DEPARTMENT OF BIOTECHNOLOGY, CHEMISTRY AND PHARMACY

Ph.D PROGRAM IN

BIOCHEMISTRY AND MOLECULAR BIOLOGY

CYCLE XXIX

Ph.D COORDINATOR: Prof.ssa Annalisa Santucci

Analyses of hemagglutinin (HA) specific antibody responses using different serological assays after three types of Influenza vaccine in children and adults.

Scientific-Disciplinary field: BIO/11

Ph. D Thesis of:

Alessandro Manenti

Supervisor:

Prof. ssa Cosima Tatiana Baldari

Co-Supervisor:

Prof. Emanuele Montomoli

Academic Year 2016-2017

INDEX

ABSTRACT

Influenza is a contagious respiratory infection caused by a single-stranded, enveloped RNA viruses, able to cause significant morbidity and mortality all over the world, together with a significant economic burden. Vaccination strategies are the most effective methods of preventing and controlling seasonal influenza epidemics that generally occur during winter season. Every year, the composition of the vaccine has to be revaluated due to the antigenic drift mechanism inherent to the influenza viruses. In order to be licensed in the European Union and declared effective and immunogenic, every new influenza vaccine has to fulfill three criteria (at least one of the assessment should meet the requirements) (CPMP/BWP/214/96), for Haemagglutination-inhibition (HI) and/or Single Radial Haemolysis (SRH) assays. Traditionally, influenza vaccines are inactivated preparations administered as a intramuscular injection containing a standardized amount of HA influenza antigen; an alternative way of administration is the intradermal injection, evaluated in the present study. Another type of influenza vaccine, today licensed and available in Russia, U.S.A and in some European countries like Norway, is the live-attenuated influenza vaccine (LAIV), administered as a nasal spray. The actual serological assays, generally used to evaluate the immunogenicity of an influenza vaccine, are the HI and the SRH, for which correlates of protection are established. The Micro-neutralization (MN), although does not present a standardized methodology and approved correlates of protection, is the most sensitive and highest specific assay able to detect functional neutralizing antibody against the HA influenza antigen.

The present work has been divided in three main tasks. The first one is based on the set-up and standardization of a seasonal influenza growth method performed in MDCK-cell culture, with a serum free medium (SF), and a new method for virus titration in MN assay. The second one is a clinical study for the evaluation of the immunogenicity and safety of a quadrivalent intradermal seasonal influenza vaccine conducted in 150 healthy adults. The third one, is a comparative analysis, carried out on a small number of samples, with the aim to assess the difference in the magnitude of HA specific IgG subclass and IgA responses in healthy adults, children (<9 and >9 years) and health care workers (HCWs) after two different types of seasonal Influenza vaccine: LAIV (Live Cold-Adapted Influenza Vaccine) and IIV (Inactivated Influenza Vaccine). The strain chosen for the purpose of the study is the A/H3N2/Texas/50/2012 seasonal influenza strain (HA1 and HA2).

The intradermal quadrivalent vaccine, results to be comparable, in terms of immunogenicity, to the intramuscular vaccine, confirming the capability of the intradermal injection to elicit a proper immune response. All the CPMP criteria were met for all the four seasonal strains.

Moreover positive correlations were found between the results derived by the three different serological assays able to detect different anti HA-influenza antibodies in serum samples.

The LAIV vaccine was to be able to promote a stronger systemic immune response in children than in adults. In adults, TIV induces better antibody responses compared to LAIV, but comparable antibody response to that induced in LAIV vaccinated children. The different mechanism of action of LAIV *versus* TIV, may explain the relative efficacy between the two vaccines in children and adults. In children, the avidity of pre-existing serum antibodies plays a role in determining the antibody response to infection. The present results suggest that exposure history and the type of vaccine play a significant role in determining the antibody response.

1. INFLUENZA VIRUS

1.1 Overview

Influenza viruses are single-stranded, negative-sense, enveloped RNA viruses, belonging to the *Orthomyxoviridae* family (Lumb and Krug, 2001). There are three main different Influenza genera or virus types: A, B and C. Only recently it has begun to discuss about a new Influenza type: the D. Types A and B share a similar structure, if compared with Influenza C, moreover they are responsible for epidemics of respiratory illness that are often associated with increased rates of hospitalization and death (Carmell et al, 2015). Influenza type C causes a milder infection in humans, that does not cause epidemics and therefore does not have the severe public health impact of influenza A and B. Flu viruses are distinguishable on the basis of antigenic differences between their matrix and nucleoproteins (M and NP); moreover, these types of flu viruses differ with respect to host range, variability of the surface glycoproteins, genome organization and morphology. Influenza C viruses, which is substantially different from the A and B viruses, is of little importance for human influenza infections, causing only a mild common-cold like disease.

Influenza A and B are the responsible for global outbreak of illness among humans and the targets of licensed seasonal influenza vaccine (Katz et al. 2011). Influenza A viruses are responsible also for the infection of a wide variety of avian and mammalian species (Webster RG et al. 1992). On the other hand influenza B viruses are human pathogens rarely isolated in other species (Ostheraus AD et al. 2000). Influenza A strain, widely distributed in many different avian species all around the world, are maintained by asymptomatic infections, generally and frequently reported from aquatic bird belonging to the order of *Anseriformes* (duck, geese, swans) and *Charadriformes* (gulls and tenes); more than 105 species have been identified as harboring influenza A virus (Munster et al, 2007). The way of transmission in these species is through a fecal-oral route and by infecting the epithelial cells of the lower intestinal tract. Influenza A viruses have been isolated in many mammalian species, including humans, pigs, dogs, horses, sales and whales (Landolt and Olsen, 2007). Some phylogenic evidences suggest how all mammalian Influenza A viruses ultimately derive from the avian pool (Wright et al, 2007).

The current nomenclature system for Influenza A viruses includes the host of origin, the geographic location of first isolation, strain number and year of isolation (WHO, 1980). The HA and NA subtypes of influenza A viruses are specified in parentheses, e.g. A/California/7/2009 (H1N1). Due to the fact that there are no subtypes of Influenza B viruses, no specification is given in this case. Moreover, as the B viruses primarily infect humans, the only other animals known to be susceptible to influenza B infection are the seals and the ferrets (Osterhaus et al, 2000; Jakeman 1994), the host of origin is not mentioned in the influenza B viruses nomenclature e.g. B/Brisbane/60/2009.

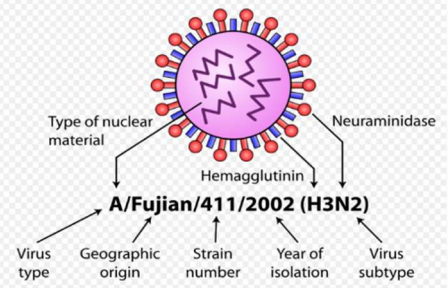


Figure 1: International Nomenclature system for Influenza A type. *Source: WHO.*

1.2 Influenza Virus Structure

Influenza viruses are spherical, or sometimes pleomorphic particles that ranging from 80 to 120 nm in diameter (Fig.2 – Fig.3) (Ruigrok RWH, 1998). Influenza viruses consist of eight independent RNA strands of negative polarity, which codify for 10 proteins.

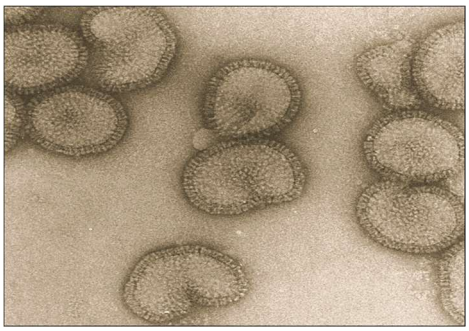


Figure 2: Electron microscopy image that shows the shape of some influenza virus particles. *Source: www.BizHealt.com*

Influenza viruses type A are enclosed in a lipid membrane (enveloped viruses), which emerges from the rod-shaped structures, from which there are the two surface antigens, two viral glycoproteins of extreme pathogenic and preventive importance, under which influenza viruses type A are classified:

Haemoagglutinin – HA *Neuraminidase - NA*

The lipid membrane contains two kind of M proteins (M1 and M2), which are involved in the stabilization and assembly of the virus. Type A flu viruses are divided into different subtypes on the basis of genetic and serologic differences: 16 different subtypes of Haemoagglutinin (HA) (from H1 to H16) and 9 subtypes of Neuraminidase (NA) (from N1 to N9) have been found. In humans, virus A, those are characterized by three types of HA (H1,H2 and H3) and two type of NA (N1 and N2). A different combination between the three types of HA and the 2 types of NA can occur. The most common types, responsible for the most recent pandemics are H1N1, H2N2 and H3N2.

The circulating flu viruses type B are not divided into subtypes like the types A viruses but they may belong to two different genetic lineages:

Yamagata Lineage

Victoria Lineage

One of the major characteristics of the Influenza A viruses is their ability to undergo antigenic change, which essentially can occur in two different ways as following described (Fig. 4).

Antigenic drift: it results from the accumulation of point mutations in the HA and NA genes during viral replication resulting in amino acid substitution in antigenic sites. These changes may render the new strain different enough to at least partially avoid the immunity induced by a previous strain infection. Thus, new influenza epidemics may arise. Antigenic drift of established human virus subtypes necessitates regular update of the composition of the annual influenza vaccine.

Antigenic Shift: Influenza viruses type A can also undergo to a more abrupt change. By definition, a shift has occurred when an influenza type A virus emerges among humans bearing either a HA protein or a combination of HA and NA proteins that have not been circulating among the human

population in recent years. Following the three major mechanisms by which antigenic shift can occur are reported:

- A virus bearing new HA and NA proteins car arise through the genetic reassortment of non-human and human influenza viruses
- An influenza virus from other animals can infect a human directly without undergoing genetic reassortment
- A non-human virus may be passed from one animal species through an intermediate animal host to humans.

Antigenic drift occurs continuously over the time whereas antigenic shift occurs infrequently and in an unpredictable way. Due to the fact that antigenic shift results in the emergence of a new influenza virus, a large portion of the world's population will have no antibodies against it. If the new strain is capable of causing illness in humans and sustained chins of human-to-human transmission, it could have the potential to spread worldwide, causing a new Influenza pandemic.

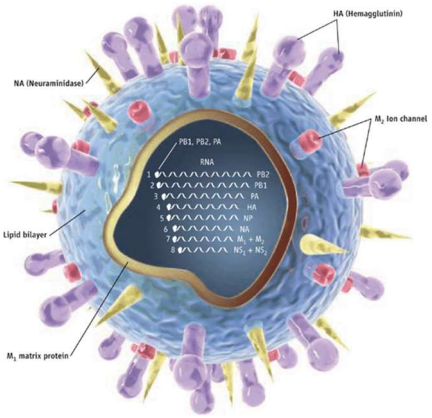


Figure 3: Structure of the Influenza virus indicating the HA and NA antigens, M1 matrix protein, M2 Ion channel, the lipid bilayer and the eight RNA segments coding for viral protein. *Source: WHO Manual for Laboratory diagnosis and virological surveillance of influenza*.

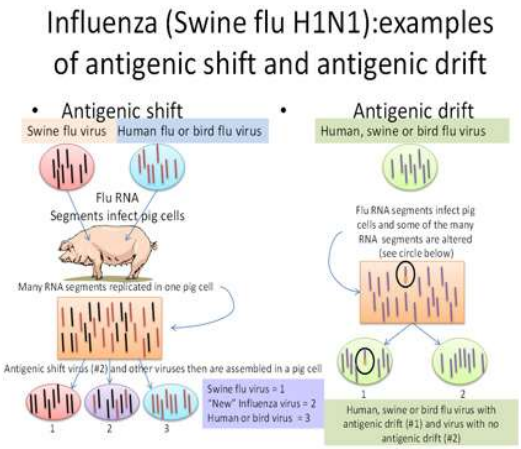


Figure 4: Example of Influenza H1N1 Antigenic Shift and Drift. Source: http://www.emedicinehealth.com/swine_flu/page3_em.htm

1.3 Influenza Envelope Proteins: "HA & NA"

HA- Haemoagglutinin

The two glycoproteins of the influenza virus membrane, HA and NA, both recognize sialic acid. HA is a trimer of identical subunits (Fig.5), each of which contains two polypeptides that result from proteolytic cleavage of a single precursor. Cleavage of the precursor is essential for activation of membrane fusion potential and hence infectivity. HA is an essential viral antigen of the influenza vaccine, moreover, it constitutes the primary target for neutralizing antibodies (Steinhauer, 1998). The main function of HA is to mediate the attachment and the fusion between the virus particle and the membrane of the target cell. It is synthesized in the infected cell as a single polypeptide chain (HA0) with a length of approximately 560 AA, which is consequently cleaved into two subunits, *HA1* and *HA2* (Steinhauer et al. 1998). These subunits remain covalently linked through disulphide bonds. Cleavage of HA0 is essential for the membrane fusion between the viral envelope and the host cell membrane. The HA spike protrudes approximately 13.5 nm from the viral surface (Wilson et al.1981). HA1 and HA2 appear in the structure of the spike as distinct subunits. HA1, the globular domain or the head domain, is responsible for the viral binding to its cellular sialic acid receptor, the receptor-binding pocket being located close to the very tip of the molecule. HA is the primary viral antigen of the host's antibody response and the only antigen including a virus-neutralization response. HA2 forms the fibrous stem of the viral spike.

From a Phylogenic point of view, there are two groups of HA (Air, 1981):

Group 1 contains: H1, H2, H5,H6, H8, H9, H11, H12, H13 and H16;

Group 2 contains: H3, H4, H7, H10, H14 and H15.

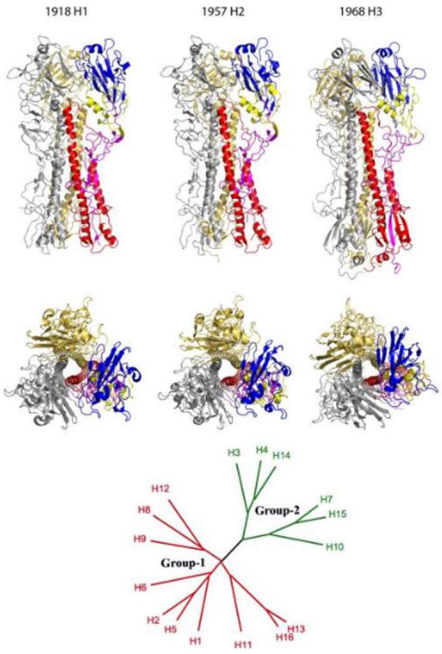


Figure 5: Crystal structures and phylogenetic organization of HAs. The upper and middle figures show orthogonal views of the H1, H2, and H3 HAs in ribbon representation. The lower part of the figure shows a phylogenetic tree containing the 16 subtypes of HA that fall into two distinct groups. As well as local variations in structure, there are significant differences in rigid body orientation of subdomains between HAs in the groups. *Source: Gamblin and Skehel, 2010.*

NA-Neuraminidase

The second envelope glycoprotein NA is characterized by enzymatic activity, cleaving sialic acid residues from glycoproteins or glycolipids (Colman PM, 1998). In absence of this protein with enzymatic characteristic, the infection would be limited to one replication cycle and the disease would be self-limiting. The 3D structure of NA consists of several domains: the cytoplasmic, trans-membrane, “head”, and also “steam”, connecting the head to the trans-membrane domain. On the viral surface, NA resembles a homo-tetramer of a mushroom shape (Fig.6). The enzyme active site and calcium-binding domain, which stabilize the enzyme structure at low pH values, are

situated in the head of NA (Takahashi T. et al., 2003). Data indicate that NA is relevant at different stage of infection. Firstly, it is considered that it helps the virus in the first approach to the target cells by cleaving sialic acids from the mucins of respiratory tract (Matrosovich et al, 2004). Secondly, it may take part in the fusion of viral and cell membranes. Thirtly, it facilitates budding of new virions by preventing their aggregation caused by the interaction of the HA of the first virus with the sialylated glycans of the second one (Wagner et al. 2000). In addition data suggest that NA amplifies HA haemagglutinating activity through the cleavage of the terminal neuraminic acid residues of the oligosaccharides surrounding the receptor-binding site of HA.

Also NA forms phylogenetically 2 groups:

Group 1 - N1, N4, N5 and N8;

Group 2 - N2, N3, N6, N7, and N9;

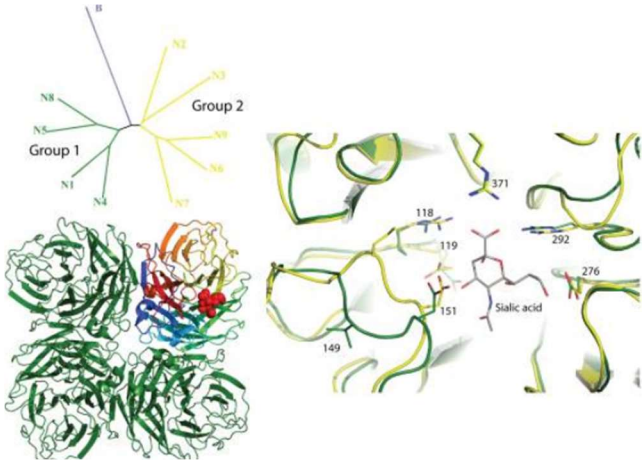


Figure 6: Phylogenetic organization and crystal structures of NA with a phylogenetic tree of the nine NA subtypes of influenza A and with NA from influenza B. The lower part of the panel shows a ribbon representation of an NA tetramer viewed along the 4-fold axis. In the right part of the figure there is a detailed view of the NA active site in an overlap between a group 1 structure (in green) and a group 2 structures (in yellow), with some key side chains shown in ball-and-stick representation. *Source: Gamblin and Skehel, 2010.*

1.4 Viral Core Structure

The influenza virus genome, is composed by eight segments of negative-sense single–stranded RNA. Each segment is associated with multiple copies of nucleoproteins (NP) and with the viral

transcriptase consisting of RNA polymerase component PB1, PB2, and PA, thus forming the RNP complex. The RNPs are surrounded by a layer of matrix protein called M1, which is the most abundant structural protein of influenza virus (Lamb, 2001). RNA segments from 1 to 6 of type A viruses encode a single protein each. The segment number 7 encodes two proteins, M1 and M2, with overlapping reading frame. The segment number 8 encodes for two non-structural proteins called NS1 and NS2 in the same way with an overlapping reading frame. NS1 protein is not present in viral particles but it is dominant into the infected cells.

Influenza A virus RNA segments and the proteins they encode		
RNA segment (no. of nucleotides)	Gene product (no. of amino acids)	Molecules per virion
1 (2341)	Polymerase PB2 (759)	30–60
2 (2341)	Polymerase PB1 (757)	30–60
3 (2233)	Polymerase PA (716)	30–60
4 (1778)	Haemagglutinin (566)	500
5 (1565)	Nucleoprotein (498)	1000
6 (1413)	Neuraminidase (454)	100
7 (1027)	Matrix protein M1 (252)	3000
	Matrix protein M2 (97)	20–60
8 (890)	Non-structural proteins	
	• NS1 (230)	–
	• NS2 (121)	130–200

Table 1: List of the RNA segments and proteins that encode for the Influenza A viruses. *Source: Influenza, Sec. Edition, Elsavier 2006.*

1.5 Virus Replication Cycle

Influenza viruses can replicate only in living cells. In this thesis, only the infection cycle in humans will be considered in detail, but this process of infection can be generalized for many other organisms. In humans, the primary targets for Influenza viruses are the epithelial cells located in the upper and lower respiratory tract.

The influenza virus life cycle can be divided into five different stage (Fig.9): (1) Receptor binding and virus entry into the host cell; (2) Entry of the vRNPs (viral ribonucleoproteins) into the nucleus; (3) transcription and replication of the viral genome; (4) assembly and release of new viral particles (Samji, 2009).

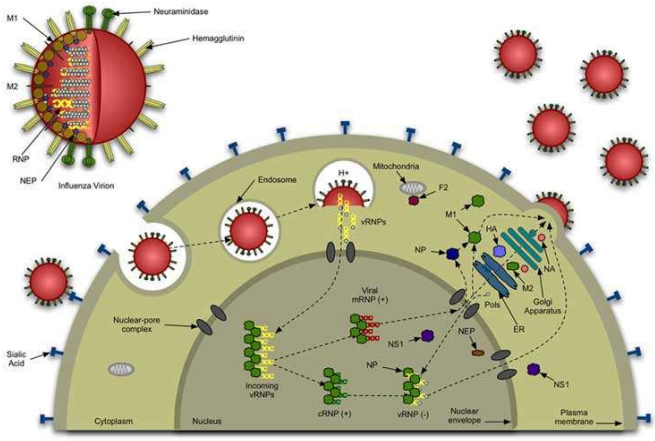


Figure 7: Image showing the mechanism of infection, replication and release of new influenza viral particles from an infected cell. The HA antigen mediates the attachment to the target cell, while the NA mediates the release of new viral particles from the infected cell. Source: http://www.itqb.unl.pt/labs/protein-modelling/activities/haemagglutinin

1) The viral HA binds to sialic acid residues on glycoproteins or glycolipids present on the surface of these cells. Human Influenza viruses prefer binding to sialic acid attached to galactose in an α2,6 configuration, whereas avian viruses prefer the sialic acid attached to galactose in an α2,3 linkage (Ito et al, 1998). This difference results to be the basis for the inefficient transmission of avian influenza viruses to humans. Upon binding to the host cell's sialic acid residues, receptor-mediated endocytosis occurs and the virus enters into the host cell in an endosome. This is a general mechanism, used by the cells to internalize macromolecular complexes before being degraded by hydrolytic enzymes. The influenza virus genome is able to escape from the degradation through the fusion of the viral envelope with the endosomal membrane allowing the access into the cell cytosol.

The fusion process is mediated by the low pH present inside the endosome, and maintained by proton pumps. This is an important and crucial step in the viral infection mechanism. At low pH, a major conformational change in the HA spike is induced, and thanks to this conformational modification the exposure of the HA2 peptide mediates the fusion (Fig.8, Fig.9). This "fusion" peptide inserts itself into the endosomal membrane, bringing both the viral and endosomal membrane into contact with each other. The acid environment of the endosome is not only important for the induction of conformational change in the HA but also to open up the M2 ion channel. M2 is a type III trans-membrane protein that forms tetramers. Trans-membrane domains form a channel that acts as a proton selective ion channel (Holisinger et al, 1991).

Opening the M2 channels acidifies the viral core. This acidic environment present in the virion permits the release of the vRNP from M1 rendering vRNP free to enter into the host cell cytoplasm.

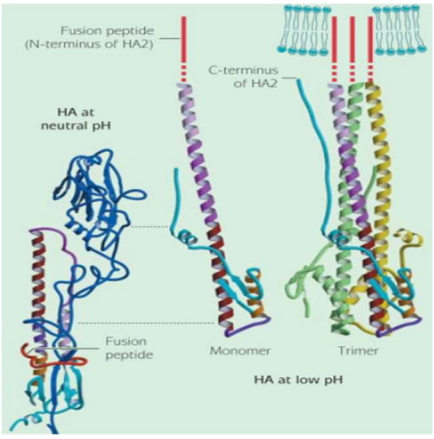


Figure 8: Conformational changes in the HA protein occurring at the pH of membrane fusion. The figure shows an HA monomer at neutral pH and the low-pH form of HA2 both as a monomer and as a trimer. The long helices are represented in equivalent positions. As a result of the acid-induced conformational change in the molecule, the HA2 fusion peptides move upwards by about 10 nm to the tip of the trimer, such that they may insert into the endosomal membrane. Then the protein folds and induces membrane fusion, the fusion peptide and the C-terminal membrane anchor ultimately ending up in the same fused membrane. *Source: Bullough PA et al. 1994.*

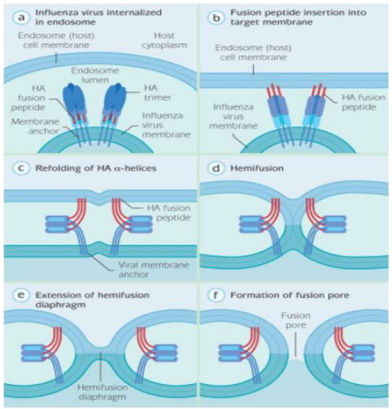


Figure 9: Supposed mechanism of HA-mediated fusion between the influenza virus membrane and the endosomal membrane, involving the formation of a hemifusion diaphragm. The principal step in this process is the relocation of the fusion peptides of HA2 to the upper part of the HA trimer

such that they can penetrate the endosomal membrane (b); this stage is drived by the low pH present in the endosome *Source: Adapted from Cross KJ et al., 2000.*

2) The transcription and the replication of the viral genome occurs in the nucleus, therefore, after being released into the cytoplasm, the vRNP must enter the nucleus. Within the nucleus, the negative viral RNA segments, are transcribed into positive-sense messenger (mRNA) by the transcriptase (consisting of PB1, PB2 and PA) carried with the RNPs. All the proteins that make up the vRNPs have known nuclear localization signals (NLSs) that can bind to the cellular nucleus import machinery and thus enter into the nucleus.

3) As mentioned above, the viral RNA segments once entered into the nucleus, are transcribed in mRNA, by a transcriptase. The transcriptase, in a process called “cap snatching”, is able to remove short cap regions from cellular mRNAs as primers allowing the start of viral mRNA synthesis. These cap regions are required for efficient binding of ribosomes to the RNA. In this way, this mechanism inhibits the synthesis of cellular proteins for the production of viral components. The mRNAs are transported back to cytoplasm and then translated into protein. The negative-sense viral RNAs also serve as templates for the production of cDNA, which in turns direct the synthesis of multiple new copies of negative-sense RNAs. These segments are transported back to the cytosol in order to assembly new virus particles. The synthesis of the viral envelope proteins HA, NA and M2 starts in the cytosol already during synthesis, the growing polypeptide chains are transported to the endoplasmic reticulum where the proteins are glycosylated and folded into trimmers and tetramers (Braakman, 1991). Through the passage in the Golgi apparatus these proteins undergo to some modifications, such as the formation of disulphide linkages and modification of the oligosaccharide side chain. The synthesis and folding of viral core proteins occur in the cytosol.

4) After the attachment of RNPs and M1 protein to the internal part of the plasmatic membrane of the cell, new viral particles are assembled. This process generally occurs in the apical part of the epithelial cells where the two external protein HA and NA are ordinated in the external layer of the membrane. As a result a new offspring of viral particles are released into the respiratory system from the infected cell which will face lysis.

