (HI) assay for H1N1pdm09. A positive result was defined as CFT>32 or HI ≥ 40. Due to the low pre-existing immunity against Influenza A(H1N1)pdm09 in the population, one serum sample with a positive result was considered sufficient for laboratory confirmation of Influenza A(H1N1)pdm09 disease [19-21].

Statistical analysis

Epidemiological analysis describing the patient population were conducted using PASW (version 18.0), while the graphics were produced using Matlab R2010b. Data quality assurance was undertaken through implementing standard data entry checks and manual checking of entered data against the hard-copy case report forms (CRF). Assessment of risk factors for severe disease was conducted through single variable analysis using the Exact Chi-square test for categorical variables. The general significance level was set to 0.05. To handle multiple testing effects, we used a Bonferroni adjustment for testing risk factors versus hospital stay, leading to sign level 0.0045. The length of hospital stay was defined as the difference between discharge date and admission date, and defined as 1 if they were the same day. Severe disease was defined as a hospital stay >2 days. The moving median was computed for each day from the median length of hospital stay of all patients admitted during the subsequent 10 days. The moving median was used to highlight time trends.

Results

The study population

This study reports on adult patients (aged 15-93 years old, median 44 years old) admitted to HUH with confirmed Influenza A(H1N1) pdm09 during the study period. All 129 patients included either met the clinical case definition (n=119) and/or had laboratory confirmed disease (n=69). The majority of patients were ≤65 years old (81.4%) and 24 patients (18.6%) were > 65 years old (Table 1) The female to male ratio was ~ 1:1 in all age groups, except for 20-50 year olds, where it was 1.6:1.

Vaccination and length of hospital stay

Mass vaccination of frontline HCWs and patients in risk groups commenced in week 43 (Figure 1). Within two weeks approximately 3000 frontline HCWs at the hospital were vaccinated. In the Infectious Disease Unit, >95% of doctors and nurses were immunized by week 45

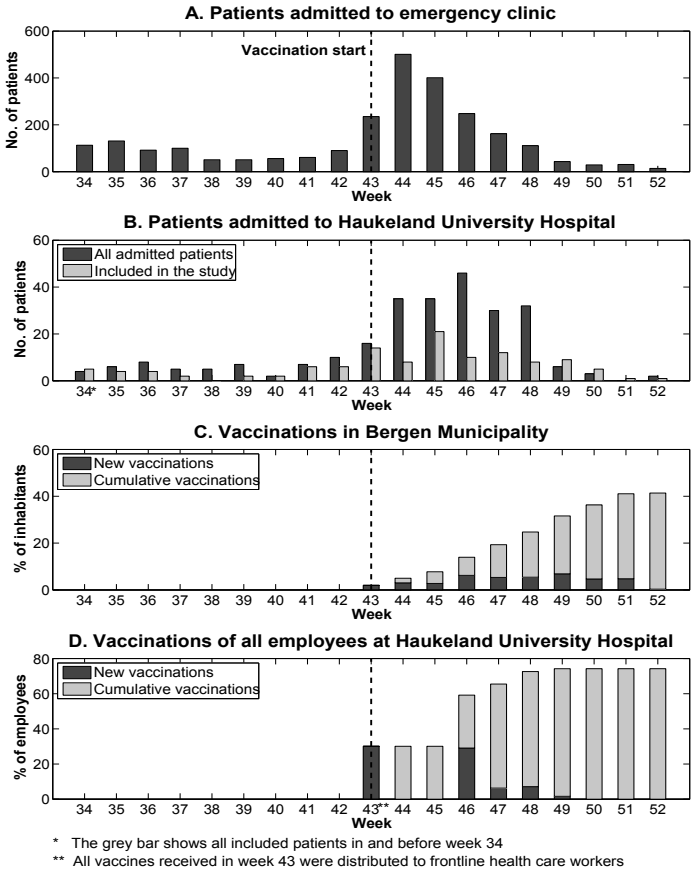

Figure 1: The time course of the pandemic and the dotted line marks the start of vaccination.