2. INFLUENZA TRANSMISSION, EPIDEMICS AND PANDEMICS

2.1 Influenza Transmission

Influenza Viruses are the major causes of infection of the upper respiratory tracts, moreover they are responsible for the annually epidemics that occurs generally during winter seasons. Generally, about 5-10% of the population gets infected by influenza virus type A and B.

In human, the infection and the transmission occurs mainly via aerosol (<0.5 μm particles) and droplets (0.5 μm particles) produced by talking, coughing and sneezing (Bridges et al., 2003). Shedding of flu Virus can occur from one day before the symptom development and generally continues for three to five days (Bridges et al, 2003). Influenza can also cause a sort of asymptomatic infection in some people, and they can also continue the shedding of the virus to other people (Foy et al., 1987). Influenza occurs all over the world, with an annual global attack rate estimated to be 5-10% in adults and 20-30% in children.

The economic burden of the current outbreaks (epidemics) changes in terms of hospitalization and deaths. In the Unites States, for instance, infection from Influenza leads to the hospitalization of more than 200,000people every year and counts something like 36,000 deaths derived from complication correlates with Influenza (Lofgren et al, 2007).

2.2 Influenza Epidemic

In temperate regions epidemics occur almost exclusively in the winter months from October to April in the northern hemisphere, whereas from April to October in the southern hemisphere. It is not completely clear from what derives this seasonal regularity in the occurrence of influenza outbreaks; on the other hand, in tropical countries influenza may be present all year round (Simonsen et al, 2011).

Despite their annual seasonal character, influenza epidemics are unpredictable (Kilbourne, 2006). It is impossible to say when precisely they will start and how long they will last.

The magnitude of an epidemic in any given year is a suitable interplay between the preexisting population immunity, the extent of antigenic drift of the virus and intrinsic virulence of the new virus variant.

Viral particles replicate in the upper respiratory tract and then they are released outside as small aerosol drops through sneezing, coughing or more simply talking, as reported before. The incubation time is short taking between 1-4 days. It seems that the virus shedding occurs during cold seasons because of the immunity defenses are weakened in these periods by the cold and by the overcrowding of people in closed space. All these things can favour influenza spreading and infection.

2.3 Influenza Pandemic

Influenza Viruses are able to cause also pandemics that occur when a new virus subtype arise, characterized by a HA completely different from those circulated before (Fig.10) (Webster and Laver, 1972).

The first description of an influenza pandemic dates to the year 1510 (Morens et al, 2010).

If we take into account that the immunity towards the Influenza Virus is mainly conferred by anti-HA antibodies (Santiago et al. 2012), it is clear that the emergence of a new HA confers the naïve status to the human population for the new virus subtype, allowing its uncontrolled spreading in all the world population. A virus type (H1N1, H2N2, H3N2) have all caused influenza pandemics in the last centuries, below there are the names attributed to pandemic, the virus type and the year.

Spanish Flu (A/H1N1) in 1918

Asian Flu (AH2N2) in 1957

Hong Kong Flu (A/H3N2) in 1968

A/H1N1 virus type, from swine origin, caused the latest pandemic in 2009. The outbreak started in Mexico but the virus spread reached quickly many other countries. The severity of the illness was like is normally observed for a seasonal influenza (Cowling et al. 2010). One different point with respect to a classic seasonal influenza, which can cause morbidity and mortality mainly in elder people, this H1N1pdm virus caused disease in many young and healthy people. One explanation can be found if we take into account that many elderly people had pre-existing immunity towards this pandemic strain (CfDCaP, 2009).

Influenza viruses can rarely cross the specie barriers from animals to humans leading to a new pandemic danger. The A/H5N1 strain is an example, and it has caused alarm since the first report of transmission from avian species to humans occurred in 1997. It can cause a very high rate of

case-fatality (60%) (WHO, Cumulative number of Confirmed Human cases of avian Influenza A/(H5N1).

The severe symptoms caused by H5N1 strain include acute respiratory distress and multi organ failure often with a fatal outcame after two weeks from the onset of illness (Beigel, 2005).

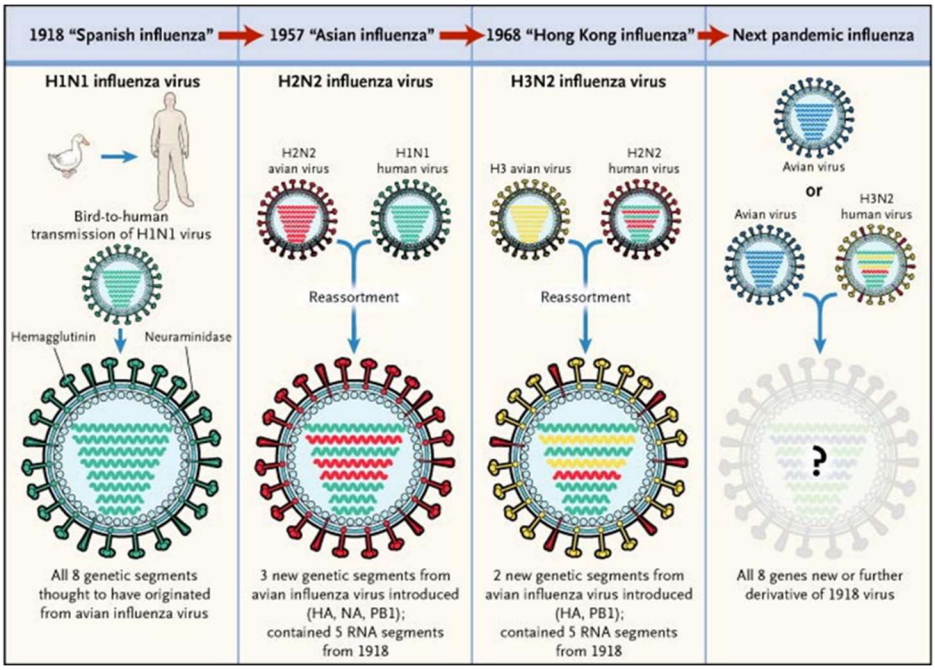


Figure 10: Image showing the two mechanisms whereby Pandemic Influenza originates from Blesche R. 2005

Pandemic Phases

The definition of pandemic that gained international acceptance is reported below:

"an epidemic occurring worldwide, or over a large area, surpassing international borders and which affects a large number of people" (Last, 2001).

The arising of a new Influenza virus type A differing from the others must meet these three requirements in order to cause a pandemic:

It must infect humans

It must be able to cause disease

It must be able to spread from one person to another

Pandemic phases have been defined by the WHO in 1999, end updated in 2005 (Fig.11) (WHO, 2008). These steps, reported below, provided reference points for nations in preparedness for a possible pandemic, and for programming an appropriate response.

Pandemic phase:

Step 1: no viruses circulating among animals have been reported to cause infections in humans

Step 2: an animal influenza virus circulating among tamed or wild animals is known to have caused infections in humans, and is therefore considered a potential pandemic threat.

Step 3: an animal or human-animal influenza reassortant virus has caused sporadic cases or small clusters of disease in people, but has not resulted in human-to-human transmission sufficient to sustain community-level outbreaks. Limited human-to-human transmission may occur under some circumstances, for example, when there is close contact between an infected person and an unprotected caregiver. However, limited transmission under such restricted circumstances does not indicate that the virus has gained the level of transmissibility among humans necessary to cause a pandemic.

Step 4: characterized by verified human-to-human transmission of an animal or human-animal influenza reassortant virus able to cause "community-level outbreaks." The ability to cause sustained disease outbreaks in a community marks a significant upwards shift in the risk for a pandemic. Any country that suspects or has verified such an event should urgently consult with WHO so that the situation can be jointly assessed and a decision made by the affected country if implementation of a rapid pandemic containment operation is warranted. Phase 4 indicates a significant increase in risk of a pandemic but does not necessarily mean that a pandemic is a forgone conclusion.

Step 5: characterized by human-to-human spread of the virus into at least two countries in one WHO region. While most countries will not be affected at this stage, the declaration of Phase 5 is a strong signal that a pandemic is imminent and that the time to finalize the organization, communication, and implementation of the planned mitigation measures is short.

Step 6: the pandemic phase is characterized by community level outbreaks in at least one other country in a different WHO region in addition to the criteria defined in Phase 5. Designation of this phase will indicate that a global pandemic is under way.

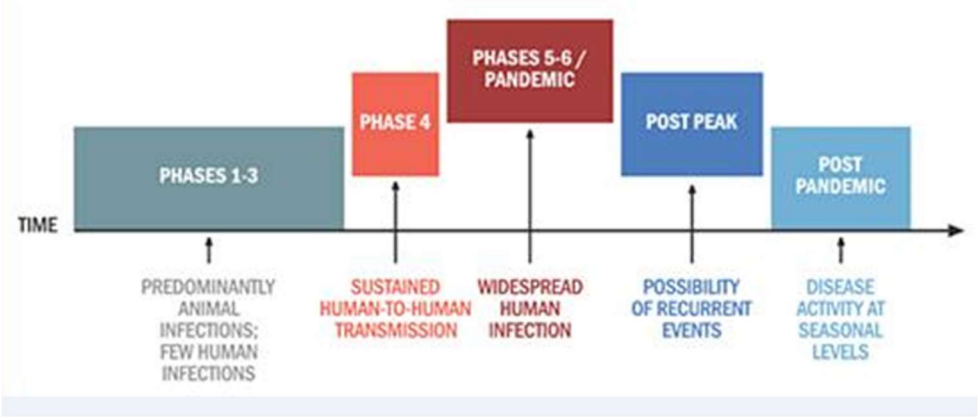


Figure 11: Pandemic phases from the Current WHO phase of pandemic alert. *Source: www.WHO.int*

3 INFLUENZA VACCINE

3.1 Vaccine overview

Influenza Vaccines are the most effective tools today available in order to reduce the morbidity and mortality derived by the influenza infection, especially for people whose are at high risk of complication, such as young children and older adults. The US military personnel, in 1945, was the first population-sample to use an inactivated influenza vaccine (Meiklejon, 1994). Today, influenza vaccination is recommended every year, with trivalent inactivated vaccine for all people aged starting from 6 months, or live attenuated influenza vaccine for healthy non-pregnant people aged 2-49 years. Children have high rates of seasonal influenza infection and illness; moreover, children under two years of age and infants show the highest risk of hospitalization and influenza-associated complications (Izurieta et al, 2000). Age ≥65 years is considered itself as a high risk factor for a more severe disease (Scheifele et al, 2013) in addition to the immunosenescence that can play an important role as well. Secondary bacterial pneumonia is a frequent complication of influenza infection in elderly people. Several trivalent influenza vaccine formulations have been modified with the aim to try to enhance protection for those classes of people. Seasonal trivalent

influenza vaccines generally contain two strains from type A viruses (H1N1 and H3N2) and one strain from type B. They must be updated yearly, with the aim to reflect the antigenic characteristics of circulating viruses. The first generation of vaccines against influenza were developed in the 1940s consisting of partially purified inactivated influenza viruses grown in embryonated hens' eggs. In 1960s, the introduction of zonal centrifugation resulted in a more effective separation between influenza virus and the egg proteins (Gerin et al, 1969; Reimer et al, 1967). The subsequent introduction of inactivated split and subunit formulations led to a lower reactogenicity. Two different types of seasonal influenza vaccines are licensed today: an inactivated (killed) preparation that is normally injected intramuscularly (IIV) and an attenuated influenza vaccine normally delivered intra-nasally as a spray nebulization (LAIV) (Fig.12) (Katz et al, 2011). The efficacy of either type of vaccine depends on several factors, not least the antigenic match between the vaccine strain and the circulating virus, the age and the health status of the vaccine recipients (Edwards et al., 1994).

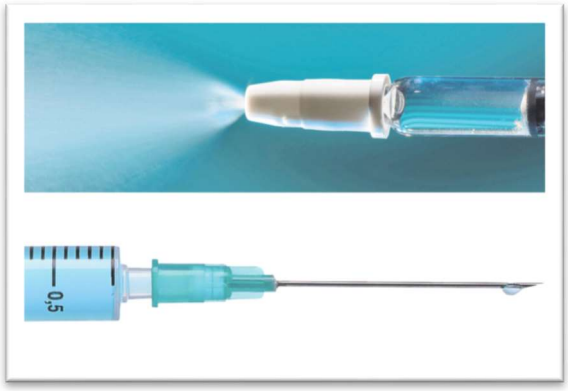


Figure 12: Two different influenza vaccines are available today. The Live Attenuated Influenza Vaccines (LAIV) (in the upper part of the figure) are administered through a spray nebulization constituted by a live, but attenuated, Influenza virus unable to cause disease. The Inactivated Influenza Vaccines (IIV) (in the lower part of the figure) are generally administered through an intramuscular injection and made with a killed virus. *Source: http://www.wsj.com/articles/for-your-next-flu-vaccine-will-it-be-shot-or-nasal-spray-1415659373.*

3.2 Inactivate Influenza Vaccine (IIV)

Inactivate Influenza Vaccine (IIV) are generally trivalent, containing a mixture of Influenza type A and B viruses. It is common practice to use reassortant strains for vaccine production that give

high yields of appropriate surface antigen. These reassortant strains present the surface glycoproteins (HA and NA) of the circulating epidemic viruses of the year, but bring the internal protein of a standardized production strain, eliminating much of the risk associated with handling pathogen strain. Flu viruses are generally grown in embryonated chicken eggs or more recently in SCRIVI PER ESTESO (MDCK) or SCRIVI PER ESTESO(VERO) cell cultures, for the production of vaccine. Below are reported the three kinds of IIV today available:

- *The whole virus vaccine*
- *The split virus vaccine*
- *The subunit vaccine*

In subunit vaccines, the two main influenza virus antigens have been purified by removing the other viral components, whereas in split vaccine the virus has been disrupted by a detergent. The whole virus vaccines contain purified whole virus. Subunit and split vaccine were initially developed to overcome the adverse reaction associated with whole virion preparations, moreover they were able to elicit a satisfactory antibody response (Jennings et al, 1981; Parkman et al, 1977).

3.3 Live Attenuated Vaccine (LAIV)

Live attenuated influenza vaccine, also called LAIV, are trivalent intranasal spray vaccine. This kind of vaccines are made with attenuated viruses able to replicate efficiency only at the temperature characterizing the nasal mucosa without causing clinical disease; for this reason they are also called Cold-Adapted viruses. The vaccine strains are generated by reassortment. More specifically, they consist of the six internal genes from a cold adapted, temperature sensitive master strain, generated by passaging at progressively lower temperature, together with the HA and NA genes from the circulating strain (Palese, 2006). The master strain contains mutations in the PB2, PB1 and NP genes, which allow efficient replication of the vaccine virus at lower temperatures (present in the upper respiratory tract) and prevent replication at higher temperature resulting in an attenuated phenotype (Katz, 2006). These LAIV viruses are able to elicit an immune response by replicating at the cooler temperatures of the nasal epithelium but do not cause clinical symptoms associated with replication at the warmer temperatures of the lower respiratory tract. Along with

IIV, LAIV vaccines are manufactured using the three influenza virus strains recommended by the WHO for the northern hemisphere.

Below there are described the main manufacturing processes followed for LAIV production (Available on internet at:http://astrazeneca.ca/en/our-medicines/en-Products-AZ) (Fig.13)

COLD-ADAPTED: in this way, virus are only able to replicate at the cooler temperature of the nasopharyngeal mucosa. They replicate efficiently at 25° C, a temperature that is restrictive for the replication of many wild type Flu viruses.

TEMPERATURE SENSITIVE: they are unable to replicate at the warmer temperature of the lower airways and lungs.

ATTENUATED: They are unable to cause clinical influenza disease.

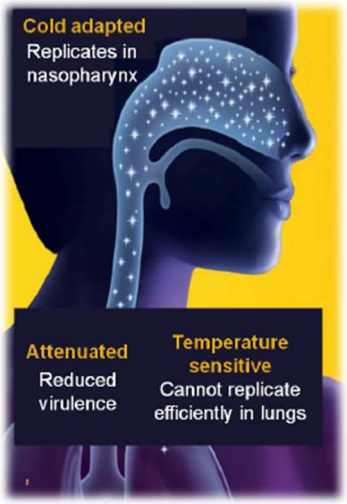


Figure 11: Main manufacturing process that modifies the influenza virus for the LAIV preparation; from http://astrazeneca.ca/en/our-medicines/en-Products-AZ.

3.4 Vaccine efficacy

It is possible to define the "Vaccine efficacy" as the ability of a given vaccine to prevent disease in vaccinated individuals, with emphasis on the effective reduction of the disease produced by the vaccine (Dunning, 2006). Influenza Vaccine, both IIV and LAIV, are effective in preventing the serious outcomes of influenza infection and complications in elderly people, and significantly

reduce hospital admission for influenza and pneumonia (Rivetti et al. 2006). However, it has been observed that there is no significant decrease in influenza infection among vaccinated elderly people (Rivetti et al, 2006) if we compare the higher rates of protection found in children and healthy adults after vaccination (Smith et al, 2006; Demicheli et al, 2004).

The difference in the efficacy between IIV and LAIV in children can be attributed to the different mechanisms of action of the two vaccines. LAIV vaccine provides a strong mucosal immune response (>IgA response), while IIV provides a more systemic immune response (>IgG). A more robust mucosal immune response could be more effective against a milder infection (Ambrose et al, 2014). The immune response upon LAIV vaccination is thought to mimic the immune response after infection. The serum antibody titers induced after LAIV vaccination are nevertheless lower than for IIV (Sasaki et al, 2007); but on the other hand LAIV vaccine induces a more significant IgA response, which contributes to protection (Treanor et al, 2003).

4. THE IMMUNE SYSTEM

4.1 Introduction

The main function of the immune system is to prevent or limit infections by several microorganisms, such as bacteria, viruses, fungi and parasites. This “system” consists of cells and molecules with specialized roles in defending the host.

The immune response can be divided into innate and acquired/adaptive. The innate immunity is provided by phagocytic cells (neutrophils, monocytes and macrophages) which are cells that release inflammatory mediators and Natural Killer cells (NKs). The innate immune response is non-specific and it has no memory in case of a second exposure to the same microorganism. The molecular components of the innate response include the complement, acute-phase protein and cytokines. On the other hand, the adaptive immune response, as the name suggests, is specific and can provide memory for a specific antigen; the adaptive response involves the proliferation of specific B and T cells (Delves, 2000). Innate and adaptive immune response generally work in synchrony to fight and eradicate different pathogens.

4.2 Innate Immunity

The innate immune system is the first line of defense of our body against several pathogens. Physical barriers, such as the skin and mucosal membranes, specialized cells, such as respiratory ciliated cells especially for Influenza viruses moving the virus away from potential target cells, and molecules are the weapons of the innate immunity and the. After these first “physical” barriers, the next line of defense is represented by the cells of the innate immune system: macrophages, dendritic cells, granulocytes and NKs. The cells of the innate immune system carry intracellularly and on the surface specific Pattern Recognition Receptors (PRRs) able to recognize pathogens-associated molecular patterns (PAMPs) of the virus (Janeway and Medzhitov, 2002; Saito and Gale, 2007). There are several different PRRs that are necessary in the detection of viruses like the Toll-like receptors (TLRs) TLR3 and TLR7, which are important for influenza (Wang et al, 2007). The binding of PAMPs to the specific PRR starts an intracellular signaling cascade with the purpose to express interferons Interferon (IFN)-α and -β and proinflammatory cytokines. The INF-α can activate dendritic cells, a key component of innate immunity due to their behaviour as antigen-presenting cells (APCs) leading to the activation of the acquired immune response. Despite the innate immunity is a really efficient system, in certain cases, it is unable to deal with the infection, and so the activation of an adaptive immune response becomes necessary.

4.3 Adaptive Immunity

The adaptive immunity can be divided into two major classes: cell-mediated immunity and antibody-mediated immunity, both highly specific for the invading organism (Fig. 14). The process by which these host defenses originate can be summarized in three actions: the recognition of the foreign organisms by specific immune cells, the activation of these immune cells to produce a specific response and the response itself, that specifically targets the organism for destruction.

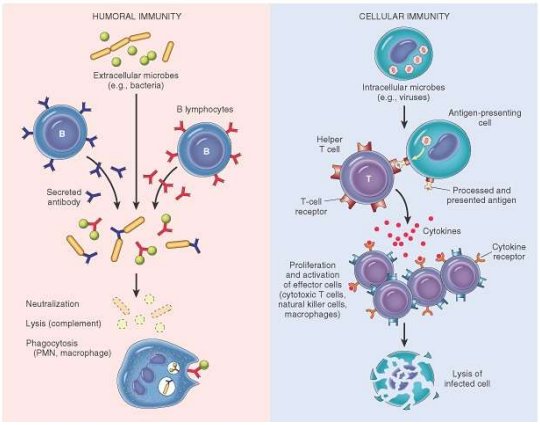


Figure 14: Simple image that shows the two main ways of the adaptive immune system showing the antibody production from the B cells and the activation and proliferation of the cytotoxic T cells. *Source: Elsevier Science, USA, 2002.*

Cell-Mediated Immunity - T cells are the protagonists for the cellular immunity. They can be classified as ($CD4^+$) and $CD8^+$ according to their expressed surface molecules. CD4 cells are also called T "helper" (Th) cells, because their principal role is the activation of B and T cells. In particular, the response towards viruses is mediated by Th1 cells, which produce the cytokine IFN-γ and activate macrophage effector function. On the other hand, CD8 cells are called "cytotoxic" cells (Tc) for their ability to kill infected cells. Another subpopulation of T cells, called "regulatory" cells (T-reg) can express both CD4 and CD8 molecules and are involved in the down-regulation of immune response after the pathogen neutralization (Romagnani, 2006). T cells recognize epitopes associated with the major histocompatibility complex (MHC) proteins. MHC is a gene complex that codify for the MHC I and MHC II proteins, whose purpose is to present pathogen peptides to the immune system. The two classes of MHC proteins present different antigen types; MHC I generally present endogenously synthesized antigens, such as viral proteins, whereas MHC II mainly present peptides from non-living particles and antigens of phagocytized microorganisms (Bontrop, 2006). Below it is reported an example regarding influenza virus infection. Dendritic cells can control the magnitude of Influenza specific T-cell immunity; in fact, the respiratory tract presents an extensive network of dendritic cell population, both in the lung and in the draining lymph node. In order to acquire the Influenza virus antigen, these cells may be directly infected with the virus. The glycoproteins of the viral envelope appear on the surface of the infected cell associated with the MHC I proteins. A cytotoxic T cell binds the viral antigen-class I MHC protein complex via its antigen-specific receptor and its stimulation through Interleukin-2 (IL-2) produced by Th cells induces the generation a clone of cells. These cytotoxic T cells specifically kill influenza virus

infected cells by recognizing viral antigen complexed with MHC protein on the cell surface and release perforins that destroy the membrane of the infected cells.

Antibody-mediated immunity - Humoral immunity is mediated by antibodies secreted by differentiated B cells. Antibodies are globulin proteins, called Immunoglobulins (Ig),that react specifically with the antigen inducing their production. They make up about 20% of the blood plasma proteins. Blood contains three type of globulins, alpha (α), beta (β) and gamma (ϒ), based on their electrophoretic migration rate. Antibodies are gamma globulins. There are five classes of antibodies in humans: IgG, IgM, IgA, IgD and IgE, with the IgM, IgG and IgA being the most important for viral immunity. The IgG class (Fig. 15) can be further divided into subclasses, named IgG1, IgG2, IgG3 and IgG4 (Mestes and Hughes, 2004)). The four IgG subclasses constitute respectively about 65%, 23%, 8% and 4% of the total serum IgG. They differ in structure, half-life and function. IgG1 and IgG3 are the most important in complement fixation and antibody-dependent cellular cytotoxicity, which have a role in virus neutralization (Frasca et al, 2013). Previous studies have shown that the major subclasses of IgG detected in serum after influenza infection or vaccination is IgG1 followed by IgG2 (Chen et al 2011) or IgG3 (El-Madhun et al, 1999). Levels of IgG4 are usually lower than those characterizing the other subclasses. B cells can switch from producing one class of Ig to another one in an unidirectional process called switching. Before being activated by an antigen, mature B cells express both IgM and IgD molecules as membrane bound antigen receptors, but after the interaction with an antigen, some cells are activated to secrete IgM, whereas others switch to produce IgG, IgA and IgE Abs (Goldsby et al, 2003). Human IgA Abs are the most abundant class secreted at the mucosal surface. They are divided into two classes: IgA1 and IgA2, which constitute 85% and 15% of the total IgA, respectively. They play their main role at mucosal surface level, but their fucntion in systemic immunity has not been elucidated yet (Roitt, 1994). The mucosal surface, covering both the aerodigestive and urogenital tracts, contains a highly specialized innate and adaptive mucosal immune systems, which represents the first line of defense against pathogens.

Below a table (Tab.3) illustrating the major functions of the different classes of Abs is reported.

Immunoglobulin	Major Funtions
IgG	Main antibody involved in the secondary response Opsonizes bacteria, making them easier to phagocytize Fixes complement Neutralizes bacterial toxins and viruses Crosses the placenta
IgA	Prevents attachment of virus and bacteria to mucous membranes Does not fix complement
IgM	Produced in the primary response to an antigen Fixes complement. Does not cross the placenta Antigen receptor on the B cell surface
IgE	Mediates immediate hypersensitivity by causing release of mediators from mast cells and basophils upon exposure to antigen (allergen) Defends against worm infections by causing release of enzymes from eosinophils Does not fix complement
IgD	Uncertain functions. Found on the surface of many B cells as well as in serum

Table 3: Major functions of the four classes of Immunoglobulin in human

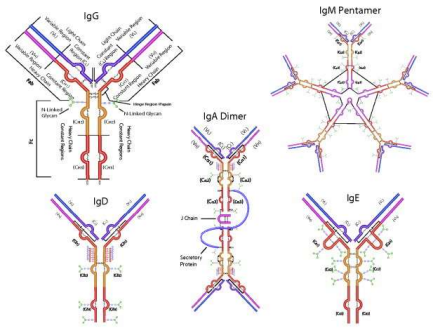


Figure 15: Molecular structure of the different antibody classes Source: http://www.sigmaaldrich.com/content/dam/sigma-aldrich/life-science/cell-biology/antibodies/main-immunoglobulin-classes.jpg

The relative role of IgM, IgG and IgA Abs in protecting against replication is still no clearly demonstrated and no strong consensus has emerged. This could be attributed to the different

mechanisms of protection against infection. In 1970 it was demonstrated that a serum HI titre above 40 was associated with protection against influenza infection in humans (Kendal, 1970). Due to the fact that IgG constitutes a large proportion of serum Ab, they must have a big role in protecting against diseases. Influenza viruses replicate in the epithelial cells of the respiratory tract, therefore an important role should be played by the IgA Abs, which are actively secreted across the mucosa. On the other hand, inactivated vaccines, that induce a more strong IgG Abs response, are nevertheless effective in preventing illness in humans (Cox et al 2004). It results that the prevention from illness is a more complex immunological process involving a complex interaction of the infection and the immune response than indirect prevents viral shedding.

5. CORRELATES OF PROTECTION AND INFLUENZA SEROLOGICAL ASSAYS

5.1 Introduction

Current IIV or LAIV are trivalent or quadrivalent and contain well standardized amount of the major virus surface antigen, the HA; generally at 15µg HA per strain. Every years, accordingly to the WHO strain recommendation about the circulating virus for the year, vaccines are updated by manufacturers. In 1996 the European Medicine Agency (EMA) Committee for Medicinal Products for human use (CHMP) published the regulatory requirements for annual updating of Seasonal Influenza Vaccine (Answorth, 2003). These criteria require vaccine manufacturers to conduct different studies each year, with the purpose to examine the immunogenicity and safety of the vaccine. The annual vaccine update requires the testing of pre- and post-vaccination serum samples from two different groups (18-60 years old and over 60 years old) and at least 50 samples per group.

The serological assays recommended by the CHMP are: the Haemagglutination-inhibition assay (HI) and the Single Radial Haemolysis assay (SRH), wherein an HI titer ≥40 and a SRH area of ≥25mm^2, are considered as seroprotective.

The CHMP has defined three main criteria that need to be fulfilled, and for the seasonal vaccine registration in the European Union at least one of the assessments should meet the indicated requirements (Cox, 2013):

1. *The Seronconversion rate (SCR) (at least 4-fold increase in titres between pre and post vaccinated serum):* it should be >40% in people between 18 and 60 years old and >30% in people over 60 years.

2. *Geometric Mean Increase (ratio of pre and post vaccination):* of >2,5 in 18-60 years old and >2 for over 60s.
3. *Proportion of subject with an HI titre ≥40 or SRH area ≥ 25mm^2*: should be >70% in 18-60 years old and >60% in 60 years old.

Another important immune-assay, generally used along with the recommended HI and SRH, is the Micro-Neutralization assay (MN), which is the gold standard for confirmation but correlate of protection are not established so far. The comprehensive results from HI, SRH and MN assays represent the basis for the assessment of vaccine immunogenicity (EMEA, 2006).

The USA Food and Drug Administration (FDA) uses the same mentioned criteria but with defines the lower bound of the 95% confidence interval that should be greater or equal to the GMT and SCR criteria.

There are other important assays that can be used to characterize the Abs response generally used in influenza field. The ELISA-Enzyme Linked ImmunoSorbent Assay, in order to detect the different Concentration and Subclass of Ig and the Neuraminidase Assay with the aim to detect functional Abs directed against the NA influenza antigen.

Test	**HI**	**SRH**	**Age group: 18-60 years**	**Age group: > 60 years**
Geometric Mean Ratio (pre to post-vaccination)			> 2.5	>2
Seroprotection	Titer ≥ 40	≥ 25mm^2	>70% of subjects	> 60%of subjects
Seroconversion or significant increase	Negative at pre-vacc. And post-vacc titer ≥ 40	Negative at pre-vacc. And post-vacc titer ≥ 25mm^2	> 40% of subjects	>30% of subjects

Table 4: Serological criteria to meet CPMP/BWP/214/96

5.2 Correlates of Protection

Although there is great importance of this concept in vaccine science, the appropriate definition of the correlates of protection still remains confuse. We can define a correlate of protection as a

“Laboratory marker of Immune response that correlates with the protection from disease” or, as suggested by Plotkin and Gilbert (Plotkin and Gilbert, 2012), “a correlate reflects a statistical relation between an immune marker and protection but does not necessary imply causal agency of the marker”. The concept is based on the capacity of a given vaccine to elicit antibodies in its recipients. As stated by Hobson et al. (Hobson et al 1972) a HI titer of 1:40 is generally associated with 50% protection against infection compared to HI titers <1:10. This cut-off was established after challenged persons presenting pre-challenge serum HI titer between 18-36 and observing a 50% of protection from infection. Other studies (Potter and Oxford, 1979) showed a clear relationship between HI titer before infection and the percentage of people infected. We should consider that this level is only 50%-70% protective and it should be regarded as relative rather than absolute. Moreover, in the field of correlates of protection, we have to take under consideration the difference between young and elderly people because of IgG serum Abs correlate well only for the group of people under 50 years (Plotkin, 2010).

5.3 HI - Haemagglutination-inhibition assay

HI is the assay most widely used for the detection of specific anti HA-Influenza antibodies; it is in fact named the “Gold Standard”. It was developed by Hirst in 1941 (Hirst, 1941; Hirst 1942), who discovered the ability of influenza viruses to agglutinate the red blood cells (RBCs). Subsequently, the test was improved, and modified by Salk (Salk, 1944). Influenza virus HA binds to terminal sialic acid of glycoproteins and glycolipids that serve as receptor on the cell membranes. Human influenza viruses bind mainly α2,6-linked sialic acid; on the other hand, avian viruses bind predominantly α2,3 linked sialic acid, with the linkage typically to a galactose residue (Rogers et al, 1983). Antibodies directed against HA antigen, that bind or block the receptor binding site, can inhibit haemagglutination. Species and quality of the RBCs are important variable for results. Generally, turkey, chicken, guinea pig and human type 0 RBCs are preferred with seasonal influenza viruses, while RBCs from horse are preferred for HI when avian viruses like H5N1 are used(Stephenson et al, 2003). Briefly (Fig. 16), sera are incubated overnight with receptor-destrying enzyme (RDE) with the aim to remove all sialic-containing glycans that can mimic the binding of influenza-specific anti-HA, giving false positive. Two fold serial dilutions of serum are than combined with an equal volume of influenza virus containing 4 haemagglutination units (HAU; one HAU is defined as the amount of virus needed to agglutinate an equal volume of RBCs

suspension). After generally 1h of incubation, the RBC suspension is then added and the HI end point titer is considered as the reciprocal of the highest dilution of serum that inhibits haemagglutination. HI is a good assay in order to screen a large number of samples and only BSL2 containment is needed, also for pandemic strain. HI is both FDA (Food and Drug Administration) and EMA approved. Although the HI assay is a simple assay to be performed and easy to automate, there are a lot of international standardization studies that have shown large variation in HI titers among different laboratories (Wood et al, 1994; Stephenson et al, 2009; Wagner et al, 2012).

There is the need to standardize laboratory protocols and to establish international standard to minimize inter-laboratory variations, although the principal cause of variation has been identified in the source of erythrocytes.

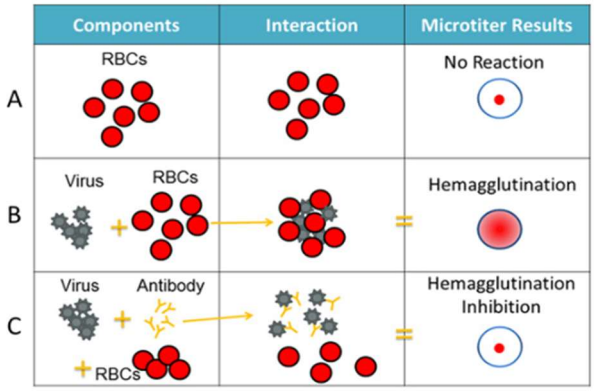


Figure 16: Principle of haemagglutination-inhibition assay: if the serum contains Influenza specific Abs, the haemagglutination of RBCs is inhibited. *Source: http://microbeonline.com/wp-content/uploads/2014/12/Hemagglutination-Inhibition-image.jpg.*

5.4 SRH - Single Radial Haemolysis assay

The Single Radial Haemolysis Assay (SRH), developed by Shild in 1975 (Shild et al, 1975), is based on passive haemolysis of erythrocytes mediated by complement and induced by the antibody-antigen complex. The haemolysis area produced on the agarose plate correlates with the concentration of anti-influenza Abs present in serum (Morley et al 1995). There are many advantages associated with the SRH: it is suitable for a large number of samples, no sera treatment is needed, only a half hour of incubation at 56°C to obtain the complement inactivation and a small volume of serum is required for the test (Trombetta et al, 2014). Another important

advantage is its safety since the test is performed with inactivated virus. For Influenza A Viruses, there is a good correlation between results obtained with SRH and HI, instead SRH is more sensitive for B strain (Wood et al, 1994). Briefly, serum sample are incubated for 30 minutes at 56°C in order to inactivate the complement. Undiluted serum samples are added to wells in a prepared immunodiffusion agarose plate (Figure 17) and allowed to diffuse at 4°C overnight. The following day the complement-mediated lysis of RBCs wherein anti-influenza Abs are present is performed by an incubation at 37°C.

SRH assay, along with HI, is officially recognized by the EMA.

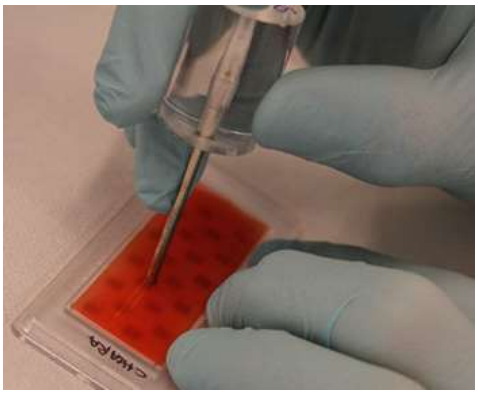


Figure 17: Immunodiffusion agarose plate used for SRH assay. *Source: VisMederi, 2014.*

5.5 MN - Micro Neutralization assay

The Micro Neutralization (MN), also named Virus Neutralization assay, represents a reliable test that can detect a wide range of functional neutralizing influenza-Abs, blocking virus attachment/entry in mammalian cells (Trombetta et al, 2014). As it has been shown in animal models (Veguilla et al, 2001; Sui et al, 2009)) Ab-mediated neutralization can predict the prevention of infection, while HI correlates with disease prevention. Although no standardized protocols and correlates of protection have been defined for MN, it is reported as a more sensitive assay than the HI and SRH for seasonal strains and H5N1 pandemic strain (de Jong, 2003). Generally, MN assay, is based on the inhibition of the cytopathic effect in MDCK, but its main drawback is that it require 4/5 days for obtaining the results. On the other hand, a faster MN, combined with an ELISA assay as read-out, requires only two days (WHO- Manual for the Laboratory diagnosis and virological surveillance of influenza). The strength of the MN assay is that it can detect even very small quantity of Influenza Abs, allowing good discrimination between pre-

and post- vaccination titer when the difference is small (Stephenson et al, 2007). One of the major limitation for the MN assay is the necessity to handle live wild-type viruses and the associated costs for high level biocontainment (Biosafety Level 3 Laboratories – BLS3) and personnel training. One solution for these problems could be the MN application with Pseudo-particle viruses that do not require biocontainment facilities. Pseudo-types virus particles can attach to the target cells, entry and after the reverse transcription and integration express a reporter gene, such as luciferase gene. Because of there are not common reference protocol for the MN procedure, there is a quite high inter-laboratory variation with assay endpoint determination discrepancies and limited knowledge of correlate of protection. Although MN is widely used in many laboratories, the main limitation of using this assay to the vaccine immunogenicity assessment is the lack of universal accepted protective threshold for neutralizing Abs. As reported by Tsang et al, 2014) an MN titer ≥ 40 was associated with 49% protection against PCR-confirmed H3N2 infection. Below (Figure 18) is reported a simple flow chart with the aim to illustrate the main passage for the MN test.

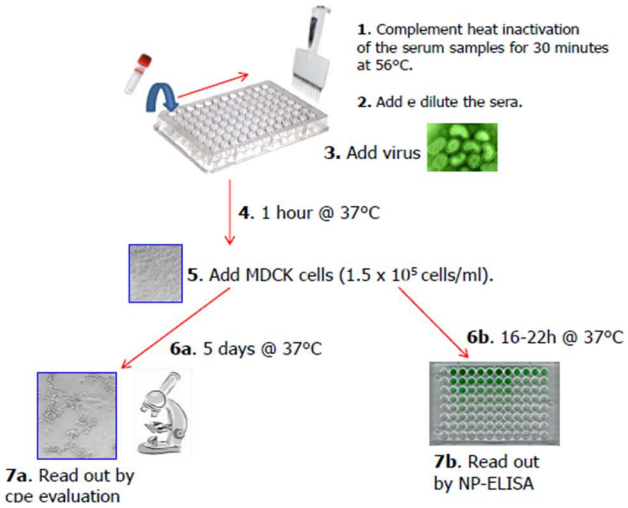


Figure 18: Flow Chart of the main passages of the MN assay with two different methods of read-out: **7a)** read out through an optical microscope to evaluate the CPE on the cells platform after 5 days of incubation; **7b)** read out through an ELISA method. *Source: WHO, 2011.*

5.6 ELISA- Enzyme Linked Immunosorbent assay

Through the ELISA assay is possible to measure, with high accuracy, the concentrations of different classes of human Abs in serum samples or in nasal washes, that are able to bind influenza whole virus or for example purified recombinant HA1 antigen. There are many different protocols available today to perform the ELISA assay but its principle remains always the same. The antigen (whole virus or HA) is coated on a 96-microtiter well plate followed by the sequential addition of serum samples, that should contain Abs against the antigen coated. Secondary conjugated Ab to detect Igs and a substrate for the colorimetric detection of the binding are then added. All these points are divided by 1h of incubation and washing steps (Figure 19).

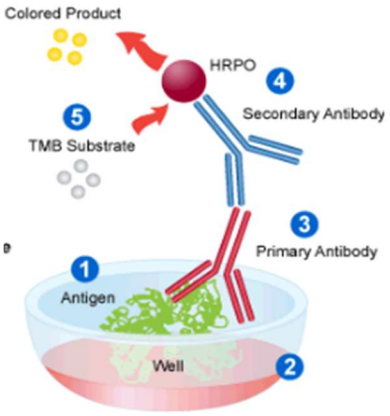


Figure 19: *Indirect* ELISA for the Ig Class or Subclass. The 96-well plate is coated with the influenza antigen overnight. The day after, the serum is diluted and added to each well. After 1 h of incubation the Secondary Antibody (Anti Human Antibody) HRP conjugated is added and incubated for 1h. TMP substrate is then added for colorimetric detection. At this point the plate is ready for the Optical Density reading. *Source: http://www.leinco.com/indirect_elisa.*

One of the advantages of the ELISA assay is that it can detect and measure serum and mucosal IgM, IgG and IgA subclasses before and after influenza vaccination (Greenbaum et al, 2004). Moreover, it is also possible to modify the assay using low concentrations of a denaturing agent, such as Sodium Thiocyanate (NaSCN), in order to evaluate the Ab-avidity: how strength is the interaction between antibody and an antigen (Brokstad et al. 1995). Moreover, with the use of recombinant HA1 domain rather than the full-length HA as a coating antigen, the specificity of the assay is improved (Alverez et al, 2010).

5.7 NA assay

All the assays above mentioned can measure anti-influenza Ab directed against the HA viral antigen, that is responsible for initiating the cell infection by contacting the sialic acid receptors on target cells and for the internalization and virus-cell fusion (Newman et al, 2006). On the other

hand, NA, constituting the second most important influenza virus antigen, present silidase activity and it is responsible to allow the release of new virus particles from the infected cell, moreover NA play a role also in the initial stage of infection by digesting decoy receptors that can block the access of viral particles to the epithelial cells of the respiratory tract (Mastrosovich et al, 2004).

It has been proved that NA immunity plays an important role in reducing the morbidity and mortality, limiting the spread to the other susceptible individuals (Johansson and Cox, 2011).

Presently, there are two main serological assays that can be used in order to detect functional anti NA-Abs: the Enzyme-Linked Lectin Assay (ELLA) and the ThioBarbithuric acid Assay (TBA).

The ELLA Assay is a more sensitive and reliable method if compared with the TBA assay, which requires also the handle of hazardous chemical reagents. ELLA is a plate-based assay that can allow the testing of a large number of samples and is therefore the method of choice for measuring NA-specific Ab in human samples (Eichelberger et al, 2014). In order to avoid interference with anti-HA Abs that could be present in tested sera, HA mismatched virus reassortants (Sandbulte et al, 2009) and detergent treated viruses (Cate et al, 2010) are generally used as NA sources.

6. AIMS OF THE STUDY

This PhD work has been focused on three main tasks:

1. The first one was based on the set-up and standardization of a seasonal influenza growth method in MDCK-cell culture with a serum free medium (SF), and a new method for virus titration, with the aim to find the proper viral quantity to be used in the Virus-Neutralization CPE-based assay. Four seasonal influenza strains were propagated, titrated and used in the clinical study described in the task number two.

2. The second one is a clinical study for the evaluation of the immunogenicity and safety of a quadrivalent intradermal seasonal Influenza Vaccine involving 150 healthy subjects. The assays used in this part are the MN assay with the four propagated viruses (task 1), the HI assay and the SRH assay. This part of the present PhD work has been completely carried out inside the VisMederi Enterprise Laboratories and in collaboration with the Department of Molecular Epidemiology at the University of Siena.

3. The third one is a comparative analysis, carried out on a small number of samples, with the aim to assess the difference in the magnitude of HA specific IgG subclass and IgA responses in healthy adult, children (<9 and >9 years) and health care workers (HCWs) after two different kind of seasonal Influenza vaccines, LAIV (Live Cold-Adapted Influenza Vaccine) and IIV (Inactivated Influenza Vaccine) against the *A/H3N2/Texas/50/2012* seasonal influenza strain (HA1 and HA2). This study involved the necessity to generate, standardize and validate a new ELISA-based serological assay for the detection of the IgG and IgA subclasses in serum samples. Part of this project was carried out during the foreign period of the PhD program at the Houkeland University Hospital and at the Influenza Center in Bergen, Norway.

7. TASK 1

7.1 Introduction

7.1.1 MDCK cell line

MDCK cell line, developed in 1958 by S.H.Madin and N.B.Darby, derived from the kidney of a normal cocker spaniel. In 1966 Gaush et al. (Gaush et al, 1966) characterized this kind of cell line by its growth rate, immunologic and cytogenetic properties, as well as their susceptibility to several A and B viruses. MDCK cells are the preferred host for the isolation and characterization of Influenza A and B Viruses but not Influenza C viruses, due to the incompatibility of sialic acid moieties on the cell surface with the viral receptor specificity. Moreover, it was found that human influenza viruses isolated and propagated in MDCK retain their original antigenic properties, making this cell line a suitable substrate for the selection of influenza vaccine strain candidate for vaccine development. MDCK cells bear both α-2,3 and α-2,6- sialosides on their surface; in this way this kind of cell line supports easily the isolation of influenza viruses shed from the mammalian cells that line the human respiratory tract with no obligate change in receptor specificity (Katz et al, 1987).

7.1.2 Serum free Medium & UltraMDCK

Serum free media are not necessarily fully defined media. They typically contain low molecular weight digests like for instance yeast extract, plant or animal tissue hydrolysate. Hydrolysates mostly contain free amino acids and peptides, but also vitamins, mineral and undefined components. Similar to fetal calf serum these poorly defined components are subject to significant lot-to-lot variation (Genzel et al, 2006).

Only few data are available concerning the composition of serum-free or protein-free media. Adaptation of cells for growth in serum free medium has been reported for different cells, often with a reduced growth rate or a comparatively high death rate.

UltraMDCK™ (Lonza) is a Serum-free Renal Cell Medium, setted up to support the growth of Madin-Darby Canine Kidney (MDCK) cells at low and high plating densities. UltraMDCK™ Medium contains low levels of recombinant human insulin and bovine transferrin, yielding a very low protein formulation. MDCK cells grown and adapted in UlraMDCK medium share different characteristics respect to the serum containing cultures; smaller and more densely packed.

Furthermore, cell cultures can stay confluent for at least two weeks without medium change. There are also several advantages derived from the use of a serum free medium:

- Superior growth rate and characteristics without the growth inhibitors generally found in serum
- Simplification in purification procedure
- A defined growth environment without the inconsistencies and concerns of serum

Before using the cell for the viral growth or for MN cell suspension, several passages must be performed in the UltraMDCK medium, in order to accustom cell to a serum-free medium.

7.1.3 Cytopathic effect

In 1954 J.F.Enders proposed to classify viruses into groups of those that caused degeneration, those that cause formation of inclusion bodies and cell degeneration, those that cause formation of multinucleated cells (syncytia) and those that caused no cytopathic effect.

During the time that synthesis of viral components is occurring in the infected cell, the cell goes through characteristic biochemical and morphological changes. Progression of these changes is most readily observed in cell culture, where infection of cells is more easily synchronized and where the cells can be observed and sampled frequently during the course of infection. All morphological changes occurring in cells and caused by viral infection are called cytopathic effects (CPE) (Figure 1); the responsible virus is said to be cytopathogenic. The degree of visible damage/lysis to cells caused by viral infection varies with type of virus, type of host cells, multiplicity of infection (MOI), and other factors. Some viruses cause very little or no CPE in cells of their natural host.

Some CPE can be readily observed in unfixed unstained cells under low power (10X objective with 10X ocular for 100X magnification) of the light microscope, with the condenser down and the iris diaphragm partly closed to obtain the contrast needed for viewing translucent cells.

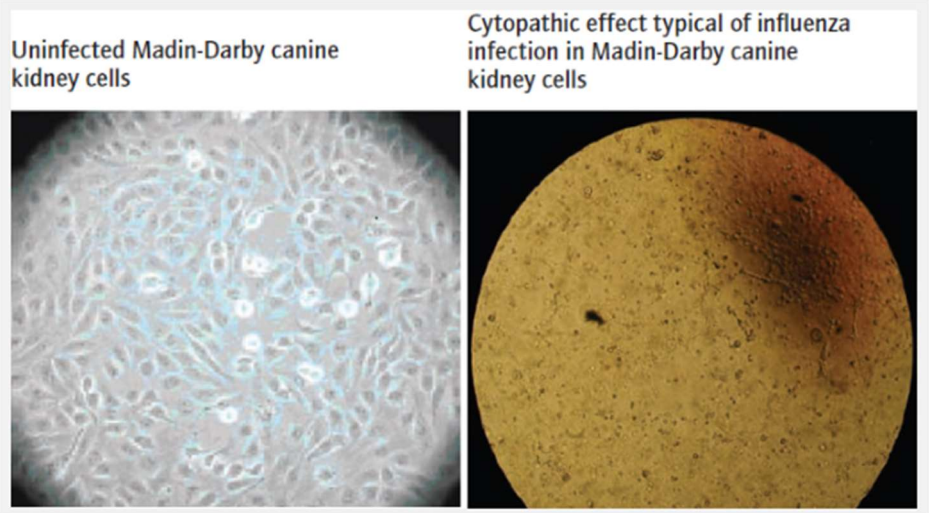


Figure 20: Difference between uninfected MDCK cells and the presence of the cytopathic effect derived from an Influenza virus infection. *Source: www.WHO*.int.

7.2 Scope, Set-up and Viral growth method

The Influenza virus growth has different purpose:

- Is the first step in the manufacture of new cell-based influenza vaccine; influenza vaccine have traditionally been prepared in embryonated chicken eggs, but there are several problems associated with the use of eggs, including the variability in their susceptibility to influenza infection. Cell culture however, is far more sensible to a large scale production.

- Is the first step in order to isolate an Influenza Strain from human/animal sample.

- It is functional in order to produce from few microliter of the original stock of the virus a big amount of the same, able to cover a whole, and large clinical trial in Micro-Neutralization assay.

Each Influenza strain has its own growing temporal conditions.

The incubation time for influenza is typically about 48h, but can vary between 24-96h, possibly owing to the size (virion quantity) of the initial inoculum. Cell infection started by adsorption of the virions to the cell surface. The influenza virus haemagglutinin (HA) is responsible for binding the sialic acid receptor on the surface of epithelial cells providing a strong bond, facilitating the

adsorption of virions into the cell. This results in receptor-mediated endocytosis of the virus particles approximately 20 min after infection.

Once inside the cell, the virions begin replicating, using the machinery and buildings materials that would normally be used by the host cell to maintain its function. The period between successful infection of the cell and the productive release of viral progeny is often called the “eclipse phase”. Just as it did upon cell entry, the HA on the surface of the virions will again bind the sialic acid receptor. The virus Neuraminidase (NA) is responsible for cleaving the sialic acid receptors on the surface of the cell to allow the newly-produced influenza virions to be released and go on to infect other cells. Successive cycles of infection quickly result in an exponential growth of viral titer, which peacks around 3-5 days post infection.

In order to find the best condition of propagation, and be able to define the growth curve for each seasonal strain, is necessary to monitor the flasks in which the virus is growing daily by the observation of the cytopatic effect (CPE) produced in the cell monolayer of the flask, and take under consideration the evolution of the haemagglutination titre of the supernatant and the viral titre by the $TCID_{50.}$ titration.

The table below is an example of the daily monitoring of growth for the H1N1/California strain.

Initially, to understand the growth pattern for each viral strain, it is advisable to monitor the flasks for a period ranging between 3 and 5/6 days.

The better size flask to use in this part of the viral growth set-up is $25cm^2$, due to the fact that they are more manageable and they require a small amount of medium.

Strain: **A/H1N1/California**
HA titer of working seed: **20230**
TCID50 titer of working seed: **3.5**
Infection date: **xx/yy/ZZZZ**
Startin dilution: **1:1000**

Nº of Propagation: 1												
CONDITIONS	Day 2 (after 24H)			Day 3 (after 48h)			Day 4 (after 72)			Day 5 (after 96)		
	CPE	HA (1ml)	TCID50/ml	CPE	HA(1ml)	TCID50/ml	CPE	HA(1ml)	TCID50/ml	CPE	HA(1ml)	TCID50/ml
FLASK- $175cm^2$	5%	0	0	25%	1788.6	2	65-70%	4550	2.5	80%	4550	3.5

Table5: Example of recording sheet used for the monitoring of the viral growth.

It is a necessary condition to follow day by day the growth of the virus in order to be able to collect it at the maximum titer before the decrease.

The HI titer, on the other hand, does not decrease as the viral titer, but when achieve the maximum it remains at the plateau, because of the HI titer measure the quantity of the Haemoagglutinin antigen and not the live virus functionality.