A: Patients consulting the emergency clinic with influenza-like-illness.
B: The number of patients admitted with suspected H1N1pdm09 to HUH (dark grey bars) and patients included in the study (light grey bars). There are more patients included than admitted in week 34 and 43, due to patients being recruited from other departments or delayed inclusion.
C: The number of patients in at risk groups immunized in Bergen. HCWs resident in Bergen are included in the numbers and the figure represents only people < 65 years of age. Light grey bars are the cumulative number of vaccinations; dark grey bars are the number of new vaccinations each week.
D: The number of health care workers immunized at HUH

(Personal communication, Matron Beate Haaland, Infectious disease Unit, Medical department, HUH). In week 46 the hospital received a second batch of vaccine and vaccination of hospital employees in general continued (Figure 1). In the municipality of Bergen approximately 15 000 people in the WHO defined risk groups were immunized in weeks 43-45, amounting to 1/3 of the estimated 30-40000 people at increased risk of severe disease in Bergen (Personal communication, Senior consultant Dr. Øystein Søbstad, Department of Infection Control, The municipality of Bergen).

Only 25 (19.4%) of admitted patients had received the pandemic vaccine prior to hospitalization, and 12 patients (9.5%) had received the seasonal 2009-2010 inactivated influenza vaccine (Table 1). Despite an increase in number of hospitalizations, there was a significant reduction in length of hospital stay one week after vaccination commenced, in patients admitted with confirmed Influenza A(H1N1)pdm09 (p=0.029) (Figure 2). This reduction was maintained throughout the rest of the study.

Clinical and diagnostic findings

Symptoms reported upon admission included: fever > 38°C (93%), cough (65.9%), general malaise (58.9%), vomiting/diarrhea (38.8%), dyspnea (37.2%), and clinically suspected pneumonia (10.9%) (Table 1). Only one patient presented with organ failure. The median duration of symptoms before hospitalization was 3 days.

Chronic cardiovascular disease (including hypertension) was the most common underlying co-morbidity, followed by chronic pulmonary disease including COPD and immunosuppression

	N					
Total Cases of H1N1 disease	129 (100)					
Clinically confirmed (1)	119 (92.2)					
Lab confirmed (2)	70 (54.3)					
Confirmed (clinically and lab)	69 (53.5)					
	Total		Female		Male	
	N (%)		N (%)		N (%)	
Age (median)	44.1 (15.1-93.2)		38.9 (15.9-92.8)		51.9 (15.1-93.2)	
<20 years	10	(7.8)%	5	(7.1)%	5	(8.5)%
20-50 years	59	(45.7)%	37	(52.9)%	22	(37.3)%
50-65 years	36	(27.9)%	18	(25.7)%	18	(30.5)%
>65 years	24	(18.6)%	10	(14.3)%	14	(23.7)%
Hospital stay (mean)	3 range	(1-125)%	23.2	(15.2-47.7)%	26.3	(18.6-49.2)%
BMI (mean)	24.1	(15.2-49.2)%	-	-	-	-
Smokers	33	(91.7)%	17	(24.3)%	16	(27.1)%
H1N1pdm09-vaccination	25	(19.4)%	14	(20.0)%	11	(18.5)%
Seasonal influenza vaccination	12	(9.5)%	6	(9.0)%	6	(10.2)%
Clinical features of patients under admission	Total		Lab confirmed		Clinically confirmed	Clinically and Lab confirmed
	N (%)		N (%)		N (%)	N (%)
Total	129 (100.0)		70 (100.0)		119 (100.0)	69 (100.0)
Fever Temperature > 38°C	120 (93.0)		61 (87.1)		119 (100)	60 (87.0)
Cough	85 (65.9)		51 (72.9)		79 (66.4)	45 (65.2)
General malaise (including headache)	76 (58.9)		40 (42.9)		71 (59.7)	35 (50.7)
Myalgia	71 (55.0)		38 (54.3)		69 (58.0)	36 (52.2)
Gastrointestinal symptoms	50 (38.8)		21 (30.0)		48 (40.3)	19 (27.5)
Sore throat	49 (38.0)		26 (37.1)		47 (39.5)	24 (34.8)
Shortness of breath (dyspnoea)	48 (37.2)		29 (41.4)		45 (37.8)	26 (37.7)
Joint aches	30 (23.3)		19 (21.1)		29 (24.4)	18 (26.1)
Rhinhorrea	25 (19.4)		15 (21.4)		23 (19.3)	13 (18.8)
Clinical pneumonia	14 (10.9)		6 (8.6)		14 (11.8)	6 (8.7)
Organ failure	1 (0.8)		1 (1.4)		1 (0.8)	1 (1.4)

1. Met the clinical case definition (modified CDC case definition of Influenza A(H1N1)pdm09 disease)
2. Laboratory confirmed cases had positive H1N1pdm09 rt-PCR and/or serology results

Table 1: Demographic and clinical characteristics of hospitalized patients with pandemic Influenza A (H1N1).

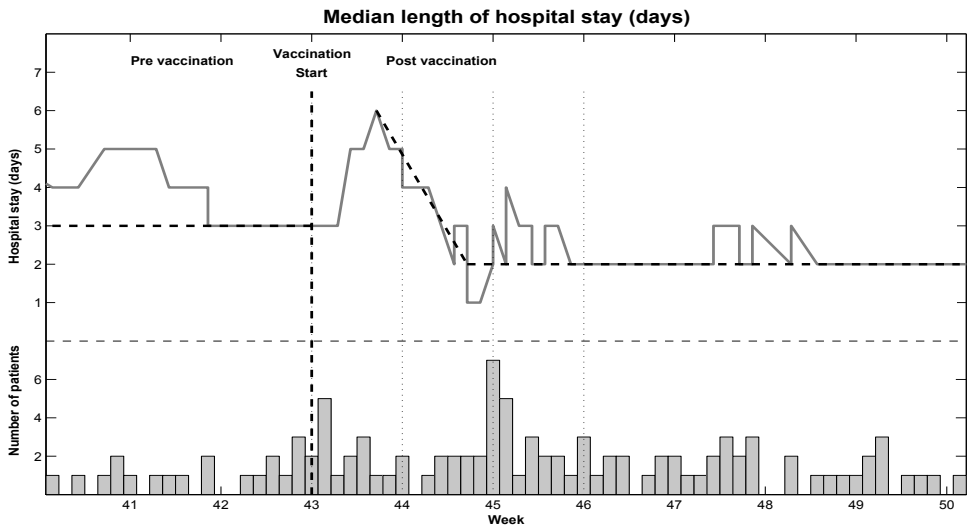

Figure 2: The median length of hospital stay (days) with regard to the timing of mass vaccination.

In the upper part of the figure, the grey line shows the median length of hospital stay of patients admitted during the following 10 days (moving median). The dotted black line illustrates the partial linear trend, showing a significant decrease in median hospital stay (days) starting one week after mass vaccination commenced (p= 0.029). In the lower part of the figure, the bar chart shows the number of admitted patients included in the study each day. (Not all admitted patients were included in the study).

(including HIV infection, bone marrow-or organ transplant) (Table 2). The mean BMI was 24 kg/m². All three patients with BMI > 40 kg/m², needed intensive care treatment (ICU). Unilateral or bilateral infiltrates were present on chest X-ray in 34 patients (26.6%) (Table 3). Of the 120 patients with fever >38°C, blood cultures were performed in 90 and 11 were positive. The most common bacteria was streptococci. There was no correlation between infiltrate shown on X- ray and positive bacteriology findings.

H1N1pdm09 rt-PCR was performed on 126 patients, and found positive in 51 patients (40.5%). Influenza A serology was performed on 47 patients, of whom 32 (68.1%) were positive. Nineteen patients with negative H1N1pdm09 rt -PCR had positive serology results. Two of the three ICU patients had three negative PCR tests before the fourth test, obtained from the lower respiratory tract, was found to be positive.

Treatment and outcome

The median length of hospital stay was three days (range 1-125 (cut-off)). A length of stay >2 days was chosen as a sign of severe disease which was associated with a significantly higher rate of infiltrates on the initial chest X-ray (29 vs. 7, p=0.001) and a tendency to more severe hypoxia (pO2<8.0 kPa) (Figure 3). Patients reporting an underlying medical condition (n=73), specifically, chronic cardiovascular disease or immunosuppression higher risk of increased hospital stay (Figure 3).

Comorbidity	Data available from N	Confirmed
		Positive finding N (%)
No comorbidity	120	47 (39.7)
1 underlying condition	120	27 (22.5)
2 underlying conditions	120	22 (18.3)
>2 underlying conditions	120	24 (20.0)
Risk factors		-
Chronic cardiovascular disease	129	37 (28.7)
Chronic pulmonary disease	129	35 (27.1)
Asthma	129	15 (11.6)
COPD	129	12 (9.3)
Immunosuppression	129	22 (17.1)
BMI>25	120	50 (41.4)
BMI>30	120	16 (13.3)
BMI>40	120	3 (2.5)
IV drug abuse	129	12 (9.3)
Malignancy	129	9 (7.0)
Chronic liver disease	129	9 (7.0)
Chronic neurological disease	129	9 (7.0)
Diabetes	129	7 (5.4)
Chronic renal disease	129	3 (2.3)
Pregnancy (1)	70	5 (7.1)

(1) Total number of women was 70

Table 2: Co-morbidities and risk factors for severe disease in patients with pandemic influenza A (H1N1)pdm09 disease.