It might be possible that, if the viruses to be propagated are egg-derived, more cycle of propagation are required in order to achieve a satisfactory viral titer. This is due to the fact that an egg-derived virus could require time in order to get used to a new substrate of growth. Generally, it takes between 2-3 cycles of propagation.

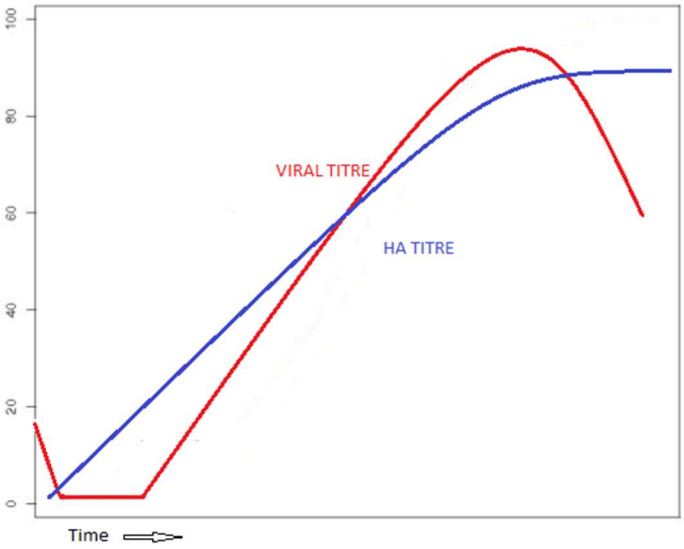


Figure 21: Viral growth curves; the viral titer (red curve) measure the amount of live virus instead the HI titer (blue curve) measure the amount of haemoagglutinin protein, this value does not give a functional data.

Method:

Ultra MDCK cells culture

- Seed 5Ml of 1 x 10^6 MDCK cell/ml in ULTRAMDCK medium supplemented with
- 2mM GLU and 100 U/Ml Pen-Step in 25cm^2 cell culture flask
- Incubate Incubate at 37°C and 5% of CO_2 in CO_2 for one day
- View cultures in 25 cm^2 flask to check confluency using an inverted microscope
- When the cell monolayer are at 80-100% confluency the flask is ready for the inoculation

Virus Inoculation

- Remove the spent medium from the flask and discard it
- Add 5 Ml of DPBS for each flask and rock gently in order to wash the cell culture
- Remove the DPBS from the flask and discard it
- Repeat the step 2) and 3) one more time
- Add 500 µl of medium plus virus diluted (from 1:10 to x) (0.5 % TPCK concentration)
- Incubate at 37° C and 5% of CO2 in CO2 incubator for 1 h
- wash the flask with DPBS
- add 5 ml of virus growth medium (0.5 TPCK final concentration)
- Incubate at 37 °C and 5% of CO2 in incubator for 2-5 days

Harvesting and storage

- Collect the virus solution in 15 ml falcon
- Centrifuge the virus solution at 1200 rpm for 3 minutes in order to precipitate cells debris
- Filtrate the virus solution in a 0.45 µm filter
- Store the virus solution at -80°C.

The condition that could be differentiated for each seasonal strain are:

- The amount of TPCK to add in the propagation medium
- Daily addiction of a percentage of TPCK or only at the beginning of the infection
- The incubation time of the flask, ranging between 45 -120 minutes
- The temperature of incubation; the H1N1 strain, could be incubated at a temperature of 35/33°C.

- The starting dilution of the Virus to be inoculated (1:10 – 1:100 – 1:500 – 1:1000 - 1:10000) depends by the initial titer of the stock to be propagated and the Moltiplicity of Infection (MOI) at which we desire to infect.

After the harversting, the virus must be immediatly stored at -80°C. It is possible also to centrifugate the virus after the harvesting in order to remove the cellular debrits, this is mainly

recommended when the virus has a low titre for the MN application, for example when the TCID50 is more or less $10^{3.5}$/1ML.

7.2.1 Calculation of the Viral Titer – TCID50

In order to calculate the titer of the propagated virus the $TCID_{50}$ assay (Tissue culture infective dose 50%) must be performed. The "$TCID_{50}$ titer" can be expressed as the viral dose that give rise to a cytopathic effect in 50% of cells in the inoculated culture; expressed as $TCID_{50}$/ml. For the $TCID_{50}$ assay, ten-fold dilution of the propagated virus must be done starting from 1:10 dilution, in ULTRAMDCK SF Medium at 0.5% TPCK as a final concentration. At the end of the dilution step, 100 μl of cell suspension (ranging between 1.5-5 $x10^5$ cell/ml) has to be added to each well. The plate must be incubated at 37°C - 5% CO2 for a maximum of 4 days. The CPE in each well is evaluated using an optical microscope. The final viral titer is determined by means of the TCID50 using the Spearman/Karber (Spearman, 1908) method on treated MDCK cells.

$$X = \frac{V}{R} \cdot 2^{(\frac{Nneg}{R} + 0,5)}$$

Sperman-Karber formula (*X*= neutralization titre; *V*= Serum startig dilution; *R*= number of repetition for each tested sample; *Nneg*= number of negative wells)

7.3 Micro-Neutralization Set-Up

In order to set-up a more sensitive and reliable MN assay, based on the UltraMDCK SF medium, several experiments were performed with the propagated virus and a Negative serum and Antiserum (serum High Positive) provided by NIBSC.

In order to check if the $TCID_{50}$ values found can match with the Micro – Neutralization assay has been designed this kind of "MN cross-test" with the Antiserum and Negative Serum. Below is reported the scheme plate of this kind of experiment (Figure22a, 22b)

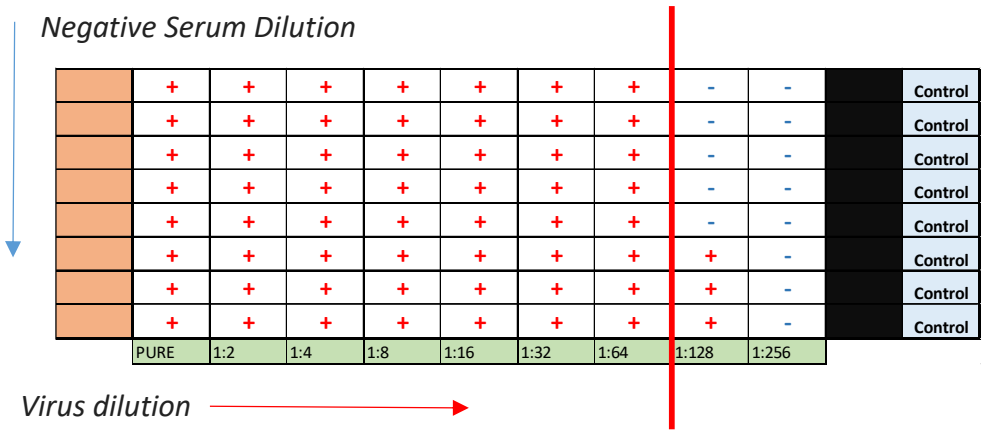


Figure22a: Scheme plate of the MN test cross with the Negative Serum dilution

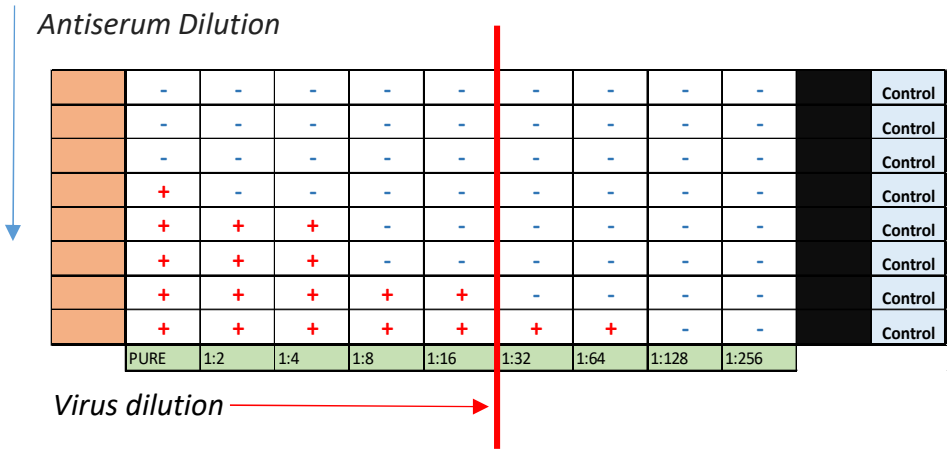


Figure 22b: Scheme plate of the MN test cross with the Antiserum dilution

As we can observe the virus is diluted with two fold dilution starting from pure, from left to right, instead the Negative serum is diluted with two fold dilution starting from 1/10 and the Antiserum is diluted with two fold dilution starting from 1/40.

From the above examples we can state that:

- For the Negative Serum plate, the virus can overcame all the nonspecific protein that can confer protection to the cell monolayer up to 1:64 virus dilution, due to the fact that the first five well of the 1:128 virus dilution results protect against the virus effect.

- For the Antiserum plate, we can say that the virus is alive and function; if we take under consideration that an Antiserum mimics a high positive serum sample, the virus certainly can infect since 1:16 dilution. The last wells of the 1:32 and 1:64 columns, that result

infected, are not take in consideration because we are not sure if the result that we see could depend by a plate effect (false positive that generally occur at the extreme wells of the plate) or for the presence of the virus.

In this case, I expect to have a dilution factor included between 1:8 and 1:16. The TCID50 results (Figure 3), combined with the Sperman-Karber (Figura 4) formula, in order to have 100$TCID_{50}$ virus infective dose per well in the MN assay, confirm this result.

+	+	+	-	-	-	-	-	-		Control
+	+	+	-	-	-	-	-	-		Control
+	+	+	-	-	-	-	-	-		Control
+	+	+	-	-	-	-	-	-		Control
+	+	+	-	-	-	-	-	-		Control
+	+	+	-	-	-	-	-	-		Control
+	+	+	-	-	-	-	-	-		Control
+	+	+	-	-	-	-	-	-		Control
1:10	1:100	10^3	10^4	10^5	10^6	10^7	10^8	10^9		

Figure 23: $TCID_{50}$ assay with a ten-fold dilution of the virus; as we can see in this case the TCID50/ml result to be $10^{4.5}$

The dilution factor results to be 14.8.

This result confirm ours previous expectations from the MN experiments with the Antiserum and Negative Serum.

7.4 Results

The figures below show the “MN cross-test”results found for the four propagated seasonal strain after six cycles of propagation in MDCK cells, after the TCID50 titration and before the MN application with sera sample.

The four propagated seasonal influenza strain are:

A/H1N1/California/7/2009 pdm09

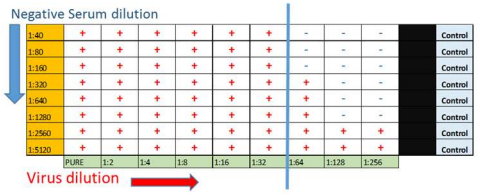
A/H3N2/Texas/50/2012;

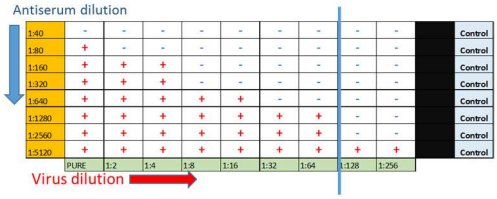
B/Brisbane/60/2008;

B/Massachussetts/02/2012.

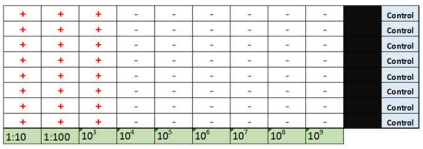
As we can observe there is complete overlap between the TCID50 results and the MN cross-test.

A - A/California/7/2009



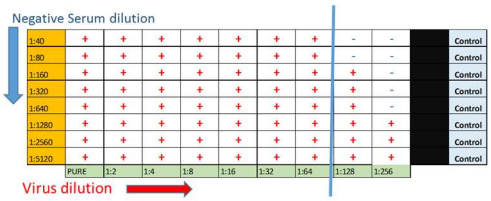


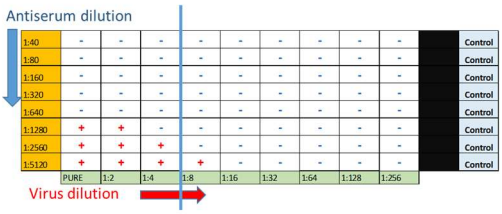
TCID50



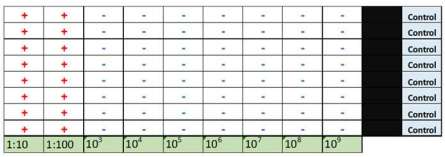
TCID50/Ml = 4.5

B - A/Texas/50/2012



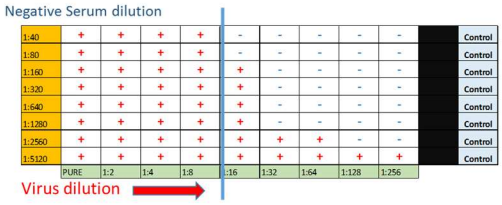


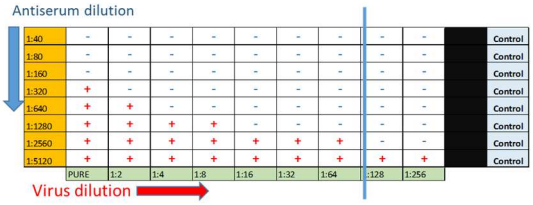
TCID50



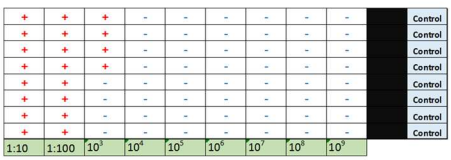
TCID50/Ml = 3.5

C - B/Brisbane/60/2008





TCID50



TCID50/Ml = 4

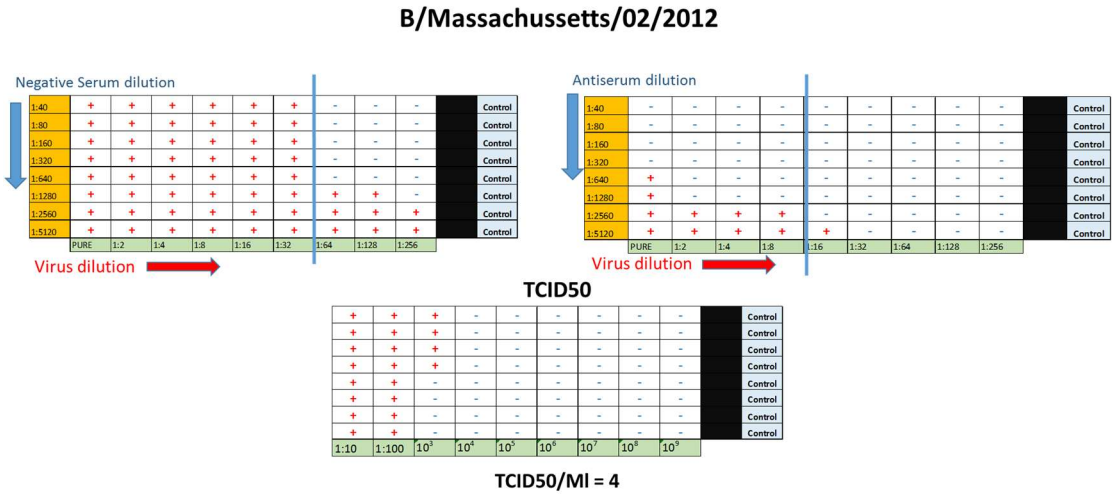


Figure 24 A,B,C and D: All the four figures show the MN cross-test method along with the classical virus titration with the TCID50 assay (Ten-fold dilution factor). The TCID50 value was calculated with the Spearman-Karber method. For all the four propagated Influenza Strain, the MN cross test confirm the functionality of the viruses at the dilution factor calculated through the viral titre.

7.5 Conclusion

The MN assay requires live infectious virus as antigen. The assay is able to detect functional antibodies (all the antibodies involved in the protection able to neutralize the virus), which are specific for the strain take under consideration. In addition, the MN protocols can be optimized to measure the antibody responses against other envelope glycoproteins, i.e. the NA. Moreover, in contrast to the HI assay, the MN seems able to detect very low antibody levels (Benne et al, 1994; Harmon et al, 1988).

The drawback of this test is the absence of recognized and well approved correlates of protection, probably due to the many variables able to modify the sensitivity of the assay; however it is recommended for quantify the neutralizing antibodies. A four-fold increase in titer after vaccination has been generally used in literature with the aim to assess the antibody response against the H5 strain (Treanor et al, 2006). Several studies show as the MN assay, compared with the HI, appear to be more suitable in order to evaluate the influenza serological response after vaccination (Granstrom and Voordouw, 2011; Wagner et al, 2012).

The “MN cross -test” that we setted and performed along with the Negative Serum (Human serum minus IgA, IgG and IgM) and the Antiserum (High positive serum from sheep), with the aim to

evaluate and validate the functionality of the propagated strain, is a very powerful quality control tool.

This kind of test allows to verify and, at the same time to discriminate, in combination with the classical TCID50, the right dilution factor to be used in the MN assay in order to achieve 100TCD50/50µl. Not always the dilution factor, only calculated accordingly with the TCID50 value, works in the MN assay, where a serum component can affect the virus functionality.

Before the use of the virus for the MN application, it would be advisable to do this kind of test, as an additional control between the TCID50 and the Micro-Neutralization. This is to exclude from the assay false positive and false negative that could affect the results of the trial and to find a more reliable sera titer as possible.

8. TASK 2

8.1 Introduction

Latest studies on the skin physiology show how this organ is potentially a good site for vaccination due to the presence of both cellular and humoral immune system components and for the simplicity of administration. The skin is comprised of three primary layers: epidermis, dermis and hypodermis. For the purpose of this work I will focus only on the dermis layer. The dermis is located between the other two layers and it is comprised of collagen, elastin and reticular fibers. It consists of two sub-layers: the papillary dermis and the reticular dermis. The papillary dermis is composed by loosely arranged connective tissue, on the other hand the reticular dermis is composed by a network of horizontally running collagen fibers, connective tissue and a very dense network of capillary blood and lymphatic vessel in which dermal dendritic cell, monocytes, lymphocytes and mast cells (Pober and Cotran, 1990).

The skin is able to generate both innate and adaptive immune responses, with the Langerhans cells in the epidermis, and the dermal dendritic cells in the dermis, that are the most important component involved in the immune response. These cells are bone marrow-derived leukocytes highly specialized in antigen-presenting properties (Cerio et al, 1989; Medzhitov et al, 1997). Body site, gender, age and ethnic origin are parameter to take under consideration for dermal vaccination (Seidenari et al, 1994).

Historically the renewed interest in intradermal immunization using a syringe injection in a controlled clinical trial was reported in 1930 by Tuft (Tuft, 1931), demonstrating the equivalent immune response and an improved in the safety using a smaller dose of typhoid vaccine when injected intra-dermally relative to subcutaneous injection.

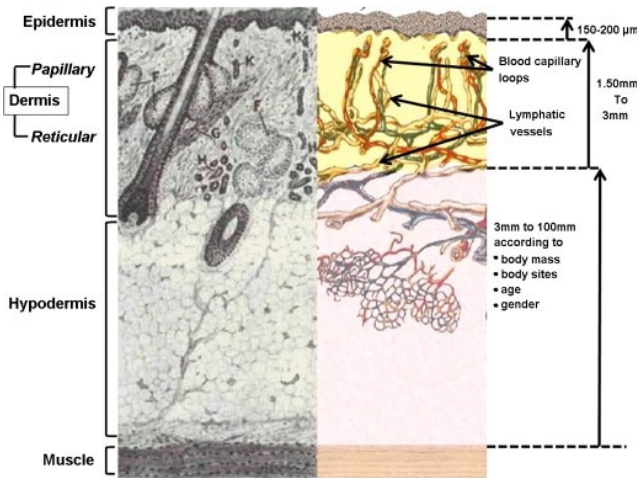


Figure 25: Skin anatomy with thickness measured by 20 MHz ultrasound echography in usual body sites for vaccine delivery. *Source: Lambert and Laurent, 2008.*

Other studies, were performed with the aim to evaluate the efficacy and efficiency of the intradermal route of vaccination reducing the vaccine dose using different available vaccines such as influenza (Van Gelder, 1947), measles (WHO, 1967) and cholera (MacBean, 1972). A study carried out by Brown et al. in 1977, demonstrated how an influenza intradermal vaccination, with one fifth of the standard dose of A/Swine/NJ/76 influenza vaccine, was able to induce antibody titers similar to those elicited after a standard intramuscular vaccination in adults (Brown et al, 1977).

8.2 Material and Methods

8.2.1 Study Design

The present study has been performed with the aim to evaluate the safety and the immunogenicity of an intradermal seasonal quandrivalent influenza vaccine in 150 healthy adults. The immunization was performed with two doses of vaccine with an interval of 21 days with 15μg of HA per dose of each antigen (A/H1N1/California/7/2009, A/H3N2/Texas/50/2012, B/Massachussets/02/2012 and B/Brisbane/60/2008). The sampling points were at Day 0 (D0) before the first dose of vaccine, at Day 21 (D21) before the second dose of vaccine, and at Day 42 (D42). The recruitment of the 150 volunteers and subsequent vaccination took place in two months (between the beginning of April and mid of June). Every sera sample has been analyzed in duplicate in different plates.

8.2.2 HI assay

Sera samples were incubated overnight with Receptor Destroying Enzyme (RDE) (1:5 proportion of sera and RDE) (Cholerae filtrate, Denka Seiken, Japan) with the aim to inactivate all non-specific inhibitors. The day after, 4 parts of Sodium Citrate for each serum volume were added. The samples are than incubated for 1h at 56°C. Sera, were then serially diluted 2-fold in a V bottom 96-well plate in saline solution, than the virus solution, with the virus diluted in order to achieve 4

Haemagglutinant units (HAU) in 25µl was added. After 1h of incubation at room temperature, the Turkey red blood cells (RBCs) (Emozoo) suspension (0.35%) was added. After 1h the plates were red.

The HI titer is defined as the reciprocal of the last serum dilution which do not contained agglutinated RBCs. Seroprotection was defined as an HI titer ≥ 40. Titers resulting < 10 (negative) were assigned 5 as a value.

8.2.3 SRH assay

The SRH assay is based on the passive haemolysis of erythrocytes (RBCs)(Emozoo) sensitized with influenza virus particles, by antibodies directed against the viral haemagglutinin in the presence of guinea pig complement.

Sera Samples were incubated at 56°C for 30 minutes in order to inactivate the complement. A red blood cells solution at 13% was prepared, along with an antigen dilution containing 2000 HAU/ml and a 1.5% agarose containing 0.1% Sodium Azide solution. The Guinea Pig solution was prepared immediately before the use. Once that the agarose solution was ready, the corresponding quantity of antigen-RBCs and Guinea Pig Complement was added; 3 ml of this solution were than spread in each SRH compressed polystyrene plate (size: 70 X 22 mm and 3 cc of volume).

The plates were than incubated 30 minutes at room temperature and 30 minutes at +4°C. With a calibrated punch, 18 holes measuring 2.256mm were made in the agarose plate. 6µl of each sera sample was seeded. The plates were incubated in a humid box at +4°C overnight. The day after the plates were incubated in water bath for 90 minutes at 37°C than the haemolysis halos were read in millimeter.

8.2.4 Cell Cultures and MN assay

MDCK cells (Sigma) were growth at 37°C, 5% CO2 in UltraMDCK SF Medium (Lonza) supplemented with 100 Ul/ML of penicillin streptomycin (Lonza). Sub confluent cultures were passages every 3-4 days (Gaush et al, 1966).

Sera samples were heat inactivated at 56°C for 30 minutes with the aim to inactivate the complement.

96-well plates ware prepared in order to cover all the sera samples of the trial, in which one plate can contain 12 sera samples. The first column was fill with 90µl of UltraMDCK SF medium (Lonza);

starting from the second to the sixth with 50μl, the seventh was leave empty and the last one (cell control) with 100μl. 10μl of each serum was seeded in duplicate in the first wells, than a two fold serial dilution was performed transferring 50μl starting from the first column to the sixth ones. 50μl of the virus solution (200TCID50/100μl) was than added in each well. After one hour of incubation at 37°C, the cell suspension (150.000 cell/ML in UltraMDCK SF medium supplemented with 1 μg/ml of TPCK-Trypsin (Sigma) and 0.2% of Fetal Bovine Serum (Lonza)) was added. The pates were than incubated at 37°C, 5% ± 1% CO2 in humidified atmosphere for a minimum of four days. At the end of the period of incubation each well was evaluated with an optical microscope in order to evaluate the presence of local lesions (CPE) derived from the infection and replication of the influenza virus in the cell lawn. When compared with the cell control wells, the wells presenting CPE were signed as positive or infected.

The Neutralization titre for each serum duplicate is calculated according to the Spearman-Karber formula.

8.2.5 Influenza Live Virus

A/H1N1/California/7/2009pdm, A/H3N2/Texas/50/2012, B/Brisbane/60/2008 and B/Massachusetts/02/2012 were obtained from the National Institute for Biological Standard (NIBSC), propagated in MDCK cells and titrated following the standard protocol of TCID50 with the addiction of the MN Cross-test as exposed in the previous task.

8.3 Results

HI results

All the 450 sera samples (150 subjects for three visits) were titred with the HI assay in order to assess the presence of HA specific Anti-Influenza Abs against each influenza strain.

For each strain was assessed a significant increase ($p < 0.0001$) in the specific anti-influenza Abs after two doses of vaccine through the HI, SRH and MN assays (Figure 26, 27 and 28). All the three CHMP criteria for the effectiveness of a seasonal influenza vaccine were met for all the four strain apart from that of the Sero-Conversion rate for the B Yamagata strain, which result under the threshold of 40% (Figure 29).

For the H1N1 and the H3N2 strain, the 20% and the 16.5% of subjects respectively, were seronegative before the first dose of vaccine, whereas the 64% and the 41,3% of subjects were seronegative for the B Yamagata and B Victoria respectively.

The High percentage of sero-protected for H1 and H3 strains is explainable for adulthood of the group considered for this study. It has been shown how an older population (respect to children or infants) presents higher antibodies concentrations able to cross-react with the H1N1 strain. This is due to structural similarities between the HA of the H1N1 2009 and other A type viruses like the H3N2 (Verguilla et al, 2011).

SRH Results

All the 450 sera samples (150 subjects for three visits) were evaluated with the SRH assay in order to assess the presence of HA specific Anti-Influenza IgG.

For the H1N1 and the H3N2 strain, the 16.6% and the 4.6% of subjects respectively, were seronegative before the first dose of vaccine, whereas the 10% and the 9.3% of subjects were seronegative for the B Yamagata and B Victoria respectively.

All the three CHMP criteria for the effectiveness of a seasonal influenza vaccine were met for all the four strain apart from that of the Mean-Geometric Increase for H3N2 and for the B Yamagata strains, which result under the threshold of 2.5 (Figure 29).

MN Results

All the sera were evaluated in Micro-Neutralization assay for the presence of function anti-influenza antibodies. A very high percentage of subjects were seronegative prior vaccination for all the four strains; 91.3% for H1N1, 28.6 % for H3N2, 85.3% for B/Yamagata and 98% for B/Victoria. Although there are no correlates of protection established for the MN assay, in the present study, I decided to take under consideration the same threshold adopted for the HI and SRH of the three CHMP criteria. The sero-conversion threshold was achieved by the H1 and H3 strains, not for both B strains, the mean geometric increase threshold was achieved by all the four strains and the sero-protection rate only by the H3 strain.

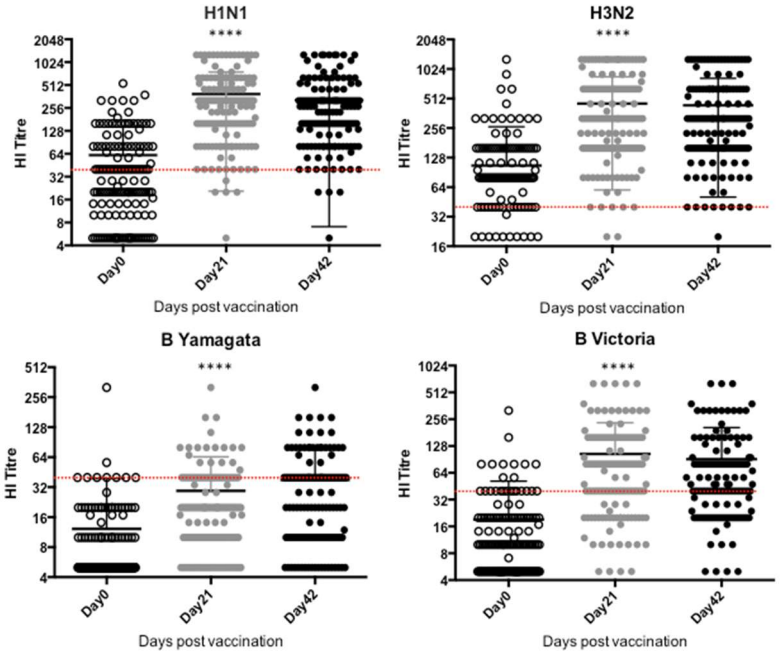


Figure 26: HI results for all the four strain after the first and the second dose of vaccine. For all the four strain there is a significant increase in the HI titers after two doses of intradermal influenza vaccine *(p<0.0001)*. The red line indicates the correlates of protection of 40 for the HI test.

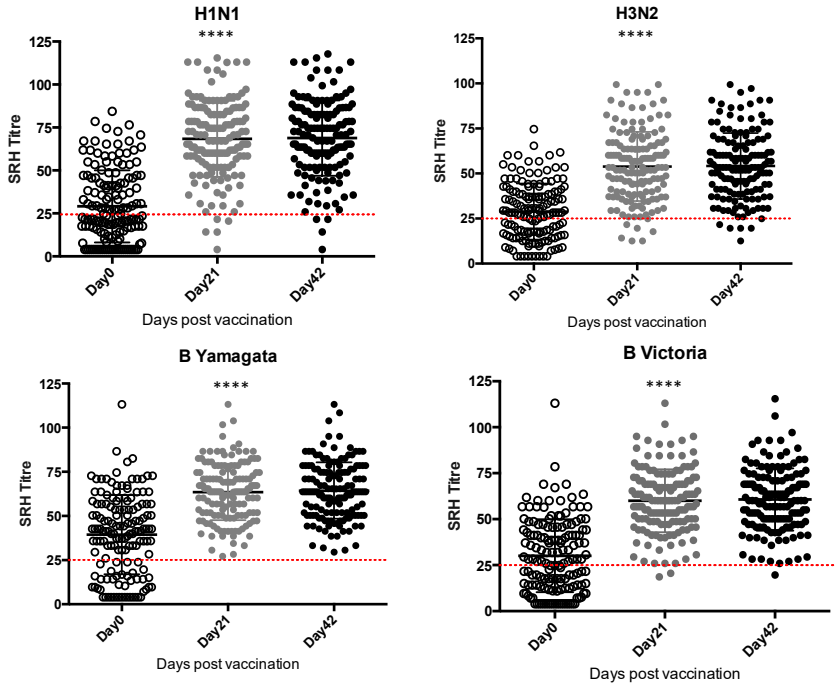


Figure 27: SRH results for all the four strain after the first and the second dose of vaccine. For all the four strain there is a significant increase in the SRH titers after two doses of intradermal influenza vaccine *(p<0.0001)*. The red line indicates the correlates of protection of 25mm^2 for the SRH test.

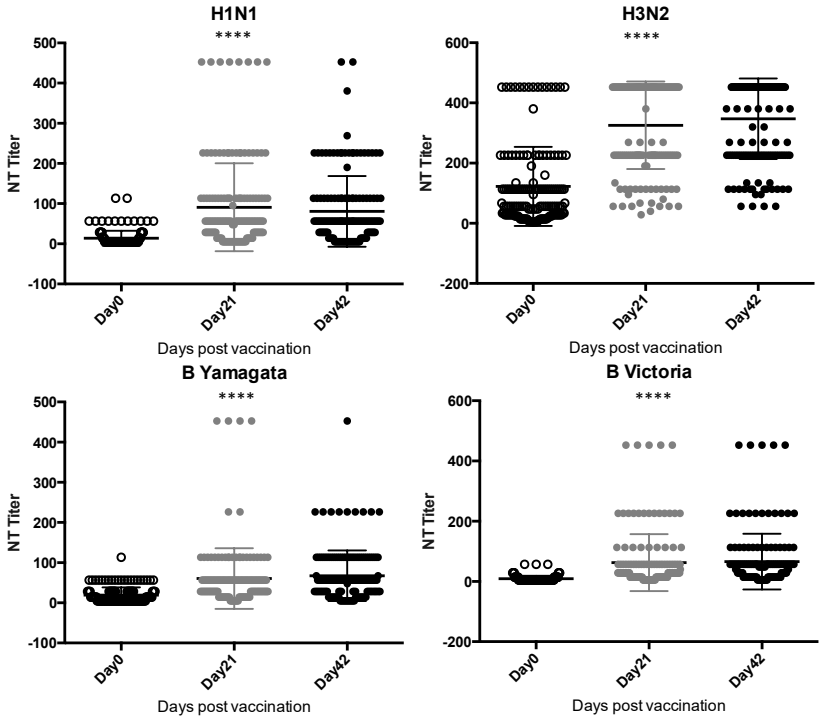


Figure 28: MN results for all the four strain after the first and the second dose of vaccine. For all the four strain there is a significant increase in the Neutralization titers after two doses of intradermal influenza vaccine *(p<0.0001).* In this case a red line is not present because of there are not correlates of protection established for the MN assay.

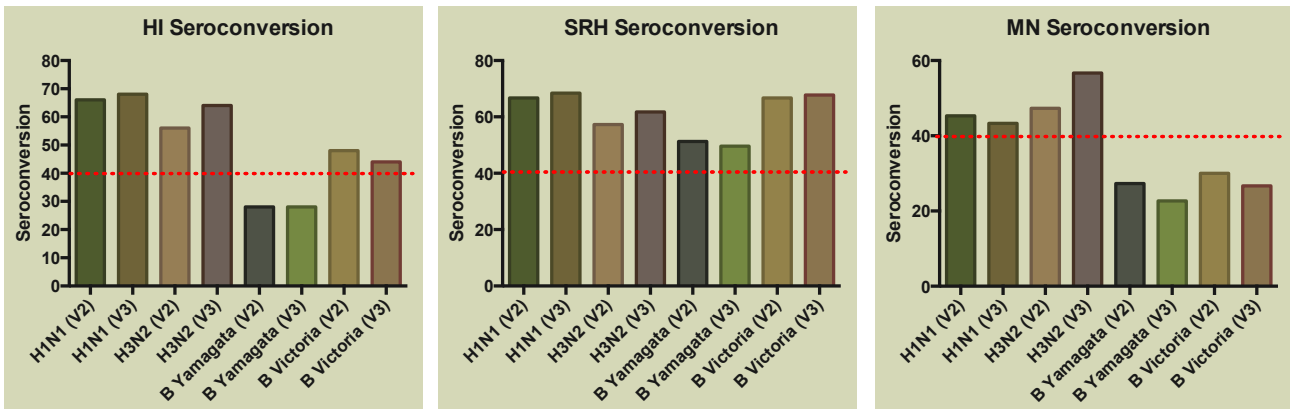


Figure 29: The seroconversion rate (SCR) is defined as at least a 4-fold increase in titer. The SCR should be >40%. For the HI only the B Yamagata does not meet the SCR criterion. For the SRH all the four strain meet the SCR criteria and for the MN both the B strain do not meet the SCR criterion.

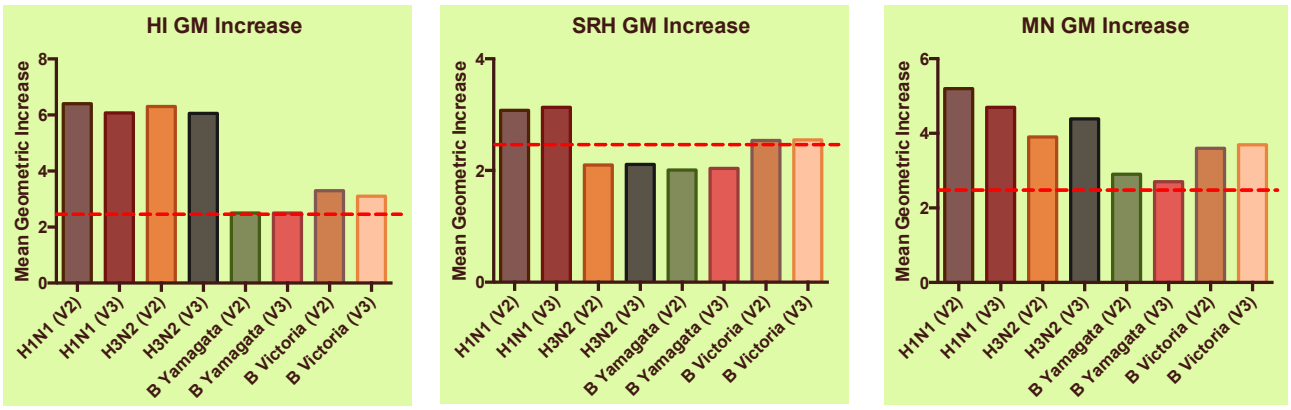


Figure 30: The Mean Geometric Increase is the ratio of pre to post vaccination, it should be >2.5. It is meet by all the four strain for the HI and MN assays but not for the H3 and B Yamagata for the SRH assay.

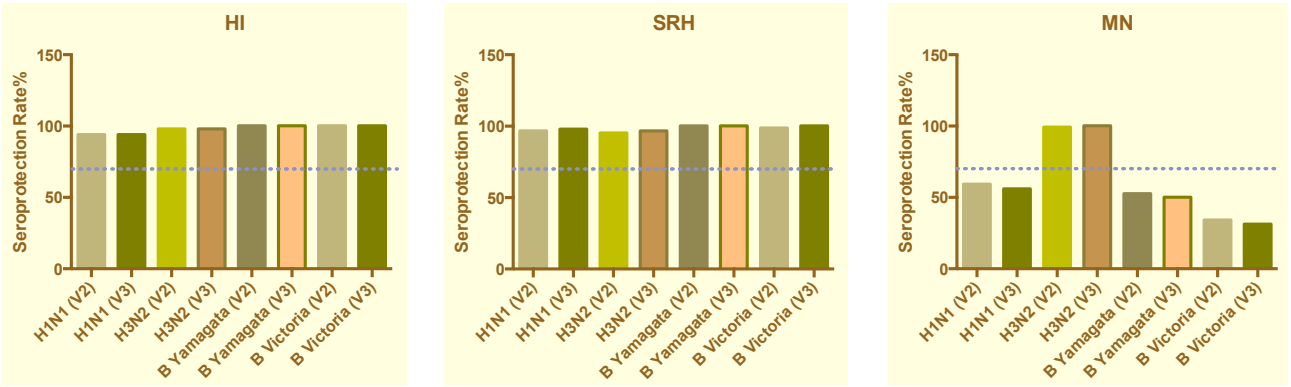
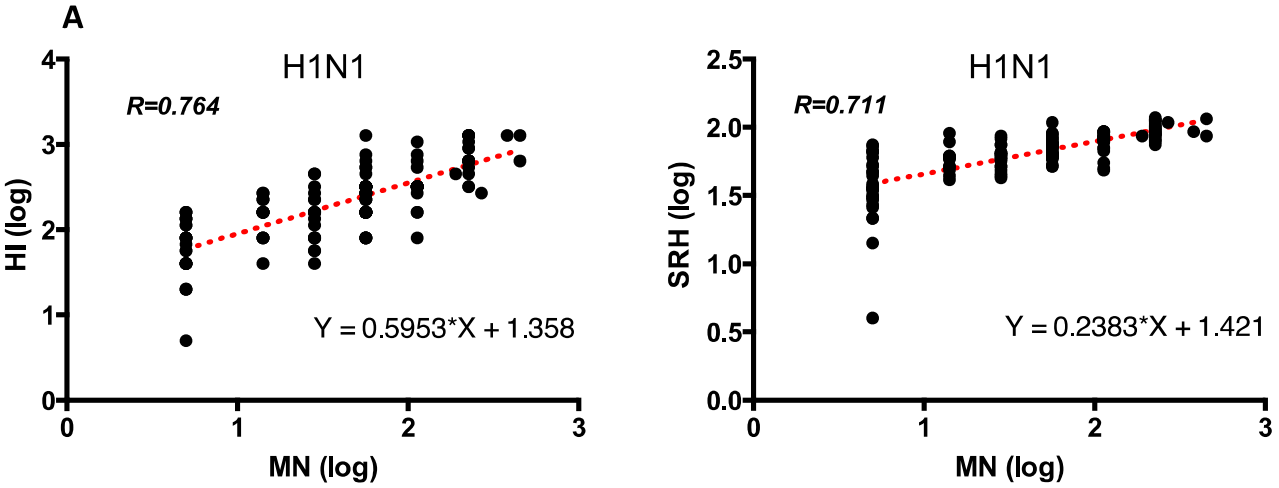


Figure 31: The Seroprotection rate expresses the proportion of subjects achieving an HI titer >40 or SRH titer > 25mm^2. It should be >70%. For the MN as no correlates are established, generally, the same cut-off of the HI is considered. It is meet by all the four strain for the HI and SRH assay but only the H3 strain reach the criterion for the MN.

Correlations

In order to evaluate whether the results obtained with the HI and SRH serological assays correlate with the MN assay, a linear regression analysis of all Visit 3 Geometric Mean Titers, log_{10}-trasformed, was performed for each influenza strain. Pearson test was performed with the aim to evaluate the goodness of correlation (Figure 32). Visits 1 were not analyzed due to the many negative titers, especially for the MN results, and Visits 2 (data not shown) presented a very similar trend respect to the Visits 3. The Pearson coefficient (R), for MN and HI was respectively 0.764 for H1N1 strain, 0.644 for H3N2 strain, 0.432 for B Yamagata strain and 0.616 for B Victoria strain; for MN and SRH was respectively 0.711 for H1N1 strain, 0.490 for H3N2 strain, 0.546 for B Yamagata strain and 0.588 for B Victoria strain. All results obtained indicate a positive and, especially for H1N1 strain, a good correlation. The lowest correlation was found between MN and HI for the B Yamagata strain.



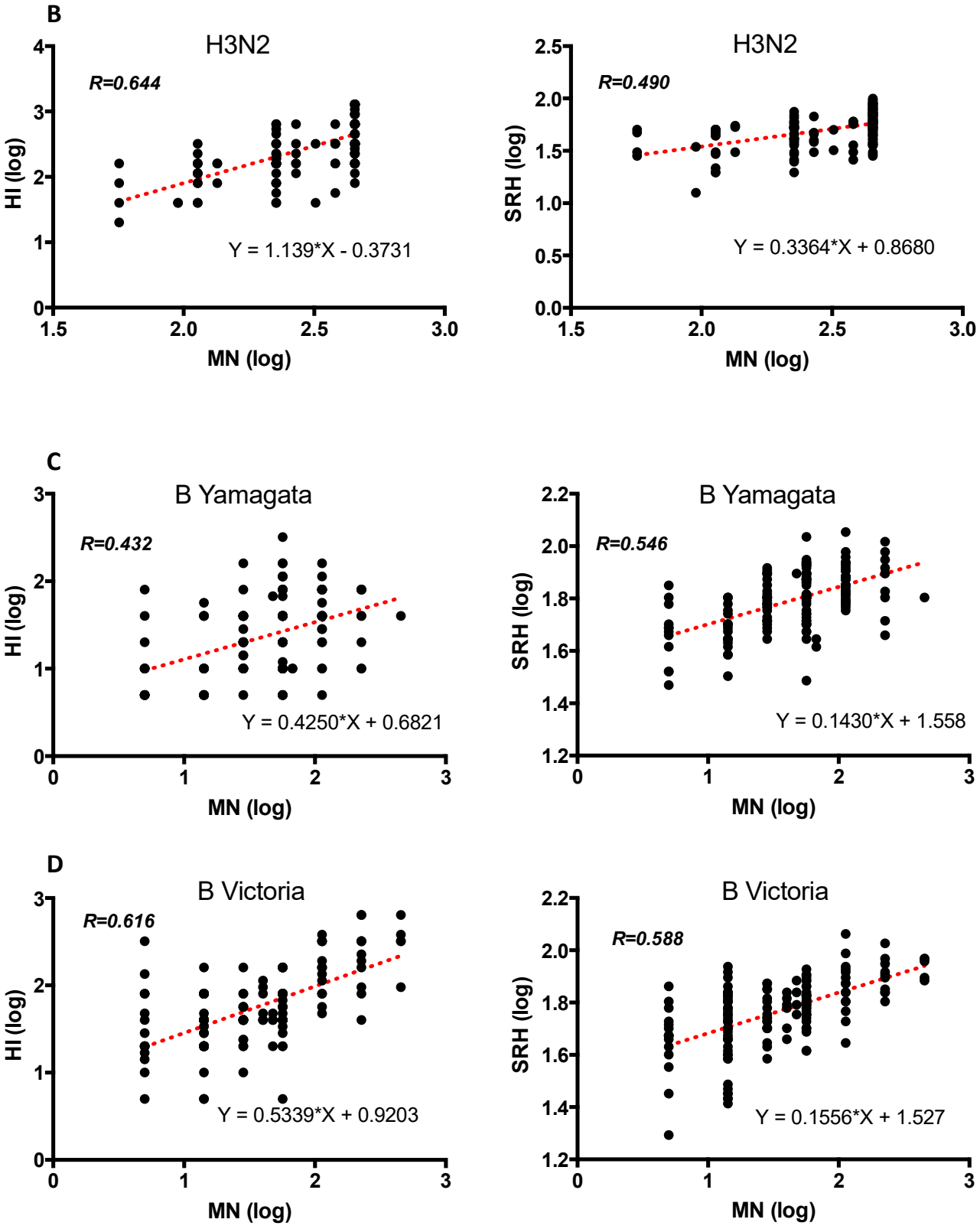


Figure 32: Linear regression analysis of MN versus HI and MN versus SRH $\log_{10}$ Geometric Mean Titers (V3) for each strain obtained from the totality of 150 subjects. (A) Linear regression for A/H1N1 influenza strain; (B) Linear regression for A/H3N2 influenza strain; (C) Linear regression for B Yamagata Lineage influenza strain; (D) Linear regression for B Victoria Lineage influenza strain.

8.4 Discussion

Influenza epidemics occur every year during the winter season in temperate climates with different rates and severity (Cox et al. 2004). Different factors are involved in the influenza-related morbidity and mortality, such as the virus type and subtype, the level of preexisting protective antibodies present in the population and the age of the target population. Children are one of the most important factors in terms of spread of influenza in the population; they are able to shed the virus longer than the adults and with higher titers in the nasopharinynx (Weinberg et al, 2004). Influenza epidemics occur every year because different strains are constantly generated through the antigenic drift mechanism (Wright and Webster, 2001).

Since 1952, the WHO initiated the "Global Influenza Surveillance Network" (GISN) with the aim to create an international network of laboratories able to provide the annual recommendations on the influenza virus to be included in the new vaccine of the year.

The surveillance data are collected every year at the WHO centers. The data give information about the predominant influenza virus currently spreading and circulating worldwide. Twice per year, a committee meets with the aim to analyze the data and provide indication on the suitable strain to be included in the next influenza vaccine (www.influenzacentre.org/centre-GISN.htm).

Two type of influenza vaccines are licensed and available today: inactivated influenza vaccine (called IIV3 or IIV4 if trivalent or quadrivalent), generally administered parenterally as intramuscular or intradermal injection, and live attenuated influenza vaccine (called LAIV) administered as a nasal spray nebulization. Concerning the IIV vaccines, they can be made with whole, split or subunit virus. Moreover in order to enhance the immunogenicity of subunit vaccine, different adjuvants with different formulation have been used (Podda, 2001).

The alternative to the intramuscular administration, the most common delivery route for inactivated influenza vaccine, is constituted by the intradermal injection, which present some advantages derived by the cost (more economy) and the avoidance of febrile reaction. Nowadays the intradermal route of administration is only widely used for Rabies and Bacille Calmette-Guérin vaccines (Hickling et al, 2011).

Currently marketed inactivated influenza vaccines contain a well standardized amount of HA antigen, whereas there is no specific requirement so far for the NA content. Focus on the HA antigen as the primary influenza virus immunogen relies on early challenge studies, conducted in man using also live attenuated strains which caused infection but not illness, which showed a

strong, positive correlation between protection from infection and serum antibodies against HA protein when measured by the hemagglutination-inhibiton (HI) test.

The HA influenza antigen, is the major antigenic determinant for the production of neutralizing antibody after natural infection or immunization.

Different factors are involved in the determination of the composition and quantity of the antibody response related to the HA influenza antigen present in inactivated vaccine; the quantities of HI and neutralizing antibody are strongly related to the quantity of the antigen present in a single dose (Mostow et al, 1970).

The actual serological assays generally used to measure and quantify specific influenza antibody include the HI, SRH, MN assay, Whestern blot and ELISA assay, but only for the HI and SRH correlates of protection are established and accepted.

In 1996 the European Medicine Agency (EMA) committee for medical products for human use (CHMP) defined the requirements for annual updating of seasonal influenza vaccine (Ainswort, 2003). The vaccine update require pre and post vaccination samples at least from 50 people between 18 and 60 years old and >60 years. Two serological test are recommended by the CHMP: the HI and the SRH for which correlates of protection are established as reported in the introduction part of this work; is possible to define a correlate of protection as an immune response that is responsible for and statistically correlated with protection.

Three criteria need to be fulfilled and at least one of the assessments should meet the indicated requirements for the vaccine registration in the European Union; this is not true for the pandemic vaccine where the new vaccine has to meet all the three criteria.

In the present study has been evaluated the immunogenicity of an intradermal quadrivalent seasonal influenza vaccine, (A/H1N1/California/7/2009pdm, A/H3N2/Texas/50/2012, B/Brisbane/60/2008, B/Massachussetts/02/2012), respect to the three CHMP criteria using three different serological assays: the HI, gold standard in the influenza field, the SRH and the CPE-based Micro-Neutralization assay. Moreover, correlations between the three serological assays have been made with the aim to evaluate and estimate the difference in the antibody detection for each assay. From the analysis of results is possible to state that the intradermal quadrivalent influenza vaccine, evaluated in the present study, in whose preparation are not included adjuvants, presents a good immunogenicity for all the four seasonal strains. All the three CHMP criteria have been fully satisfied for the A/H1N1 strain for all the three serological assays but also all the other remaining strains satisfied at least one CPMP criterion per assay. Furthermore, the

degree of accordance among the three assays, in terms of correlates of protection is good; only for the seroprotection rate there is discrepancy between HI/SRH results and MN results.

It is important to consider that generally lower serum titers in MN are expected, if compared for instance with HI titers. They do not indicate the lower sensitivity of the MN but the fact that the MN detects exclusively antibodies able to arrest the infection.

Despite the MN does not present a recognized and approved correlate of protection, a four-fold increase in titer after vaccination has been used many time with the aim to assess the immune response (Treanor et al, 2006; Bresson et al, 2006; Katz et al, 1999). Moreover, due to the high sensitivity and strain-specificity of the method, the MN is now routinely used as an important part of sero-epidemiologcal investigations concerning the detection of H5 and H7 pandemic viruses (Kajaly et al, 2008). Concerning the correlations between assays, we can observe how the best correlation has been found between the MN and the HI for the H1N1 strain, lower correlations, but always positive, were found for HI/SRH versus MN for all the other strains, especially for B Yamagata strain. These results are nevertheless in line with our expectations for many reasons: primarily the three assays chosen for this study are able to detect different classes of antibodies; eg. the SRH is able to detect the IgG1 and IgG3 involved in the complement-fixation reaction, the MN mainly the IgG1 subclass, principally involved in the neutralization and opsonization (Eidem et al, 2015). The HI assay is able to detect only antibodies directed against the HA1 domain (head domain) of the HA influenza antigen, on the other hand the MN is able to detect a wide range of antibodies included those directed against the stalk (HA2) domain (Krammer and Palese 2013), moreover, it is well known that, the HI assay it is less sensitive towards influenza B strains (Wright et al, 1980).

It is important to keep in mind that the results of these serological assays can be highly influenced by several factors such as the immune status, geographical settings, age and breed of subjects; all factors that may vary within population.

Concerning the Intradermal administrations, they are generally evaluated with the same standardized amount of antigen (15μg of HA of each strain) as present in intramuscular administration, but with a smaller volume of injection.