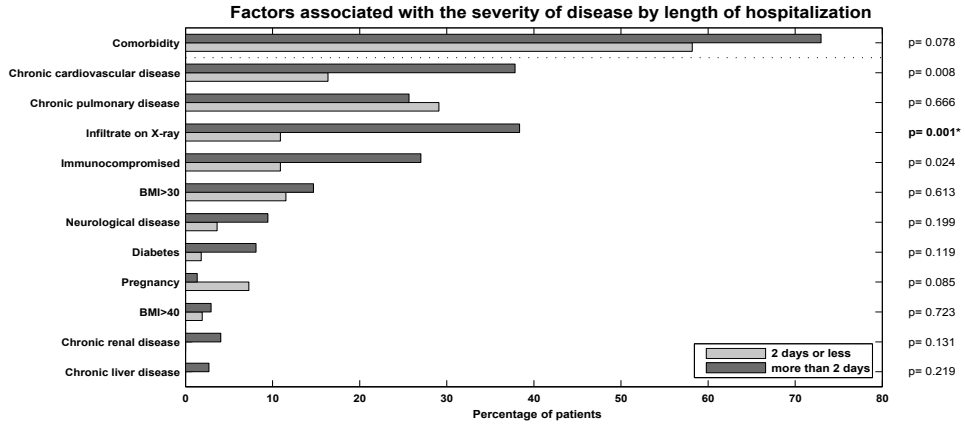

Figure 3: Risk factors associated with disease severity by length of hospital stay. The figure shows the percentage of patients with a defined risk factor divided by the length of hospital stay (≤2 days) (light grey bars) and severe illness (>2 days) (dark grey bars). The exact Chi squared test was used to evaluate the association. The p-values reported on the right column are the unadjusted p values.

Altogether 95 patients received antiviral treatment (Tamiflu) (73.6%), with a median time from onset of illness to treatment of 2 days (Table 3). In our cohort 64.4% of patients received antibiotic treatment, most commonly Penicillin, followed by Cefotaxime. Sixty-three patients (48.8%) received Oxygen treatment, of which 7 (5.5%) received non-invasive respiratory support (CPAP/BIPAP). Hypotension requiring vasopressor treatment occurred in 4 (3.1%) patients.

The three patients (2.1%) requiring ICU treatment all had fever > 38°C upon admission, were severely hypoxic (lowest had pO2 5.10 kPa), and therefore put on a ventilator (including oscillator). They were hypotensive, required vasopressor treatment, and had initial CRP counts >200. Two of the 3 patients had been infected abroad and none of them were vaccinated. They all had co-morbidities (1): COPD and obesity; (2): asthma, IV drug abuse and BMI <15 kg/m^2 and (3): cardiovascular risk, diabetes and obesity. They had CRB scores of 1,2 and 3 respectively, thus this was not a predictor of severe disease.

None of the study patients died in hospital. The majority of patients were discharged, recorded as "healthy/getting better/unchanged function in daily life". Fourteen patients were discharged with "increased need of help/support in daily life".

One patient with confirmed Influenza A(H1N1)pdm09 disease and with BMI 47 kg/ m^2, survived despite multi organ failure. This patient was hospitalized for 18 months, but followed the study for 125 days, the maximum time frame in this study. The patient was discharged to a rehabilitation center with tetra paralysis as sequelae, but cognitively intact. Despite having COPD and obesity, this patient had chosen not to be vaccinated.

Discussion

The goal of pandemic vaccination is to elicit appropriate immunological effector mechanisms to reduce viral replication and shedding, thus reduce transmission and provide protection against serious illness and death from influenza. In Bergen, Norway, the pandemic virus spread rapidly from the beginning of November 2009 and hospitalizations peaked three weeks after the onset of mass vaccination. At risk patients in the community and frontline HCWs at the hospital received the first batches of vaccine (Pandemrix®) arriving mid October in week 43.

This is a unique report on hospitalized patients with confirmed Influenza A(H1N1)pdm09 disease, where mass vaccination of the at risk population in the community and frontline HCWs at the hospital was performed prior to the peak in pandemic activity.