From our results, it appears clear that 15μg of HA per dose of each antigen, without adjuvant, are sufficient in order to elicit immune responses that are similar in the magnitude to those elicited by a full dose of an intramuscular seasonal influenza vaccine to healthy adults. This is in line with some published meta-analysis regarding the non-inferior profiles of intradermal vaccine respect to

intramuscular vaccine (Arnou et al, 2009; Marra et al, 2013; Pileggi et al, 2015). Moreover, according to the results, is possible to observe that even after the first dose the vaccine is able to elicit a proper immune response. There is no significant increase in antibody titers after the second dose for all the four antigen; but to confirm this statement we should analyze also the vaccine responses in children and elderly people, where generally a second dose is necessary to elicit a good antibody response. One of the resulting potential benefits of intradermal vaccination is the "Dose-Sparing" concept. For some intradermal vaccines have been demonstrated, for some target people, the potential of induce protective immune response with less amounts of vaccine antigen (Lambert and Laurent, 2008).

Dose-Sparing, derived from intradermal vaccination, might be of help in immunization programs by reducing the pre-injection cost of vaccine due to the fact that more doses could be obtained from the existing vaccine. Another important point of the dose sparing is the potential to make available vaccines when the level of demand suddenly increase and the supply is limited by manufacturing capacity; this concept can become particularly relevant at the beginning of a pandemic influenza. In 2009, during the A/California/H1N1/7/2009 pandemic, the vaccine was not available in many low-income countries for 8 months after the pandemic declaration (Patriage et al, 2010). In order to be able to reduce the vaccine volume per dose, new seasonal intradermal, adjuvanted, influenza vaccine should be evaluated. Despite Chi et al, 2010 reported that 60% of intradermal dose of trivalent influenza vaccine resulted in similar rates of immunogenicity as a standard dose delivered intramuscularly, this was not observed for pandemic (H5N1) intradermal vaccine (Patel et al, 2010), where in case of a pandemic the "dose-sparing" concept could become of crucial importance. The degree of dose-sparing concept for intradermal administration can not be generalized for all vaccines, but it should be evaluated for each vaccine through non-inferiority trials. Adjuvants are able to boost and amplify the immune response reducing in this way the vaccine dose (Lindblad 2004). Is possible to recognize two main mechanisms of action of adjuvants in intradermal vaccination. The first one is the assumption that adjuvants are able to delay the clearance of injected antigen from the dermis through a depot effect. This concept has been well demonstrated with the aluminum salt after an intramuscular injection; the absorption and union of the antigen with the aluminum salt, leads to a depot formation able to stretch the time of interaction between the antigen and the antigen presenting cells (APCs) (Li et al, 2007). The second main effect is the ability of the adjuvant to mobilize and activate the dendritic cells of the skin, that have encountered and captured the influenza antigen, including the migration of APCs to

lymph nodes for starting T cell responses. Another important aspect, for which it would be useful to deepen and refine the Intradermal route of administration for influenza immunization, is the relatively simple method of injection and the potential for self-administration with dedicated microinjection system, especially for health care workers. The self administration practice has been previously explored and studied, with good results for live attenuated influenza vaccine (Ambrose and Wu, 2013; Zahn et al, 2013).

This practice has many advantages; one is the potential reduction of the vaccination time and the vaccine uptake increase (Bragazzi et al, 2016). These aspects would become of crucial importance at the beginning of a possible influenza pandemic or severe influenza epidemic. Despite the more simple method of administration of the intradermal vaccines, for self-administration, is not possible without a supervision by trained stuff in order to deal with possible adverse or anaphylactic reaction. Different studies reveal how intradermal vaccine appears to be more accepted if compared with the classical intramuscular administration, and the self-administration practice more prefer compared with the nurse-led administration (Icardi et al, 2012 Coleman et al, 2012). Moreover, nowadays are available new administration approaches for the intradermal vaccine administration respect to the past, where the Mantoux technique and bifurcated needles were the main technologies available. Today new micro-needle devices are the best system in order to achieve a proper skin vaccine delivery, with precise tissue localization, along with a good degree of satisfaction of the receivers.

To conclude, intradermal influenza vaccine result to be a good and satisfactory alternative to the classical intramuscular injection.

9. TASK 3

This Task represents the Article published by the Vaccine Journal (http://dx.doi.org/10.16/j.vaccine.2016.10.024), and derived from the work done at the Influenza Center in Bergen, Norway.

9.1 Introduction

Influenza is one of the most common respiratory infections representing a cause of concern in the field of public health (Mayer and Wilke, 2015). Each year, seasonal influenza infection can cause up to 5 million severe cases and between 250,000 and 500,000 deaths worldwide (Ramakrishnan et al, 2012; WHO, 2014). Vaccination remains the most effective preventative measure against infection and limits morbidity and mortality caused by influenza. The effectiveness of influenza vaccination varies in different age groups and by vaccine formulations (Goodwin et al, 2006; Frasca et al, 2013; Ohmit et al, 2014).

Currently, there are two main types of seasonal influenza vaccines licensed in Norway: the trivalent inactivated influenza vaccine (TIV) and live attenuated influenza vaccine (LAIV). Studies that have investigated the antibody responses after TIV and LAIV vaccination have focused on the classic serological assays, such as hemagglutination-inhibition assay (HI), single radial hemolysis assay (SRH) and microneutralization assay (MN) (Schild et al, 1975; Katz et al, 2011; Trombetta et al, 2015; Stephenson et al 2007). These antibody responses are mainly directed to the major viral surface glycoprotein, hemagglutinin (HA). HA has important functions essential for infection, such as recognition of host cells' receptors and fusion of viral and endosomal membranes. Antibodies to HA are measured by classical serology as surrogate correlates of protection. However, there are no established correlates of protection for LAIV. Furthermore, there is limited data documenting the differences in systemic IgG and IgA subclass responses after vaccination in children and adults.

IgG levels are important for influenza vaccination responses and protection (Clements et al, 1986; Crum-Cianflone et al, 2012). The four subclasses of IgG; IgG1, IgG2, IgG3 and IgG4, found in humans differ in function and make up 65%, 23%, 8% and 4% of the total serum IgG, respectively (Schroeder et al, 2010). In particular, IgG1 is involved in many important immunological functions, including complement fixation along with IgG3 and opsonization, as well as virus neutralization (Frasca et al, 2013). Two IgA subclasses, IgA1 and IgA2 constitute 85% and 15% of the total IgA,

respectively (El-Madhun et al, 1999). IgA is involved in the protection in the local mucosa, including the upper respiratory tract where the influenza virus enters and causes infection (Tamura S-I and Kurata, 2004).

We conducted this study to investigate the differences in IgG subclasses and IgA antibody responses induced by LAIV in children and adults and by TIV in adults. We measured the H3N2 HA-specific response and showed that LAIV induced a stronger systemic IgG and IgA antibody response in children than in adults who received the same vaccine. However, in children, LAIV induced antibodies of comparable quantity and avidity to the antibodies induced by TIV in adults.

9.2 Materials and Methods

Study Design

All participants >12 years and parents provided written informed consent before inclusion in the study, which had ethical and regulatory approval (ClinicalTrials.gov NOT01003288 and NCT01866540). All individuals were vaccinated during the winters of 2012 and 2013. Fifteen healthy children <5 years of age received two doses of LAIV, 28 days apart, while 14 healthy children aged 10-17 years old received 1 dose of LAIV as recommended by the manufacturer. Fifteen healthy adults received a single dose of LAIV. As a comparator control, an additional 15 adults were vaccinated with the TIV. All the TIV vaccinated adults were healthcare workers and had received prior seasonal influenza vaccination. The remaining subjects who had received previous influenza vaccination have been immunized with the pandemic H1N1 vaccination only (Table 1).

The LAIV vaccine (Fluenz™) for 2012-2013 contained $10^{7,0\pm0.5}$ FFU for each influenza strain: A/California/7/2009(H1N1)pdm09-like, A/Victoria/361/2011(H3N2)-like, and B/Wisconsin/1/2010. The 2013-2014 LAIV vaccine contained A/California/7/2009 (H1N1)pdm09, A/Victoria/361/2011-H3N2-like strain (A/Texas/50/2012) and B/Massachusetts/02/2012 strains. The trivalent seasonal TIV (split-vaccine) (Vaxigrip®) containing 15 µg HA per dose of each antigen: A/California/07/2009-like virus (H1N1)pdm09, A/Texas/50/2012 (H3N2) and B/Massachusetts/02/2012. Serum samples were collected immediately prior to vaccination, and after vaccination in all groups (Fig 1). All serum samples were aliquoted, and stored at -80°C before use in the serological assays.

	Children < 5	Children 10-17yrs	LAIV vaccinated adults	TIV vaccinated adults
Number	15	14	15	15
M/F (% Male)	11/4 (73%)	3/11 (21%)	5/10 (33%)	2/13 (13%)
Age, mean (range)	3.8 (3-5)	14.2 (10-17)	34.6 (19-59)	44.9 (26-64)
Previous influenza vaccination	4 (27%)[a]	7 (50%)[a]	5 (33%)[a]	15 (100%)[b]

[a] Pandemic H1N1 vaccination in 2009; [b] Prior seasonal influenza vaccination and pandemic H1N1 vaccination in 2009

Table 1: Characteristic of study partecipants with number, gender and age.

Figure 1: Study design. Fifteen healthy children < 5years old, 14 children aged 10- 17 and 15 adults were vaccinated with Live Attenuated Influenza Vaccine (LAIV). In addition, 15 adults were vaccinated with Trivalent Inactivated Influenza Vaccine (TIV) as a positive control. Children < 5 received 2 vaccine doses, 28 days apart, while the remaining participants received 1 dose. Plasma was collected at day of vaccination (D0) in all subjects. Additional plasma was collected at D28 and D56 post-vaccination in children <5, day 28 (D28) in children 10- 17 years old, D28 in LAIV vaccinated adults and at day 21 (D21) in TIV vaccinated adults.

Hemagglutination-inhibition assay (HI)

Serum samples were treated with receptor destroying enzyme and tested in duplicate in the HI assay, using 8 hemagglutination units of the homologous H3N2 vaccine strain and 0.7% turkey red blood cells (Madhun et al, 2010). Seroprotection was defined as an HI titer ≥ 40. HI titers < 10 were assigned a value of 5 for calculation purposes.

Hemagglutinin specific IgG1 ELISA

An indirect ELISA was performed in order to determine the HA-specific IgG1, IgG2, IgG3 and IgG4 antibody concentrations in serum samples (Pedersen et al, 2014; Grohskopf et al, 2014). Ninety-

six-well plates (Nunc maxisorp, Denmark) were coated with Influenza A/Texas/50/2012 (H3N2) - HA1 6xHis tagged Hemagglutinin (1µg/ml) (eEnzyme, USA) and incubated (4°C) overnight. For the standard wells, 0.3 µg/ml of donkey anti-human IgG (Jackson ImmunoResearch, USA) was used. The plates were blocked for 1 h at 37°C with PBS containing 5% milk, 0.1 % Tween-20 and 1% of BSA. Serum samples were added at the appropriate dilution and incubated for 1 h at 37°C. Mouse anti-human IgG1, IgG2, IgG3 and IgG4 (Sigma) and HRP conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, USA) were added and incubated for 1 h. TMB substrate was added and the reaction was stopped with 0.5 N HCl after 18 minutes. The absorbance was then read at 450 nm and analyzed on Gen5 software.

Hemagglutinin specific IgA1 ELISA

An indirect ELISA was performed for the detection of the IgA1 antibodies. ELISA plates were coated as previously described for the IgG1 detection except monoclonal goat anti-human IgA (Sigma) (1 µg/ml) and HRP conjugated monoclonal mouse anti-human IgA1 Abs (SouthernBiotech) were used as detection antibodies.

IgG Avidity ELISA

Serum samples were evaluated for avidity of HA-specific IgG antibodies as previously described (Pedersen et al, 2014). ELISA plates were coated as previously described with Influenza A/Texas/50/2012-HA1 6xHis tagged Hemagglutinin (1µg/ml) (eEnzyme, USA). Serum samples were standardized to a dilution that gave an Optical Density (OD) of 0.7 ± 0.3 in a direct ELISA and 1.5M Sodium thiocyanate (NaSCN) was added 1 h after the serum, followed by 1 h of incubation. The percentage of Abs remaining after the serum treatment with 1.5M NaSCN was calculated as follow: (OD450 treated serum / OD450 untreated serum) x 100%.

Statistics Analysis

Data analysis was performed using GraphPad Prism version 5. Kruskal-Wallis test was used for multiple comparisons between the four groups. Wilcoxon and Friedman tests were used to compare pre- and post-vaccination data within each group. Correlation between the serological assays was performed using Spearman rank test. A p-value <0.05 was considered statistically significant.

9.3 Results

LAIV induces age-related H3N2 HI antibody responses in children and adults

We analyzed the seroprotective HI antibodies to H3N2 in children vaccinated with LAIV and in adults vaccinated with LAIV or TIV. Prior to vaccination, adults vaccinated with TIV had the highest HI titers (GMT 68) with 73% having pre-existing seroprotective titers (HI titers ≥ 40) compared to 40% (GMT 27.4), 43% (GMT 21.2) and 27% (GMT 12.6) in children <5 years old, children 10-17 years old and LAIV vaccinated adults, respectively (Fig 2A). Vaccination resulted in a significant increase in HI titers in all groups except for LAIV vaccinated adults ($p < 0.05$) (Fig 2A, 2B). High titers and seroprotection rates were observed after vaccination in children <5 years, children 10-17 years and TIV vaccinated adults 87% (GMT 220.5), 86% (GMT 147.3) and 87% (GMT 89), respectively. In contrast, the post-vaccination seroprotection rate in LAIV vaccinated adults was low at 40% (GMT 20.8). However, this was an increase from a pre-vaccination seroprotective rate of 27%. The HI titers induced by TIV in adults were significantly higher than those induced by LAIV in adults ($p < 0.01$) but comparable to those induced by LAIV in children. Furthermore, the post-vaccination HI titers in the LAIV vaccinated subjects negatively correlated with age (Spearman´s $r = -0.57$, $p < 0.0001$) (Fig 2B).

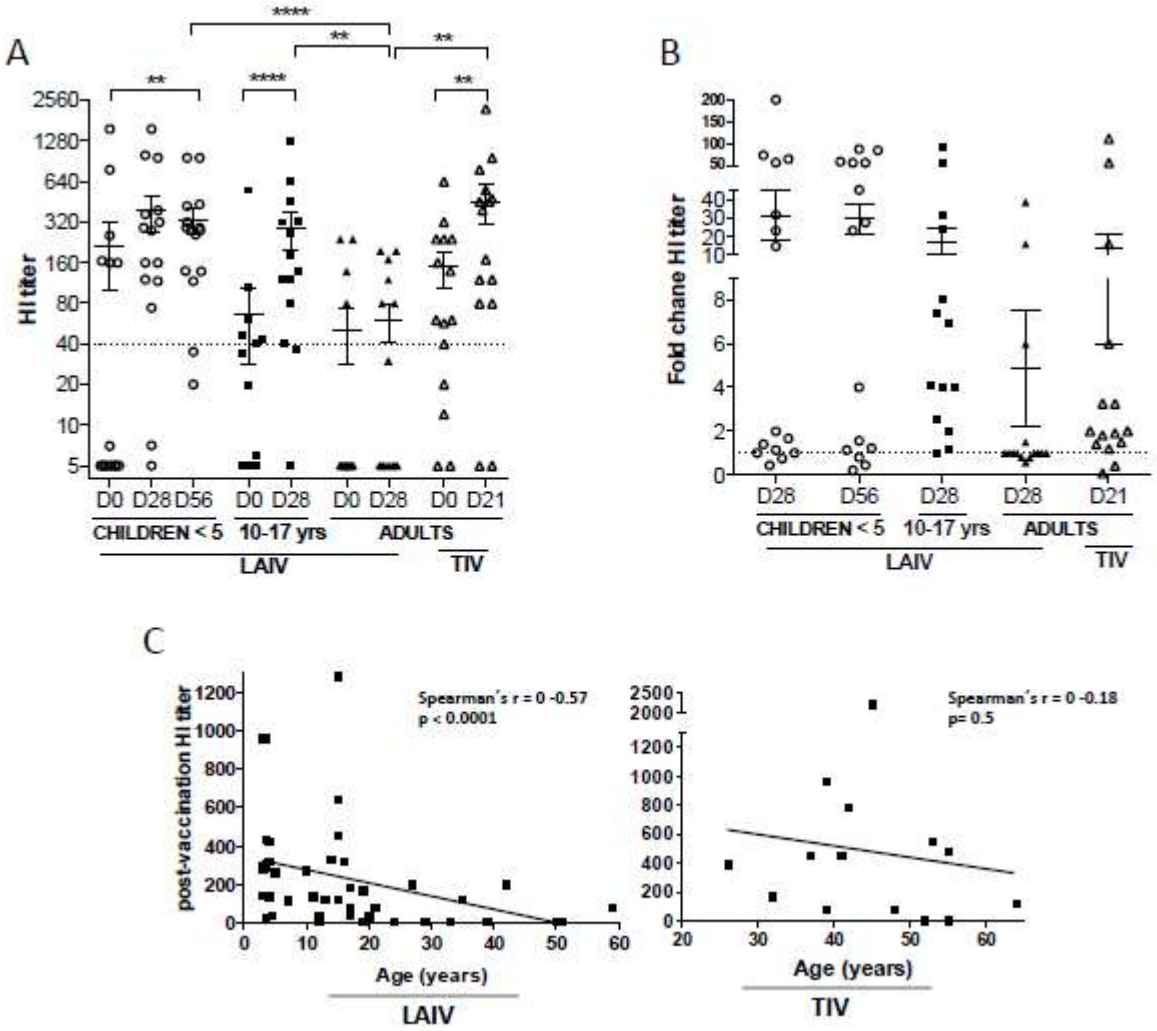


Figure 2: Hemagglutination inhibition response. (A) Geometric mean HI titers against influenza A H3N2/Texas virus pre- and post-vaccination in children < 5 (open circles), children 10-17 years old (closed circles), LAIV vaccinated adults (closed triangles) and TIV vaccinated adults (open triangle). The dotted line indicates a titer of 40, which is considered protective. Each symbol represents the GMT of all the titers at each time-point ± SEM (bars). (B) Pre- to post-vaccination fold change in HI titres. (C) Correlation between age and post-vaccination HI titres in LAIV and TIV vaccinated subjects. *, $p < 0.05$; **, $p< 0.01$; ***,$p< 0.001$, ****, $p<0.0001$

LAIV after two doses in young children and TIV in adults induced comparable hemagglutinin-specific IgG1 responses

Since the HI assay does not differentiate between the IgG subclass of antibodies, we quantified the H3N2 HA1-specific IgG1, IgG2, IgG3 and IgG4 antibodies in an ELISA assay. Pre-vaccination, HA1-specific IgG1 levels were comparable between the children and adults (Fig 3A). In children <5 years

old, a second vaccine dose was required to induce a significant increase in IgG1 levels. In children 10-17 years old one dose of LAIV was sufficient to induce a significant increase in HA1-specific IgG1 concentrations. However in adults, no change in HA1-specific IgG1 concentrations was observed after LAIV immunisation. TIV induced a significant increase in HA1-specific IgG1 in adults (Fig 3A) and hence significantly higher fold increase in IgG1 (mean 3.43 fold) than LAIV in adults (mean 0.90 fold) ($p = 0.008$) (Fig 3B). TIV induced significantly higher IgG1 than one dose of LAIV in both older children (10-17 years) and adults ($p < 0.05$) (Fig 3A).

Overall, IgG3 was detected at very low concentrations in a few subjects but was not detectable in most vaccinees (Supplementary Fig 1). HA1-specific IgG2 and IgG4 subclasses were absent in all subjects tested (data not shown).

Since most antibodies generated after vaccination are IgG specific and the HI assay detects HA specific antibodies, we analyzed the relationship between the HA1-specific IgG1 response and HI titers. We found a significant correlation between HI titers and HA1-specific IgG1 titers in LAIV vaccinated children and adults post vaccination (Spearman´s $r > 0.5$, $p < 0.01$). However, we observed no relationship between the HI titers and HA1-specific IgG1 response in TIV vaccinated adults (Supplementary Fig 2).

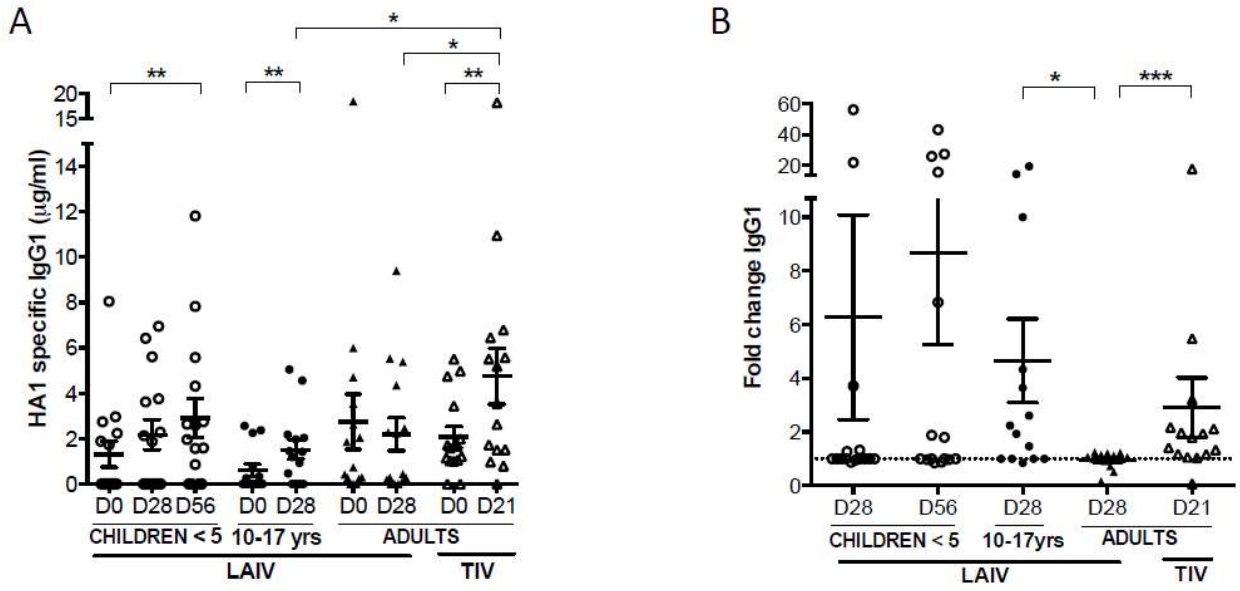


Figure 3: HA1-specific IgG1 response. (A) The concentration of HA1-specific IgG1 was measured in pre- and post-vaccination plasma of children < 5 (open circles), children 10-17 years old (closed circles), LAIV vaccinated adults (closed triangles) and TIV vaccinated adults (open triangle). Each symbol represents an

individual. (B) Fold induction of post-vaccination IgG1 concentrations over pre-vaccination IgG1 concentrations.

* indicates statistically significant differences in responses $p < 0.05$; **, $p< 0.01$; ***,$p< 0.001$.

High HA-specific IgA1 responses induced by vaccination in children but not in adults

We investigated whether LAIV or TIV induced HA1-specific IgA1 antibodies in in children and adults. Pre-vaccination, HA1-specific IgA1 was detectable in all the groups. Significantly higher HA1-specific IgA1 levels were found in children < 5 years old compared to older children aged 10-17 years ($p < 0.05$) (Fig 4A). Vaccination resulted in a significant increase in HA1-specific IgA1 concentrations in children but not in adults (Fig 4A). One dose of LAIV increased IgA1 levels in older children, while two doses were required to significantly increase the IgA1 levels in young children <5 ($p < 0.05$). Young children who received two vaccine doses had significantly higher HA1-specific IgA1 after the second dose than older children and adults who all received one vaccine dose ($p < 0.05$). Although LAIV or TIV did not result in a significant increase in IgA1 levels in adults, the IgA1 concentrations and fold changes were maintained at similar levels to those in older children (10-17 years) who also received one vaccine dose (Fig 4B).

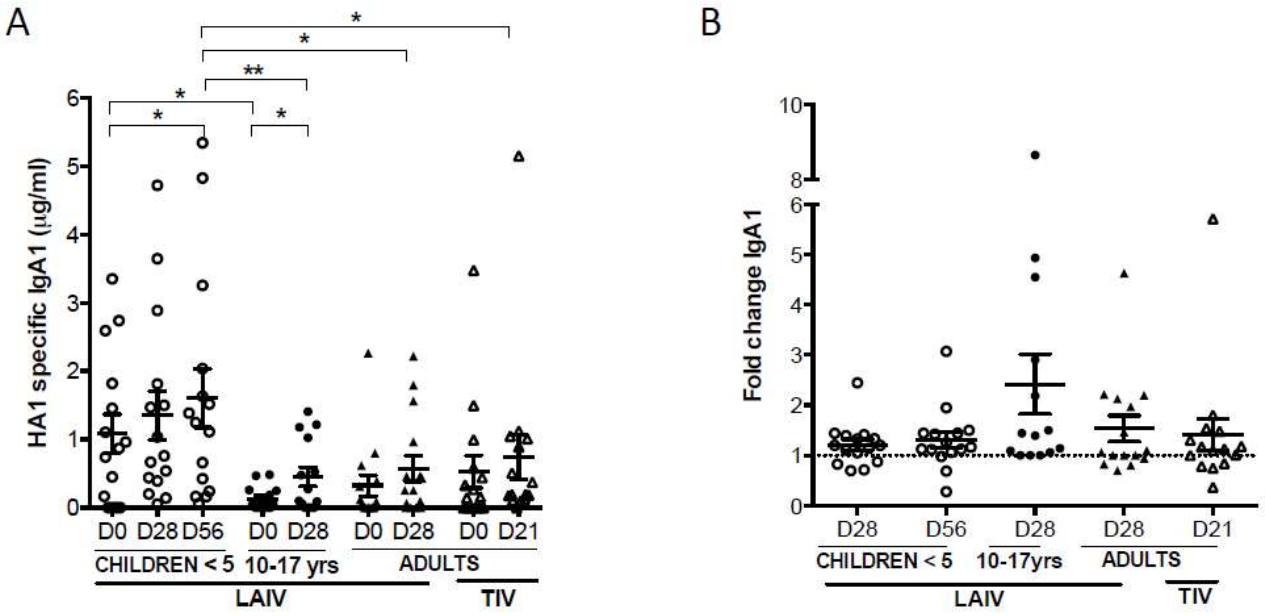


Figure 4: HA1-specific IgA1 response. (A) IgA1 antibodies specific to HA1 in children < 5 (open circles), children 10-17 years old (closed circles), LAIV vaccinated adults (closed triangles) and TIV vaccinated adults

(open triangle) pre- and post-vaccination. (B) Pre- to post-vaccination fold change in HA1 specific IgA1. *, $p < 0.05$; **, $p < 0.01$

Vaccination induced high avidity antibodies in children and adults

To assess the difference in quality of the IgG antibodies induced by LAIV and TIV in children and adults, the avidity of HA1-specific IgG antibodies was measured. The avidity was evaluated after serum treatment with a chaotropic agent, NaSCN, which allows the assessment of the strength of interaction between the antibody and the antigen. The avidity of HA1-specific IgG antibodies was comparable between young and older children, as well as between the two adult groups, pre-vaccination (Fig 5A). However, the avidity of HA1-specific IgG antibodies in older children (10-17 years) was significantly lower than that of the two adult groups ($p < 0.05$). Vaccination resulted in a significant increase in antibody avidity in children < 5 years old, children 10-17 years old and TIV vaccinated adults but not in LAIV vaccinated adults, with avidity indices of 46.8%, 33%, 37.5% and 44.4%, respectively (Fig 5A). Even though the avidity of IgG antibodies in LAIV vaccinated adults did not increase, antibodies post-vaccination were characterized by high avidity, comparable to the other three groups.

We used a cut-off avidity index of 20% to determine which subjects had high avidity antibodies. Pre-vaccination, a higher number of adults compared to children showed high avidity (>20% avidity index) antibodies. The frequency of children < 5 years old, children 10-17 years old, LAIV vaccinated adults and TIV vaccinated adults with pre-existing high avidity antibodies were 46%, 25%, 75% and 80%, respectively (Fig 5B and C). Post-vaccination, the proportion of children with high avidity IgG increased from 46% to 69% and from 25% to 75% in <5year olds and 10-17year olds, respectively. Both LAIV vaccinated and TIV vaccinated adults maintained elevated antibody avidity with 83% and 100% having high avidity antibodies, respectively (Fig 5C).

Interestingly, we found a positive correlation between the avidity of pre-existing HA-specific IgG and post vaccination HA1-specific IgG1 response in children <5 (spearman´s r =0.71, p=0.0068) and children 10-17 years old (spearman´s r =0.72, p=0.01) (Supplementary Fig 3). However, this correlation was not observed in either LAIV or TIV vaccinated adults.

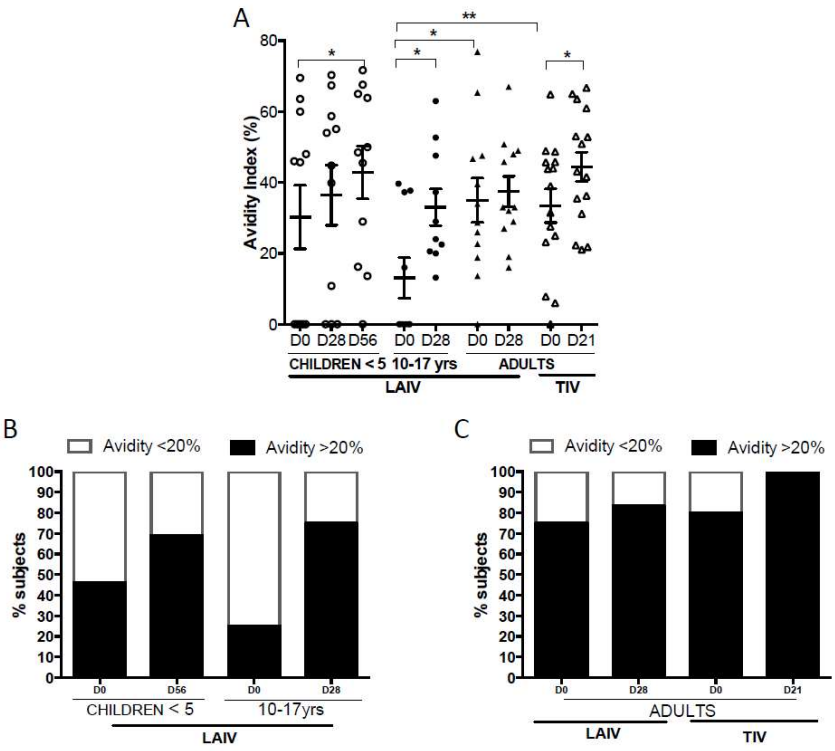


Figure 5: Hemagglutinin specific IgG antibody avidity. (A) The avidity of HA1 specific total IgG antibodies was measured in an avidity ELISA assay for children < 5 (open circles), children 10-17 years old (closed circles), LAIV vaccinated adults (closed triangles) and TIV vaccinated adults (open triangle) pre- and post-vaccination. Standardized sera were treated with 1.5M NaSCN and the percentage of HA1-specific IgG antibodies remaining bound after 1.5M NaSCN treatment was measured as (absorbance of treated samples ÷ absorbance of untreated samples) × 100%. The line represents the mean avidity index at each time-point ± SEM. The stacked column graphs represent the percentage of (B) children and (C) adults with high avidity antibodies (avidity index ≥ 20%). The percentage of subjects with antibodies with < 20% avidity are shown in white while those with avidity index ≥ 20% are shown in black.

*, $p < 0.05$; **, $p < 0.01$

9.4 Discussion

Annual influenza vaccination is the most effective method to prevent infection especially in subjects prone to develop secondary complications, such as young children, pregnant women, people with chronic medical conditions and the elderly. Among the occupational groups, influenza vaccination is recommended for farm and healthcare workers (Grohskopf et al, 2014). Both mucosal and systemic antibodies have been previously shown to be involved in the protection against influenza infection and disease (Clements et al, 1983, Clements et al, 1986a, Clements et al, 1986b). Antibodies specific to the HA1 domain of HA, which contains the receptor-binding site, are important for viral neutralization (Skehel and Wiley, 2000; Edwards and Dimmonk, 2001). However, the immune response after vaccination is dependent on several factors, including vaccine type and age of recipients (Ohmit et al, 2014, Stepanova et al, 2002, Frasca et al, 2011). Here, we evaluated the quantity as well as the quality of the antibodies specific to H3N2 HA induced by LAIV and TIV in 4 different participant groups, varying by age and vaccine type.

LAIV vaccines contain live, cold-adapted influenza viruses that only replicate in the mucosal membranes of the upper respiratory tract and do not cause disease in humans (Fleming et al, 2006; Treanor et al, 1999). LAIV promotes a robust antibody response in young children, especially in individuals that are seronegative prior to vaccination (Belshe et al, 2000; Block et al, 2008). Previous studies demonstrated that LAIV was more effective than TIV in children aged from 6 months to 17 years old (Ashkenazi et al, 2006; Fleming et al, 2006). A study that compared the efficacy and safety of LAIV versus TIV in children with recurrent respiratory tract infections showed that LAIV resulted in a 53% reduction in influenza cases by antigenically matched vaccine strains compared to TIV (Ashekenazi et al, 2006). Another study by Fleming et al. reported 35% fewer influenza cases in LAIV recipients compared to TIV recipients (Fleming et al, 2006). Although at present no immunological correlates of protection are available for LAIV, our results from the HI assay confirm that LAIV induces a significant antibody response in children of all ages.

A study by Treanor et al. showed that TIV induced higher HI titers than LAIV in adults, with titers induced by LAIV being comparable to placebo. However, after challenge with wild type virus of vaccinated adults with HI titers ≤ 8, laboratory confirmed illness occurred in 13%, 7% and 45% of TIV, LAIV and placebo vaccinated adults, respectively (Treanor et al, 1999). They demonstrated that despite low HI titers, LAIV had comparable efficacy to TIV in adults. We showed that TIV induces significantly higher HI titers than LAIV in adults, with LAIV resulting in no increase in titers.

Despite no increase in HI titres after LAIV vaccination in adults, they could still be protected by other immune mechanisms.

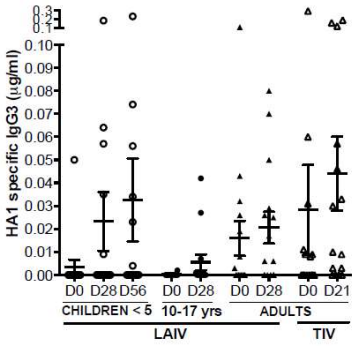
In this study, we investigated the IgG subclass response to H3N2 HA, since it represents the most dominant serum immunoglobulin class induced after vaccination (Ashkenazi et al, 2006). IgG1, along with IgG3, are the subclasses involved in critical immunologic functions, such as complement-fixation reaction, opsonization, antibody dependent cell-mediated cytotoxicity and virus neutralization (Johnson et al, 1986). In general, we detected elevated IgG1 levels in our subjects, whereas IgG3 levels were low post-vaccination. Both unvaccinated and previously vaccinated children and adults had pre-existing HA1-specific IgG1 reflecting previous exposure to antigenically similar influenza viruses (Belshe et al, 2000; Block et al, 2008).
We found that LAIV and TIV successfully induce antibody responses in subjects with no pre-existing antibodies, as well as boost pre-existing immunity. The children who received 2 doses of vaccine were all under 5 years of age and are less likely to have been previously infected with the H3N2 strain compared to adults. In Norway, seasonal influenza vaccination is only recommended for children with high-risk conditions and the children in our study were healthy and therefore not previously vaccinated. Only 27% of children <5 years old and 50% of children 10-17 years old had received pandemic H1N1 influenza vaccination in 2009. Based on the significant increase in IgG1 antibodies in children, LAIV appears to be efficient in both priming the H3N2 response in the children with no pre-existing titers and boosting this response in children with pre-existing titers.

TIV vaccine elicits a more robust increase in serum IgG antibodies, with only a minor induction of local IgA response (Brokstad et al, 1995; El-Madhun et al, 1998). Conversely, LAIV induces higher local IgA response at the nasal epithelium and lower systemic antibody responses. Since the LAIV vaccine mimics a natural infection, a local IgA response provides protection at the local mucosa where the influenza virus starts its infection cycle. Our study of systemic IgA1 response induced by vaccination revealed that whereas no change was observed in either LAIV or TIV vaccinated adults, a significant increase was found in serum IgA1 in children after LAIV. Particularly, younger children receiving two vaccine doses of LAIV showed significantly higher IgA levels compared to older children (10-17 years) who received a single dose of vaccine. The IgA1 response detected in our study could also be due to spill over of IgA from the site of vaccination at the local mucosa. However, we did not measure the nasal antibody response in the present study. A study by Boyce

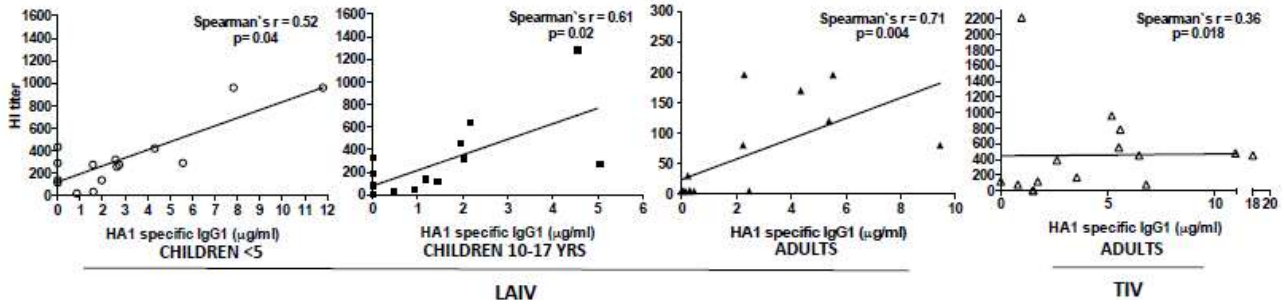
et al. demonstrated a positive correlation between nasal and serum IgG and IgA responses in adults intranasally vaccinated with inactivated vaccine (Boyce et al, 2000). Another study demonstrated elevated serum antibody titers positively correlated with nasal antibody levels. In addition, LAIV increased mucosal IgA but not systemic IgG in adults (Barria et al, 2013) (and unpublished data). Our results are in agreement with these observations, as LAIV did not induce a significant increase in systemic IgG1 or IgA1 in adults in our study. An earlier investigation showed that pre-existing nasal IgA, detected almost exclusively in subjects naturally infected or vaccinated with LAIV, was associated with protection (Johnson et al, 1986). Adults would have had a number of exposures to influenza in their lifetime either through vaccination or infection. It is plausible that pre-existing antibodies were present in the nasopharyngeal mucosa of the adults before LAIV vaccination, which may limit both intranasal infection and replication of LAIV, resulting in a lower antibody response as observed in our LAIV vaccinated adults (Mohon et al. 2014).

The pre-existing HA1-specific IgG antibodies in children 10-17 years old had low avidity compared to adults. The high antibody avidity observed pre-vaccination in a few children and most adults may be due to pre-existing memory generated by previous infection. Avidity could indicate the priming of immunological memory as vaccination results in antibody maturation and hence generation of antibodies with increased avidity as we detected in the present study (Johnson et al, 1986; Goldblatt et al, 1998). Priming and subsequent boosting of the antibody response results in a gradual increase in high affinity antibodies. Of note is that adults who received the TIV were healthcare workers who are all offered yearly influenza vaccination and also are likely to come in contact with infected patients. We have previously reported that repeated annual vaccination in healthcare workers persistently boosted the avidity of influenza-specific IgG antibodies (Eidem et al, 2015). The high avidity antibodies were maintained in adults receiving LAIV, although they did not increase in quantity or avidity post vaccination. Interestingly, the avidity of pre-existing antibodies in the sera predicted the IgG1 response in children. This suggests that vaccination and infection outcome in children likely correlates with the quality of the antibodies and their ability to restrict virus replication to the upper respiratory tract. In adults, the elevated pre-existing IgG avidity was not associated with a higher IgG1 concentration after vaccination, suggesting that a low number of high avidity memory B cells could be sufficient for the maintenance of protection in previously vaccinated or exposed individuals.

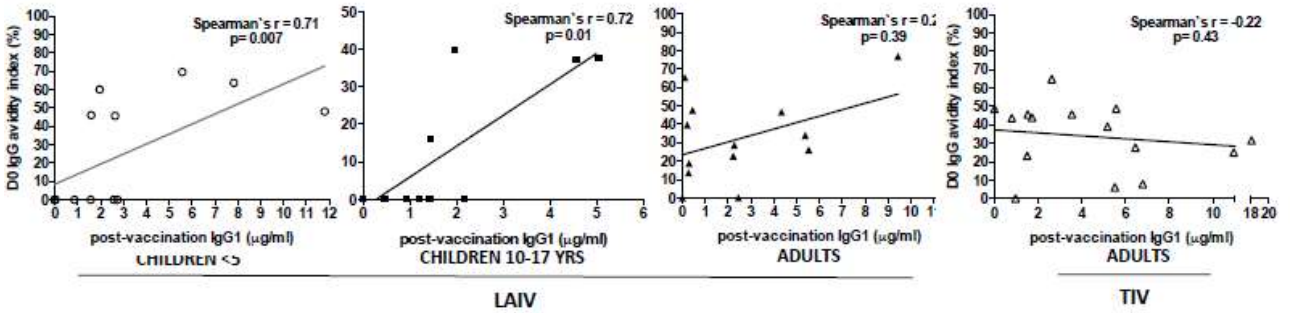
In conclusion, our findings confirm that LAIV promotes a stronger systemic antibody response in children than in adults. In adults, TIV induces better antibody responses compared to LAIV, but comparable antibody response to that induced in LAIV vaccinated children. The different mechanisms of action of LAIV versus TIV, may explain the relative efficacy between the two vaccines in children and adults. In children, the avidity of pre-existing serum antibodies likely plays a role in determining the antibody response to infection. Our results suggest that exposure history and the type of vaccine play a significant role in determining the antibody response.



Supplementary figure 1: HA1-specific IgG3 response. (A) The concentration of HA1-specific IgG3 was measured in pre- and post-vaccination plasma of children < 5 (grey circles), children 10-17years (black circles), LAIV vaccinated adults (grey triangles) and TIV vaccinated adults (open triangle). Each symbol represents an individual.



Supplementary figure 2: Correlation analysis of post-vaccination HI titers versus post-vaccinationHA1 specific IgG1 concentrations in children and adults. The Spearman rank correlation coefficients (r) and significance values are indicated on the graphs.



Supplementary figure 3. Avidity of pre-vaccination IgG antibodies plotted against the post-vaccination HA1 specific IgG1 concentrations in children and adults. The Spearman rank correlation coefficients (r) and significance values are indicated on the graphs.

10. CONCLUSIONS AND COSIDERATIONS

Among the various *weapons* available today to fight the influenza infection, vaccines are surely the best strategy of prevention from both subjective and collective point of view, especially for those people at high risk of secondary complications like young children, elderly, immune-compromised people and pregnant women. In the present study have been analyzed different types of influenza vaccine (intradermal, intramuscular and live-attenuated); each typology presents advantages and disadvantages, mainly due to the age of receivers and the vaccine composition. Generally, the intramuscular injection results to be the vaccine exploitable in broader spectrum. On the other hand, LAIV vaccines seem to result more immunogenic in young children and more simple to deliver to this target of receivers, as it is administered as a nasal spray. Another vaccine approach is the intradermal injection. Also this way of administration result to be simplest if compared to the intramuscular injection, due to the micro-needle and the possibility of self-administration at least for health care workers.

The two main classes of antiviral drugs today available are based on two main mechanisms: the ability to block the viral M2 ion channel and to inhibit the enzymatic activity of the viral neuraminidase. By recent studies, they have been associated with limited efficacy, adverse side effects (Loregian et al, 2014) and the spread of drug resistance in circulating influenza viruses.

To notice that studies have indicated how different plant extracts are able to mimic the anti-neuraminidase effect of antiviral drugs. One of these plants is sea buckthorn (SBT) (*Hippophae rhamnoides*) belonging to the family Elaeagnaceae, investigated for the capability of inhibit the influenza A/H1N1/California/7/2009 growth *in vitro* (Torelli et al, 2015).

Despite currently available influenza vaccines are not able to protect against possible and sudden pandemics derived from a genetic variations between different virus subtype, influenza vaccination has been demonstrated to be highly cost-efficient and cost-saving. For the next future, the major goal will be the development of a new *universal flu vaccine* able to provide high cross protection among different influenza type and subtype, bringing less frequent administration and more acceptance from receivers.

REFERENCES:

Ainsworth MA. 2003. New drugs and European producers of approval the European Agency for evaluation of Medicinal Product's rule. *Ugesk Laegar*. 165: 1648-9.

Air GM. 1981. Sequence relationship among the haemoagglutinin genes of 12 subtypes of Influenza A virus. *Proc Natl Acad Sci. U.S.A.* 78: 7639-7643.

Alverez MM, Lopez-Pacheco F, Aguilar-Yanez JM. 2010. Specific recognition of influenza A/H1N1/2009 antibodies in human serum: a simple virus-free ELISA method. *PLos One*. 5(4), e10176.

Ambrose CS, Wu X. 2013. The safety and effectiveness of self-administration of intranasal influenza vaccine in adults. *Vaccine*. 31(6): 857-860.

Ambrose CS, Wu X, Caspard H, Belche RB. 2014. Live attenuated influenza vaccine against influenza illness in children as a function of illness severity. *Vaccine*. 32: 5546-5548.

Arnou R, Icardi G, De Decker M, Ambrozaitis A, Kazek MP, Weber F, Van Damme P. 2013. Intradermal Influenza vaccine for older adults: a randomized controlled multicenter phase III study. *Vaccine*. 27(52): 7304-12.

Ashkenazi S, Vertruyen A, Aristegui J, Esposito S, McKeith DD, Klemola T, et al. 2006. Superior relative efficacy of live attenuated influenza vaccine compared with inactivated influenza vaccine in young children with recurrent respiratory tract infections. *Pediatr Infect Dis J.* 25(10):870-9.

Barria MI, Garrido JL, Stein C, Scher E, Ge Y, Engel SM, et al. 2013. Localized mucosal response to intranasal live attenuated influenza vaccine in adults. *J Infect Dis*. 207(1):115-24.

Beigel JH, Ferrar J, Han AM, et al. 2005. Avian Influenza A(H5N1) infection in humans. *N Engl J Med.* 353: 1374-85.

Beyer WE, Palache AM, DeJong JC, Osterhous AD. 2002. Cold adapted live influenza vaccine vs inactivated vaccine: systemic vaccine reaction, local and systemic response, and vaccine efficacy. A meta-analysis. *Vaccine*. 20: 1340-53.

Benne CA, Harmsen M, de Jong JC, Kraaijeveld CA. 1994. Neutralization enzyme immunoassay for influenza virus. *J Clin Microbiol*. 32:987-990.

Blesche R, Edward KM, Vesikant T, Black SV, Walker ER, Hultquist M, Kamble G and Connor EM. 2007. Live attenuated versus inactivated influenza vaccine in infants and young children. *N Eng J Med*. 356: (12): 1283.

Blesche R. 2005. The Origins of Pandemic Influenza — Lessons from the 1918 Virus*. N Eng J Med.*24: 353(21): 2209-11

Boyce TG, Hsu HH, Sannella EC et al. 2000. Safety and immunogenicity of adjuvanted and unadjuvanted subunit influenza vaccine administered intranasally to healthy adults. *Vaccine*. 19: 217-26.

Bontrop RE. 2006. Comparative genetics of MHC polymorphism in different primate species: duplication and deletion. *Human Immunol*. 67(6): 388-97.

Braakman I, Hoover-Litty H, Wagne KR, Helenius A. 1991. Folding of Influenza hemagglutinin in the endoplasmic reticulum. *J Cell Biol*. 144: 401-411.

Bragazzi NL, Orsi A, Ansaldi F, Gasparini R, Icardi G. 2016. Fluzone®Intra-dermal (Intanza ®/ Istivac ® intra-dermal): an updated overview. *Hum Vaccin Immunother*. 10.1080/21645515.

Bresson JL, Perrone C, Launay O, Gerdil C, Saville M, Wood J, Hoschler K, Zambon MC. 2006. Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomized trial. *Lancet.*367: 1657-1664.

Bridges CB, Kuehenert MJ and Hall CB. 2003. Trasmission of Influenza: implication for control in health care settings. *Clin Infect Dis.* 37:1094-101.

Brokstad KA, Cox RJ, Major D, Wood JM, Haaheim LR. 1995. Cross-reaction but no avidity change of the serum antibody response after influenza vaccination. *Vaccine*. 13 (16):1522-1528.

Brokstad KA, Cox RJ, Olofsson J, Jonsson R, Haaheim LRBKA. 1995. Parenteral influenza vaccination induces a rapid systemic and local immune response. 171:198.

Brown H, Kasel JA, Freeman Dm, Moise LD, Grose NP, Couch RB. 1977. The immunizing effect of influenza A/NewJeresy/76(Hsw1N1) virus vaccine administered intradermally and intramuscularly to adults. *J Infect Dis.* 136: Suppl:S466-s471.

Carnell GW, Ferrara F, Grehan K, Thompson CP and Temperton NJ. 2015. Pseudotypes-based neutralization assay for influenza: a systemic analysis. *Front. Immunol.* 6 (161).

Cate TR, Rayford Y, Kiermayr S, Hohenald C, Howard MK, Ilk R, et al. 2010. A high dosage influenza vaccine induced significantly more neuraminidase antibody than standard vaccine among elderly subject. *Vaccine*. 28(9): 2076-2079.

(CDC) CfDCaP.2009. Serum cross-reactive antibody response to a novel influenza A (H1N1) virus after vaccination with seasonal influenza vaccine. MMWR Morb Mortality Wkly Rep. 58: 521-4.

Cerio R, Griffiths CE, Cooper KD, Nickoloff BJ, Headington JT. 1997. Characterization of factor XIII positive dermal dendritic cells in normal and inflamed skin. *Br J Dermatol*. 121: 421-31.

Chen WH, Cross AS, Edelman R, Sztein MB, Blackwelder WC, Pasetti MP. 2011. Antibody and Th1-type cell-mediated immune response in elderly and young adults ummunized with the standard or a high dose influenza vaccine. *Vaccine*. 29:2865-2873.

Chi RC, Rock MT, Neuzil KM. 2010. Immunogenicity and safety of intradermal influenza vaccination in healthy older adults. *Clin Infect Dis*. 50: 1331-8.

Clements ML, Betts RF, Tierney EL, Murphy BR. 1986. Serum and nasal wash antibodies associated with resistence to experimental challenge with influenza A wild type virus. *J Clin Microb.* 24:157-60.

Clements ML, Murphy BR. 1986. Development and persistence of local and systemic antibody responses in adults given live attenuated or inactivated influenza A virus vaccine. J Clin Microbiol. 23(1):66-72.

Coleman BL, McGeer AJ, Halperin SA, Langlay JM, Shamount Y, Taddio A, Shah V, Mc Neil SA. 2012. A randomized trial comparing immunogenicity, safety, and preference for self versus nurse-administered intradermal influenza vaccine. *Vaccine*. 30(44): 6287-93.

Colman PM. 1998. Structure and function of the Neuraminidase. In: Nicholson KG, Webster RG, Hay AJ,editors. *Textbook of Influenza*. Blackwee Science . Pp65-73.

Cowling BJ, Chan KH, Fang VG, et al. 2010. Comparative Epidemiology of Pandemic and Seasonal Influenza A in Households. *N Eng J Med*. 362: 2175-2184.

Cox JR. 2013. Correlates of protection, where do we go from here? *Human vaccine and immunotherapeutics*. 9(2): 405-408.

Cox JR, Brokstad KA, Zuckerman MA, Wood JM, Haaheim LR, Oxford JS. 1994. An early humoral immune response in pheripheral blood following parenteral inactivated influenza vaccination. *Vaccine.* 12:993.

Cox JR, Brokstad KA and Ogra P. 2004. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccine. *Scand J Immunol.* 59(1): 1-15.

Crum-Cianflone NF, Collins G, Defang G, Iverson E, Eberly LE, Duplessis C, et al. 2012. Immunoglobulin G subclass levels and antibody responses to the 2009 influenza A (H1N1) monovalent vaccine among human immunodeficiency virus (HIV)-infected and HIV-uninfected adults. *ClinExpImmunol*. 168(1):135-41

de Jong JC, Palache AM, Beyer WE, Rimmelzwaan GF, Boon AC, Osterhaous AD. 2003. Haemagglutination inhibiting antibody to influenza virus. *Dev Biol.* 115: 63-73.