Despite the further increase in the number of hospitalizations, there was a significant reduction in the length of hospital stay in patients admitted with confirmed Influenza A(H1N1)pdm09 one week after mass vaccination commenced (Figure 2). This important finding we mainly attribute to the effect of vaccination. We experienced unparalleled immunization coverage of frontline HCWs at the hospital (>90%) and > a third of patients at increased risk of severe influenza in the community in weeks 43-45. We have earlier shown that protective immunity is elicited as early as 6-7 days post vaccination with adjuvant pandemic vaccine [20]. Clinical trials of candidate pandemic avian vaccines have shown 2 doses of an adjuvanted vaccine are needed to provide protective immunity. In contrast only one dose of the pandemic vaccine was required. Although no serological response was detectable in most of the population to the novel H1N1 pandemic virus, vaccines studies revealed that one dose of the adjuvanated pandemic vaccine elicited high antibody titers and hence protection within a week of vaccination. Despite the novel HA, vaccination elicited a rapid secondary response to cross reactive epitopes, hence the vaccine acted as a booster and not a priming dose of vaccine [22].

We hypothesize that the observed reduction in length of hospital stay, is due to a combined effect of lack of absenteeism of staff and reduced mass hospitalization (Figure 2). One man died prior to vaccine availability, but there were no further deaths in hospital after mass vaccination commenced.

The low HCW absenteeism possibly improved clinical confidence among medical staff and increased the ability for quick and safe patient turnover throughout the pandemic. This may also have contributed to the reduced hospital stay. It is there for likely that Norway experienced a reduced burden of the pandemic influenza disease. The burden could have been further eased, had the vaccine arrived just weeks earlier [23,24].

In our cohort, of hospitalized, infected patients, only 19% were vaccinated (compared to 45% in the general population), indicating

Diagnostic findings	Data available from	Confirmed cases	Positive finding
Infiltrate on chest radiography	128	34	(26.6)
Positive H1N1 PCR	126	51	(40.5)
Positive H1N1 serology	47	32	(68.1)
Negative PCR and positive serology	84	19	(22.6)
Positive blood culture	90	9	(10)
CRB score (1)	90	7	(85.5)
0-1	90	7	(85.5)
2	90	7	(11.1)
3-4	90	10	(3.3)
CRP count >1	128	41.5	(1-593)
Oxygen saturation at presentation (%O_2)	108	97	(50-100)
Treatment			
Oseltavimir (Tamiflu)	129	95	(73.6)
Zanamivir (Relenza)	129	0	0
Antibiotic therapy	128	83	(64.4)
Oxygen treatment	128	63	(48.8)
Non-invasive respiratory support	127	4	(5.5)
Vasopressor treatment	129	4	(3.1)

Table 3: Diagnostic findings and clinical treatment of patients with influenza A (H1N1) pdm09.

that vaccination prevented hospitalization. This is supported by findings from Canada, which found mass vaccination to be cost effective [25,26]. Twenty-five patients were vaccinated with Pandemrix® prior to hospital admission of which 11 patients had confirmed Influenza A(H1N1)pdm09. This indicates that they probably were infected and vaccinated simultaneously, without suitable time to gain protective immunity. Modeling of the effect of mass vaccination on preventing viral spread in the Norwegian community, suggested that vaccination did not commence in time to strongly influence the pandemic [24]. However, the western part of Norway, where our study was performed, experienced a slightly later wave of pandemic influenza and importantly focuses on patients requiring hospitalization due to influenza infection.

The length of hospital stay may be seen as a parameter of severity of disease as well as the ability of the hospital to rapidly handle the mass influx of patients with milder disease within the hospital´s normal capacity. These findings suggest that the pandemic vaccine provided protection from influenza illness, avoiding hospitalization in patients at highest risk of severe disease who would have required longer hospital stay (Figure 2) [5,25-27].

Timely vaccination has been estimated to have significant effect on morbidity, ICU need and mortality [2,4,25,28-30]. A study in Scotland concluded that the use of pandemic vaccine was associated with reduction in the burden of consultations with health care providers, emergency hospital admissions and most importantly mortality [30]. Our study and clinical experience supports this modeling. In Bergen, the early deployment of vaccine allowed rapid protection of front line HCWs, reduced absenteeism, despite experiencing substantial pandemic activity, thus allowing the integrity of the health care system to be maintained [20,25]. Our hospital did not experience the predicted severe mass hospitalization and avoided setting pandemic emergency plans in action such as cancelling elective surgery. A case control study from nine hospitals in Berlin suggested a protective effect of the pandemic vaccine for the prevention of hospitalization despite low vaccination coverage [31].

There is an on-going discussion in the USA regarding mandatory influenza vaccination of HCWs, and it would be interesting to compare absenteeism and patients' length of hospital stay in institutions that conducted mandatory or voluntary vaccination of HCWs, respectively [4,5,28,29,32].

This study has several limitations. The cohort represented 129 of the 233 adults admitted with suspected pandemic influenza, as we only included patients with confirmed disease, and were obliged to include only patients who provided written informed consent. Some information may therefore have been lost. Some patients may have gone undetected if initially admitted to another ward, or if they did not have fever or respiratory symptoms. In the later stages of the pandemic, the proportion of prospectively included cases decreased due to capacity limitations. Inclusion was performed retrospectively for approximately 25% of patients. The study was conducted during the heat of the pandemic without the capacity to collect convalescence serological samples. Although the study design was not primarily intended to evaluate the effect of vaccination per se, our study indicates that the Influenza A(H1N1)pdm09 pandemic peaked at a lower level than anticipated, possibly due to mass vaccination.

To lessen the burden on health care services, pharmacists could temporarily prescribe anti-neuraminidase drugs with effect from November 3rd 2009 (week 43). Furthermore, people with suspected pandemic Influenza infection were advised to stay at home for 7 days on paid sick leave, not requiring a doctor's note. Both measures contributed to lower levels of viral shedding in the community, however, the direct impact on reduced disease burden has not been evaluated in this study.

Data from the national Institute of Publlic Health (NIPH) in Norway show that approximately 1300 patients were hospitalized, nearly 200 were treated in ICU and 32 patients died from virologically confirmed Influenza A(H1N1) pdm09 only 1 of which had been vaccinated [33]. Although potentially lethal, Influenza A(H1N1)pdm09 generally caused mild disease, reflected in the short length of hospital stay in this study population. Patients were often young, otherwise healthy adults, who for a limited period were in need of observation, oxygen, antiviral treatment, and/or antibiotics.

Individuals ≥ 50 years showed a lower incidence of Influenza A(H1N1)pdm09 infection, probably due to partial pre-existing immunity from previous exposure to H1N1 infection [19,21] The present study included hospitalized adults (mean age 44.1 yrs.), adding to the growing body of literature showing a marked lower age distribution for Influenza A(H1N1)pdm09 infected patients than seasonal influenza, where the vast majority of infected patients are >65 years. The lower age distribution has been calculated to increase the estimated numbers of years of life lost (YLL) by 3-5 times compared to seasonal influenza, to 9.7 million years. Globally, South East Asia was the region with the highest YLL [34].

The study population consisted of more women than men and thus differs from the nationally reported equal gender distributions in Norway and reports from other countries where male patients dominated suspected Influenza A(H1N1)pdm09 cases [11,35-37]. The difference may be due to Norway having one of the highest percentages worldwide of working women (84%), hence influencing women to seek medical care when ill, with a female/ male ratio of 4.7:3.0. Furthermore, the female to male ratio in our cohort was almost 1 in all age groups, except for the 20-50 years old where this ratio doubled, reflecting that women are more often the main caretakers of young children, and hence more exposed to infection.

Early reports of the Influenza A(H1N1)pdm09 disease found that obesity was a risk factor for severe disease, though the mechanism remains uncertain [38,23,39]. There were a marked lower proportion of obese patients (13.3%) in our study than reported elsewhere (32-58%) [7,36,39-41], reflected by only 7% of adults (>16 years) in Norway being defined as obese [7,30,33-35]. However the most obese patient in this study was also the most severely ill, surviving total organ failure.

The clinical findings mirror reports from other countries that fever, dry cough and general malaise were the predominant symptoms upon hospital admission [35,39-43]. Furthermore, the majority of patients had underlying co-morbidities, most commonly cardiovascular disease.

In agreement with other reports, we found that the incidence of laboratory confirmed cases greatly underestimate the impact of the disease [43]. At the peak of the pandemic, rt-PCR test results were delayed due to overwhelming demand and limited laboratory capacity, resulting in the low number of positive PCR tests. The WHO global mortality numbers for the pandemic are based on reported laboratory confirmed pandemic-associated deaths. Less than 12% were reported to the WHO from the most densely populated regions [34]. Recent modeling has indicated that the WHO estimated global mortality from pandemic Influenza could be underestimated by more than a tenfold (15 times) and 51% of pandemic deaths estimated in Southeast Asia and Africa [34]. The disproportionate burden of pandemic disease born

by Africa and Southeast Asia emphasizes the importance of equitable distribution of future vaccines.

In conclusion, Norway was one of the first European countries to receive the pandemic vaccine, which arrived prior to the peak of the pandemic. This study observed a reduction in hospital stay in patients with confirmed pandemic influenza, whilst experiencing high immunization coverage of frontline HCWs at the hospital and at risk patients in the community. This suggests that mass vaccination of these prioritized groups commenced in time to elicit protection from serious effects of influenza, securing the integrity of our healthcare system.

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The Influence of Tonsillectomy on Total Serum Antibody Levels

To the Editor

Tonsillectomy, the surgical removal of the palatine tonsils, is recommended for patients with recurrent tonsillitis or tonsillar hypertrophia, as these medical conditions can cause chronic pain, repeated antibiotic use and airway obstruction such as obstructive sleep apnoea syndrome (OSAS) and secondary otitis media or speech impairment [1–3]. The palatine tonsils are glands of lymphoid tissue located in the upper aerodigestive tract, forming part of the Waldeyer's lymphatic ring and therefore playing an important role in mucosal immunity.

Although tonsillectomy has been performed for many years, some believe that the procedure increases the patient's risk of infection as it involves removal of major parts of lymphoid tissue where B and T cells can be stimulated and differentiated, consequently impairing both humoral and cellular immunity [4, 5]. Conversely, recurrent tonsillitis leads to fibrosis and atrophy in the tonsils, hence decreasing the amount of lymphoid tissue [6, 7]. As a result, the palatine tonsils lose their local immune function even if they are not removed. This is not thought to compromise the general immune system, and it has been postulated that other tonsils in the Waldeyer's ring may take up the function of the palatine tonsils, decreasing the chances of upper respiratory infections in children where these tonsils are fibrotic or removed [8–10].

The controversy whether tonsillectomy affects the immune system is still a topic of debate among the scientific community [11, 12]. In an attempt to help clarifying the outcome of that debate, we present a study where we have analysed whether tonsillectomy affects the long-term levels of total serum IgG, IgA and IgM, and consequently the immune system in general.

We measured the total IgG, IgA and IgM levels in serum collected from 45 children (3–17 years old) with recurrent tonsillitis or tonsillar hypertrophia at various time points, before and up to 1 year after tonsillectomy. Plasma samples were collected at the Department of Oto-Rhino-Laryngology, Head and Neck surgery, Haukeland University Hospital (Bergen, Norway) during the 2012–2013 influenza season. These patients were enrolled in a clinical trial of Fluenz© (AstraZenica, London, UK), a live attenuated Influenza vaccine (LAIV) [13]. The study was approved by the Regional Ethics Committee and the Norwegian Medicines Agency (EUDRACT # 2012-0028 4824.

Analysing the total serum IgG, IgA and IgM levels (Fig. 1), we found no significant alteration throughout the study period, which comprised baseline (prevaccination), vaccination day, tonsillectomy day and follow-up at 28, 56, 180 and 360 days post-vaccination (ANOVA; GraphPad Prism, La Jolla, CA, USA). IgG, IgA and IgM had a baseline serum concentration of 8.2 ± 2.6, 2.8 ± 1.2 and 0.8 ± 0.2 mg/ml, respectively. Their concentrations remained stable through pre- and post-operation and at both the short- and the long-term follow-up, suggesting that tonsillectomy does not compromise systemic humoral immunity.

Although the vaccination response was not the main focus of this study, we have also monitored the anti-influenza-specific serum titres from these patients. As an example, the serum anti-H3N2 titres rose significantly after LAIV vaccination independently of tonsillectomy and were maintained up to 1 year after vaccination, suggesting that tonsillectomy does not influence the humoral response to LAIV vaccination (unpublished results, R.J. Cox). Our data are also in line with previous studies, where patients received a parenteral influenza vaccine [14, 15].

The stability of humoral immunity after tonsillectomy has earlier been reported in studies, but did not include vaccinated subjects. Pires Santos *et al.* [16] reported a non-significant decrease of IgG 1-2 months after surgery in children under 4 years of age, and a statistically significant decrease of IgG and IgA 12-14 months post-tonsillectomy, although the values were within the normal range. Dai *et al.* [17] observed decreased levels of IgG, IgA and IgM 1 month after tonsillectomy, but returning to normal levels 3 months post-surgery. However, they suggest partial tonsillectomy rather than conventional tonsillectomy as the best procedure as they found a lower impact on short-term humoral immunity when partial tonsillectomy was performed.

Importantly, others have found significant alterations of immunoglobulins earlier in short-term studies. Nasrin *et al.* [4] found IgG levels to decrease significantly 3 months post-operation compared with preoperative data but remained similar to the normal range. However, IgA and IgM did not show significant differences in this study, lasting 3 months. Similarly, Kaygusuz *et al.* [5] found a significant decrease of serum IgG, IgA and IgM 1 month post-operation compared to preoperation, although the post-operation levels did not differ significantly from those of the control group. However, the same research group

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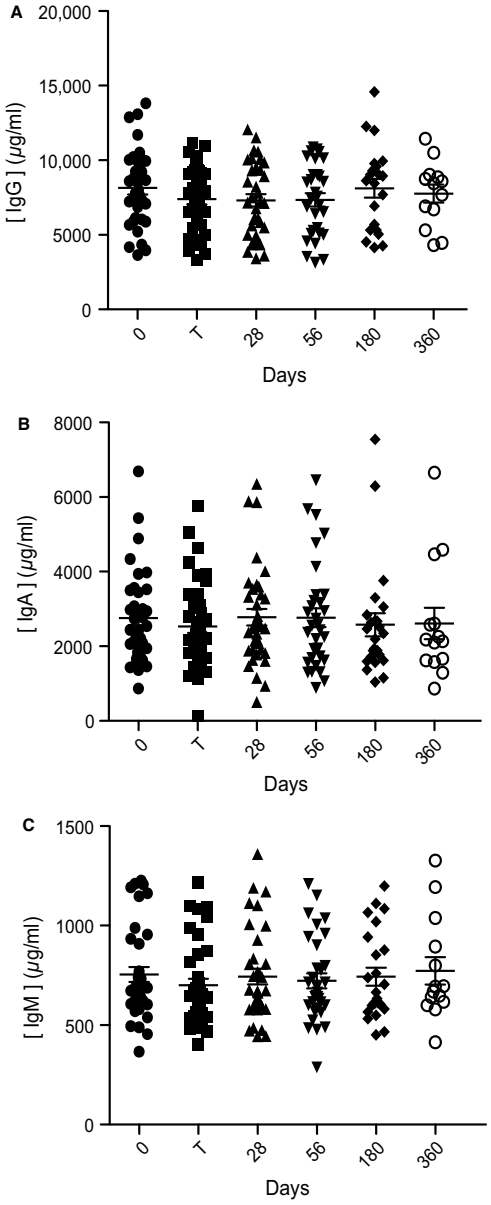

Figure 1 The total levels of serum IgG (A), IgA (B) and IgM (C) were measured by capture ELISA. Goat anti-human Ig (H+I) was used as the capture antibody (Southern Biotech, cat. no. 2010-01) and goat peroxidase-conjugated anti-human IgG, anti-human IgA or anti-human IgM immunoglobulins for detection (Sigma, cat. no. A0293, A0295, A0420, respectively). Total serum levels were measured at day 0 (the vaccination time point), at the time of tonsillectomy (T, day 3–20), and days 28, 56, 180 and 360 post-vaccination.

found a different outcome when performing a long-term follow-up study [18] collecting data from 54 months post-tonsillectomy. At that time, the levels of IgG, IgA and IgM from tonsillectomized patients were not significantly different from those found in healthy controls.

The outcome of several studies suggests that humoral immunity may be slightly impaired short term, that is the first month after tonsillectomy, due to a minor decrease in especially IgG and IgA serum levels, although these values return to normal levels after a few months. Hence, the long-term humoral immunity is not compromised.

The recovery of humoral immunity may be due to redundancy, with other tonsils from the Waldeyer's ring acquiring the function of the palatine tonsils and compensating for their removal. Another important issue is when tonsillectomy is indicated due to chronic tonsillitis. The recurrent inflammation of the palatine tonsils focuses the immune system to be extremely active in this area. Tonsillectomy removes this important focus of infection, redirecting the immune system towards other ENT areas potentially infected by airborne or foodborne pathogens.

In our study with 45 very young children, serum levels of IgG, IgA and IgM did not change significantly after tonsillectomy in either the short term or long term. Our paediatric clinical trial adds to the evidence that tonsillectomy does not compromise systemic humoral immunity nor the specific immunoglobulin response to LAIV vaccination. Hence, both surgery and LAIV vaccination can be performed within a short time period.

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