Delves PJ, Roitt IM. 2000. The immune system. First of two parts. *N Eng J Med.* 343(1):37-49.

Demicheli V, Rivetti D, Deeks JJ, Jefferson TO. 2004. Vaccine for preventing influenza in healthy adults. *Cochrane Database Syst Rev*. 3:CD001269.

DiLillo JD, Tan GS, Palese P and Ravetch JV. 2014. Broadly neutralizing hemagglutinin stalk-specific antibodies require FcyR interactions for protein against influenza virus in vivo. *Nat Med.* 20(2): 143-151.

Dunning AJ. 2006. A model for immunological correlates of protection. *Stat Med.* 25: 1485-1497.

Edwards KM. et al. 1994. A randomized controlled trial of cold-adapted and inactivated vaccines for the prevention of influenza A disease. *J Infect Dis.* 169(1) 68-76.

Edwards MJ, Dimmock NJ. 2001. A haemagglutinin (HA1)-specific FAb neutralizes influenza A virus by inhibiting fusion activity. *Journal of General Virology*. 82:1387-95.

Eicheberger MN, WanH. 2014. Influenza Neuraminidase as a Vaccine Antigen In: Micheal BA Oldstone, Richard W Compans, editors. *Curr Top Microbiol Immunol*. Switzerland: Springer International Publishing; pp 275-299.

Eidem S, Tete SM, Jul-Larsen A, Hoscheler K, Montomoli E, Brokstad KA,Cox RJ. 2015. Persistence and avidity maturation antibodies to A(H1N1)pdm09 in health care workers following repeated annual vaccination. Vaccine. http://dx.doi.org/10.1016/J.Vaccine.2015.05.081.

Ekiert DC et al. 2009. Antibody recognition of a highly conserved influenza virus epitope. *Science.* 324:246-251.

El-Madhun AS, Cox RJ, Haaheim LR. 1999. The effect of age and natural priming on the IgG and IgA subclass responses after parenteral influenza vaccination. *J Infect Dis*. 180: 1356-1360.

EMEA/CHMP/VWP. Guideline on Influenza Vaccine prepared from Viruses with the potential to cause a pandemic and intended for use outside of the core dissier context. 263499/2006.

Fleming DM, Crovari P, Wahn U, Klemola T, Schlesinger Y, Langussis A, et al. 2006. Comparison of the efficacy and safety of live attenuated cold-adapted influenza vaccine, trivalent, with trivalent inactivated influenza virus vaccine in children and adolescents with asthma. *Pediatr Infect Dis J*.25(10):860-9.

Frasca D, Diaz A, Romero M, Mendez VN, Landin MA and Blomberg BB. 2013. Effects of age on H1N1-specific serum IgG1 and IgG3 levels evaluated during the 2011 and 2012 influenza vaccine season. *Immunity & Ageing*. 10:14.

Foy HM, Cooney MK, Allan ID and Albrecht JK. 1987. Influenza B in households: virus shedding without symptoms or antibody response. *Am J Epidemiol*. 126:506-15.

Frasca D, Diaz A, Romero M, Mendez VM, Landin AM and Blomberg BB. 2013. Effects of age on H1N1-specific serum IgG1 and IgG3 levels evaluated during the 2011-2012 influenza vaccine season. *Immunity & Ageing*. 10:14.

Gaush CR, Hard WL, Smith TF. 1966. Characterization of an established line of canine kidney cells (MDCK). *Proc Soc Exp Biol Med.* 122:931-5.

Genzel Y, Fischer M and Reichl U. 2006. Serum-free influenza virus production avoiding washing steps and medium exchange in large-scale microcarrier culture. *Vaccine*. 24: 3261-3272.

Gerin JL, Anderson NG. 1969. Purification of influenza virus in the K-II zonal centrifuge. *Nature*. 221 (5187):1255-6.

Goldbatt D, Pinto Vaz ARJP, and Miller E. 1998. Antibody avidity as a surrogate marker of successful priming by Haemophilus influenza tybe b conjugate vaccine following infant immunization. *J. Infect. Dis.* 177: 1112-5.

Goldsby RA, Kindt TJ, Osborne BA, Kuby J. 2003. B Cell generation, activation and differentiation. *Immunology*. Fifth ed. New York: W.H. Freeman and Company. 247-75.

Goodwin K, Viboud C, Simonsen L. Antibody response to influenza vaccination in the elderly: a quantitative review. *Vaccine*. 24: 1159-1169.

Greenbaum E, Engelhard D, Levy R, Schlezinger M, Morag A, Zakay-Rones Z. 2004. Mucosal (SIgA) and serum (IgG) immunologic responses in young adults following intranasal administration of one or two doses of inactivated trivalent anti-influenza vaccine. *Vaccine*. 22(20): 2566-2577.

Grohskopf LA, Olsen SJ, Sokolow LZ, Bresee JS, Cox NJ, Broder KR et al. 2014. Prevention and control of seasonal influenza with vaccine. Recommendation of the advisory committee on immunization prectices (ACIP)-United States 2014-15 Influenza Season. *Am J Transplant*. 14 (12):2906-13.

Harmon MW, Rota PA, Walls HH, Kendal AP. 1988. Antibody response in humans to influenza virus type B host cell-derived variants after vaccination with standard (egg-derived) vaccine or natural infection. *J Clin Microbiol* 26: 333-337.

Hickling JK, Jones KR, Friede M, Zehrung D, Chen D, Kristensen D. 2011. Intradermal delivery of vaccines: potential benefits and current challenges. *Bulletin of the WHO*. 89: 221-226.

Hirst GK. 1941. The agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. *Science*. 94 (2427): 22-23.

Hirst GK. 1942. The quantitative determination of influenza virus and antibodies by means of red cell agglutination. *J Exp Med*. 75(1):49-64.

Hobson D, Curry RL, Beare AS, Ward-Gardner A. 1972. The role of serum haemagglutinin-inhibition antibody in protection against challenge infection with influenza A2 and B viruses. *J Hygi*. 70: 767-77.

Holisinger LJ, Lamb RA. 1991. Influenza virus M2 integral membrane protein is a homotetramer stabilized by formation of disulfide bonds. *Virology*. 183(1): 32-43.

Hong Kong Measles Vaccine Committee. 1967. Comparative trial of live attenuated measles vaccine in Hong Kong by Intramuscolar and intradermal injection. Bull WHO. 36: 375-84.

Icardi G, Orsi A, Ceravolo A, Ansaldi F. 2012. Current evidence on intradermal influenza vaccines administered by Soluvia licensed micro injection system. *Hum Vaccin Immunother*. 8(1): 67-75.

Ito T, Couceiro JN, Kelm S, et al.1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *J Virol*. 72: 7367-7373.

Izurieta H, Thompson WW, Karmaranz P. et al. 2000. Influenza and rates of hospitalization for respiratory disease among infants and young children. *N Eng J Med*. 342: 232-239.

Janeway CA Jr., Medzhitov R. 2002. Innate immune recognition. *Ann Rev Immunol*. 20: 129-216.

Jennings R, Potter CW, Massey PM, Duerden BI, Martin J, Bevan AM, 1981. Responses of volunteers to inactivated influenza virus vaccine. *J Hyg (Lond).* 86(1):1-16.

Jakeman KJ, Tisdale M, Russell S, Leone A, Sweet C (August 1994). "Efficacy of 2'-deoxy-2'-fluororibosides against influenza A and B viruses in ferrets". *Antimicrob. Agents Chemother*. 38 (8): 1864–7.

Johansson BE and Cox MM. 2011. Influenza viral neuraminidase: the forgotten antigen. *Expert Rev vaccines*. 10(12): 1683- 1695.

Johnson PR, Feldman S, Thompson JM, Mahoney JD, Wright PF. 1986. Immunity to influenza A virus infection in young children: a comparison of natural infection, live cold-adapted vaccine, and inactivated vaccine. *J Infect Dis*. 154(1):121-7.

Katz MJ, Naeve CW, Webster RG. 1987. Host cell-mediated variation in H3N2 influenza viruses. *Virology.* 156:386-395.

Katz MJ, Lim W, Bridges CB et al. 1999. Antibody response in individuals infected with avian influenza A(H5N1) viruses and detection of anti- H5 antibody among household and social contacts. *J Infect Dis.* 180:1763-1770.

Katz MJ, Garg S, Sambhara S. 2006. Influenza vaccine: current and future strategies. In: Kawaoka Y. ed. *Influenza Virology*. First edition Norfolk: Caister Academic Press. 203-208.

Katz MJ, Hancock K. and Xu X. 2011. Serologic assay for influenza surveillance, diagnosis and vaccine evaluation. *Exp Rev*. 9(6), 699-683.

Kayali G, Setterquist SF, Capuano AW, Myers KP, Gill JS, Gray JC. 2008. Testing human sera for antibodies against avian influenza virus: horse RBC haemagglutination inhibition vs. microneutralizaion assays. *J Clin Virol*. 43(1): 73-78.

Kendal AP, Pereira MS and Skehel J. 1982. Concepts and procedures for laboratory-based influenza surveillance. Publication no. B17-35. Center for Disease control, Atlanta, Ga, 1982.

Kilbourne ED 2006. Influenza Pasndemics of the 20th century. *Emerg. Inf. Dis*. 12:15-22.

Krammer F, Palese P. 2013. Influenza virus hemagglutinin stalk-based antibodies and vaccine. *Curr Opin Virol.*

Krystal M, Elliott RM, Benz EW, Young JF, Palese P. 1982. Evolution of Influenza A and B viruses: conservation of structural features in the hemagglutinin genes. *Proc Natl Acad Sci U S A*. 79:4800-4804.

Lamb RA, Krug RG. 2001. Orthomixoviridae: the viruses and their replication. *Fields Virology*. 4th edn. Lippincott Williams & Wilkins. Pp 1487-1531.

Lambert PH, Laurent PE. 2008. Intradermal vaccine delivery: will new delivery system transform vaccine administration? *Vaccine.* 26: 3197-208.

Last JM. 2001. International Epidemiological Association: A dictionary of epidemiology. Oxford Univ Press.

LI CK, Rappuoli R and Xu XNj. 2013. Correlates of protection against influenza infection in human: on path to universal vaccine? *Curr Opin Immunol.* 24: 470-476.

Li H, Nookala S, ReF. 2004. Aluminium hydroxide adjuvants active caspase-1 and induce IL-1 induce IL-1 beta and IL-18 release. *J Immunol.* 178: 5271-6.

Linbad EB. 2004. Aluminium adjuvants: in retrospect and prospect. *Vaccine.* 22: 3658-68.w

Lofgren E, Fefferman NH, Naumov YN, Gorski J, Naumova N,. 2007. Influenza Seasonality: underlying causes and modeling theories. *J Virol.* 81(11): 5429-36.

Loregian A, Mercorelli B, Nannetti G, et al. 2014. Antiviral strategies against influenza virus: towards new therapeutic approaches. *Cell Mol Life Sci*. 71:3659-83.

Lugovstev VY, Melny KD, Weir JP. 2013. Heterogeneity of MDCK cell-line and its applicability for influenza virus research. *PLoS ONE*. 8: e75014.

MacBean AM, Angle AN, Compaore P, Foster SO, McCormack WM. 1972. Comparison of intradermal and subcutaneous routes of cholare vaccine administration. *Lancet.* 1:527-9.

Madhun AS, Akselsen PE, Sjursen H, Pedersen G, Svindland S, Nostbakken JK, et al. 2010. An adjuvanted pandemic influenza H1N1 vaccine provides early and long term protection in health care workers. *Vaccine*. 29(2):266-73.

Marra F, Young F, Richardson K, Marra CA. 2013. A Meta-analysis of intradermal versus intramuscular influenza vaccine: immunogenicity and adverse events. Influenza Other Resp Viruses. 7: 584-603.

Mastrosovich MN, Mastrosovich TY, Gray T, Roberts NA, Klenk HD. 2004. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. *J Virol*. 78: 12665-12667.

Mayer AG and Wilke CO. 2015. Geometric constrains dominate the antigenic evolution of influenza H3N2 haemagglutinin. *PLOS Phatogens*. 11(5):e1004940.

Medzhitov R, Janeway Jr CA. 1997. Innate immunity: impact on the adaptative immune response. *Curr Opin Immunol.* 9:4.

Meiklejon G. Commission on Influenza. The histories of the commission. Falls Church, VA: the borden Institute, office of the surgeon General, Department of the Army, 1994.

Mestes J and Hughes CC. 2004. Of mice and not man: differences between mouse and human immunology. *J of Immunol*. 172(5): 2731-8.

Mohn KG, Bredholt G, Brokstad KA, Pathirana RD, Aarstad HJ, Tondel C, et al. 2014. Longevity of B-Cell and T-Cell Responses After Live Attenuated Influenza Vaccination in Children. *J Infect Dis*.

Monto AS, Ohmit SE, Petrie JG, Johnson E, Truscon R, Teich E, et al. 2009. Comparative efficacy of inactivated and live attenuated influenza vaccines. *N Engl J Med*. 361(13):1260-7.

Morens DM, Taubenberger JK, Folkers GK and Fauci AS. 2010. Pandemic Influenza's 500th anniversary. *Clinical Infectious Disease*. 51: 1442-3.

Morley PS, Hanson LK, Bogdan JR, Townsend HG, Appleton JA, Haines DM. 1995. The relationship between single radial haemolysis, haemagglutination inhibition, and Virus Neutralization assay used to detect antibodies specific for equine influenza viruses. *Vet microbiol*. 45(1), 81.

Mostow SR, Schoenbaum SC, Dowdle WR, Coleman MT, Kaye HS, Hierholzer JC. 1970. Studies on inactivated influenza vaccine. II. Effect of increasing dosage on antibody with resistence to influenza in man. Am J Med 92:321-322.

Munster VJ, Baas C, Lexmond P, Waldenstrom J, Wallensten A, Fransson T, Rimmelzwaan GF, Beyer WE, Schutten M, Olsen B, et al. 2007. Spetial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds*. PLOS Pathog*. 3,e61.

Murphy SL et al. 2009. Diverse IgG subclass responses to Adeno-Associated Virus infection and vector administration*. J. Med. Virol*. 81: 65-74.

Newman G, KawaokaY. 2006. Host range restriction and pathogenicity in the context of Influenza pandemic. *Emerg Infect Dis*. 12(6): 881-886.

Osterhaus AD, Rimmelzwaan GF, Martina BE, Bestebroer TM, Fouchier RA. Influenza B viruses in seals. *Science*. 288(5468), 1051-1053.

Ohmit SE, Thompson MG, Petrie JG, Thaker SN, Jackson ML, Belongia EA, et al. 2014. Influenza vaccine effectiveness in the 2011-2012 season: protection against each circulating virus and the effect of prior vaccination on estimates. *Clin Infect Dis*. 58(3):319-27

Osterholm MT, Kelly NS, Sommer A, Belongia EA. 2012. Efficacy and effectiveness of influenza vaccine: a systematic review and meta analysis. *Lancet Infect Dis*. 12:36-44.

Palese P. 2006. Making better influenza virus vaccine? Emerg. J *Infect Dis*. 12(1):61-5.

Parkman PD, Hoops HE, Rastogi SC, Meyer HM. Jr. 1977. Summary of clinical trials of influenza virus vaccine in adults. *Journal of Infectious Disease*. 136 Suppl: S722-30.

Patel SM, Atmar RL, El Sahly HM, Cate TR, Kaitel WA. 2010. A phase I evaluation of inactivated influenza A/H5N1 vaccine administered by the intradermal or the intramuscular route. *Vaccine.* 28: 3025-9.

Patriage J, Kieny MP, World Health Organization H1N1 influenza Vaccine task Force. 2010. Global production of seasonal and pandemic (H1N1) influenza vaccine in 2009-2010 and comparison with previous estimates and global action plan target. *Vaccine*. 28: 4709-12.

Pederson GK, Hoschler K, Oie Solbak SM, Berdholt G, Pathirana RD, Afsar A et al. 2014. Serum IgG titers, but not avidity, correlates with neutralizing antibody response after H5N1 vaccination. *Vaccine.* 32:4550-7.

Pileggi C, Lotito F, Bianco A, Nobile CG, Pavia M. 2015. Immunogenicity and safety of intradermal influenza vaccine in immunocompromised patients: a meta-analysis of randomized controlled trials. *BMC Infect Dis.* 15:427.

Plotkin SA and Gilbert PB. 2012. Nomenclature for immune correlates of protection after vaccination. *Clinical Infectious Disease*. 54: 1615-1617.

Plotkin SA. 2010. Correlates of protection induced by vaccination. *Clinical and Vaccine Immunology*. 17: 1055-1065.

Podda A. 2001. The adjuvanted influenza vaccines with novel with novel adjuvants: experience with MF 59-adjuvanted vaccine. *Vaccine*. 19 (17-19): 2673-2680.

Pober JS, Cotran RS. 1990. Cytokine and endothelial cell biology. Physiol Rev. 76: 427.

Potter CW, Oxford JS. 1979. Determinants of immunity to influenza infection in man. *Br Med Bull*. 35: 69-75; PMID: 367490.

Ramakrishanan A, Althoff NK, Lopez AJ, Coles LC, Bream JH. 2012. Differential serum cytokine responses to inactivated and live attenuated seasonal influenza vaccine. *Cytokyne*. 60:661-666.

Reimer CB, Baker RS, Van Frank RM, Newilin TE, Cline GB, Anderson NG. 1967. Purification of large quantities of influenza virus by density gradient centrifugation. *J Virol*. 1(6):1207-16.

Rivetti D, Jefferson T, Thomas R, Rudin M, Rivetti A, Di Pietrantoni C, Demicheli V. 2006 Vaccines for preventing influenza in the elderly. *Cochrane Database Syst Rev*. 3:CD004876.

Roger GN, Paulson JC. 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 haemagglutinin based on species of origin. *Virology*. 127(2):361-373.

Roitt JM. 1994. Essential Immunology. 8th ed. Oxford, UK: Blackwell Scientific.

Romagnani S. 2006. Regulation of the T cell response. *Clin Exp. Allergy*. 36(11):1357-66.

Ruigrok RWH. Structure of influenza A,B and C viruses. In: Nicholson KG, Webster RG, Hay AJ, editors. *Textbook of Influenza*. Blackwell Science, pp.29-42.

Saito T, Gale M Jr.2007. Principles of intracellular viral recognition. *Curr Opin Immunol.* 19(1): 17-23.

Salk JE. 1944. Simplified procedure for titrating haemagglutinating capacity of influenza virus and the corresponding antibody. *J Immunol*. 49,87.

Samji T. 2009. Influenza A: understanding the Viral life cycle. *Yale J Biol and Med*. 82 : 153-159.

Sandbulte MR, Gao J, Straight TM, Eichelberger MC. 2009. A miniaturized assay for influenza neuraminidase-inhibiting antibodies utilizing reverse genetic-derived antigens. *Influenza Other Respir Virus*. 3: 233-240.

Satiago FW, Emo KL, Fitzgerald T, Treanor JJ, Topham DJ. 2012. Antigenic and immunogenic properties of recombinant hemagglutinin proteins from H1N1 A/Brisbane/59/07 and B/Florida/04/06 when produced in various protein expression system. *Vaccine*. 30(31): 4606-16.

Sasaki S, Jaimes MC, Holmes TH, Dekker CL, Mahmood K, Kemble GW, Arvin AM, Greenberg HB. 2007. Comparison of the influenza virus-specific effector and memory B-cell responses to immunization of children and adults with live attenuated or inactivated influenza virus vaccine. *J Virol*. 81(1); 215-18.

Scheifele DW, et al. 2013. Safety, immunogenicity, and tolerability of three influenza vaccines in older adults. Results of a randomized, controlled comparison. *Hum Vaccin Immunother*. 9:11; 2460-2473.

Schild GC, Pereira MS, Chakraverty P. 1972. Single-radial-hemolysis: a new method for the assay of antibody to influenza haemagglutinin. Applications for diagnosis and seroepidemiologic surveillance of influenza. *Bull World Health Organ*. 52(1):43-50.

Schroeder HW, and Cavacini L. 2010. Structure and function of immunoglobulins. J. Allergy Clin. Immunol. 125: s41-s53.

Seidenari S, Pagnomi A, Di Nardo A, Giannetti A. 1994. Echographic evaluation with image analysis of normal skin: variations according to age and sex. *Skin Pharmacol*. 7 :201-9.

Simonsen L, Viboud C, Taylor RJ, Miller MA, 2011. The epidemiology of influenza and its control. In Influenza Vaccines for the future, 2th edn, pp.27-54. Edited by Rappuoli R. & Del Giudice G. Springer.

Skehel JJ, Wiley DC. 2000.Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem*.69:531-69.

SmithS, Demicheli V, Di Pietrantoni C, Harnden AR, Jefferson T, Matheson NJ, Rivetti A. 2006. Vaccine for preventing influenza in healthy children. Cochrane Database Syst Rev. 3:CD004879.

Spearman C. 1908. The method of 'Right and Wrong Cases? (constant stimuli) without Gauss formulae. Brit *Jour of Psych*. 2.

Steinhauer DA, Wharton SA. Structure and function of the haemoagglitinin. In: Nicholson KG, Webster RG, Hay AJ, editors.1998. *Textbook of influenza*. Blackwell science. Pp29-42.

Stepanova L, Naykhin A, Kolmskog C, Jonson G, Barantceva I, Bichurina M, et al. 2002. The humoral response to live and inactivated influenza vaccines administered alone and in combination to young adults and elderly. *J Clin Virol*. 24(3):193-201.

Stephenson I, Wood JM, Nicholson KG, Zambon MC. 2003. Sialic Acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. *J Med Virol*. 70(3):391-398.

Stephenson I, Das RG, Wood JM, Katz JM. 2007. Comparison of neutralizing antibody assays for detection of antibody to influenza A/H3N2 viruses: An international collaborative study. *Vaccine*. 25: 4056-4063.

Stephenson I, Health A, Major D, Newman RW, Hoschler K, Junzi W et al. 2009. Reproducibility of serologic assay for influenza virus A (H5N1). *Emerg Infect Dis.* 15:1252-9.

Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen LM, Santelli E, Stec B, Cadwell G, Ali M, et al. 2009. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nature structural & Molecular Biology*. 16: 265-273.

Takahashi T, Suzuki T, Hidari KI-PJ, Miyamoto D, Suzuki Y. 2003. A molecular mechanism for the low pH stability of sialidase activity of Influenza A Virus N2 Neuraminidase. *FEBS Lett*. 543, 71-75.

Tamura S-i, Kurata T. 2004. Defense mechanisms against influenza virus infection in the respiratory tract mucosa. *Jpn J Infect Dis.*57(6):236.

Torelli A, Gianchecchi E, Piccirella S, Manenti A, Piccini G, Llorente Pastor E, Canovi B, Montomoli E. 2015. Sea buckthorn but extract displays activity against cell-cultured Influenza virus. *J Prev Med Hyg*. 5:56.

Treanor J, Wright PF. 2003. Immune correlates of protection against influenza in the human challenge model. In: Brown F, Haaheim LR, Wood JM, Schild GC, eds. *Laboratory correlates of immunity to influenza - a reassessment. Dev Biol*. Basel: Karger. 115:97-104.

Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M. 2006. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. N Engl J Med. 354: 1343-1351.

Trombetta CM, Perini D, Mather S, Temperton N, Montomoli E. 2014. Overview of serological techniques for influenza vaccine evaluation: past, present and future. *Vaccine*. 2(4):707.

Trombetta CM, Perini D, Vitale L, Cox RJ, Stanzani V, Piccirella S, et al. 2015. Validation of Single Radial Haemolysis assay: A reliable method to measure antibodies against influenza viruses. *JImmunolMethods*. 422:95-101.

Tsang TK, Cauchemez S, Perera RAPM, Freeman G, Fang VJ, Ip DKM, Leung MG, Peiris JSM, Cowling BJ. 2014. Association between Antivody titers and protection against Influenza virus infection within households. *J Infect Dis*. 10:1093.

Tuft L. 1931. Immunization against thyphoid fever, with particular reference to an intradermal method. *J Lab Clin Med.* 16: 552-6.

Van Gelder D, Greenspan F, Dufresne N. 1947. Influenza vaccination: comparison of intracutaneous method. *Naval Med Bull*. 47: 197-206.

Veguilla V, Hancock K, Schiffer J, Gargiullo P, Lu X, Aranio D, Brench A, Dong L, Holiday C, Liu F, et al. 2011. Sensitivity and specificity of serological assay for detection of human infection with 2009 pandemic H1N1 virus in U.S. population. *J Clin Microbiol*. 49: 2210-2215.

Wagner R, Gopfert C, Hammann J, Neuman B, Wood J, Newman R et al. 2012. Enhancing the reproducibility of serological method used to evaluate immunogenicity of pandemic H1N1 influenza vaccine-an effective EU regulatory approach. *Vaccine*. 30: 4113-22.

Wang TT and Palese P. 2011. Biochemestry. Catching a moving target. *Science*. 333:834-835.

Wang JP, Kurt-Jones EA, Finberg RW. Innate immunity to respiratory viruses. *Cell Microbiol*. 9(7): 1641-6.

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. 1992. Evolution and ecology of influenza A viruses. *Microbiol Rev.* 56(1), 152-179.

Webster RG and Laver VG. 1972. Studies on the origin of pandemic influenza. I. Antigenic analysis of A 2 influenza viruses isolated before and after the appearance of Hong Kong influenza using antisera to the isolated hemagglutinin subunits. *Virology*. 48(2):433-44

Weinberg GA, Erdman DD, Edwards KM, Hall CB, Walker FJ, Griffin MR, Schwartz B, New Vaccine Surveillance Network Study Group. 2001. Superiority of reverse-trascription polymerase chain reaction to conventional viral culture in the diagnosis of acute respiratory tract infections in children. *J Infect Dis.* 189:706-710.

Wilson IA, Skehel JJ, Wiley DC. 1981. Structure of the Haemoagglutinin membrane gluycoprotein of influenza virus at 3 Å resolution. *Nature*. 289: 366-373.

World Health Organization (WHO).2011. Manual for the Laboratory diagnosis and virological surveillance of Influenza. Available online at: http://whqlibdoc.who.int/publications/2011/9789241548090_eng.pdf.

World Health Organization (WHO). Memorandum.1980. A revised system for nomenclature of Influenza viruses. *Bull World Healt Organization*. 58: 585-591.

World Health Organization (WHO). 2008. Antigenic and genetic characterristics of H5N1 viruses and candidate H5N1 vaccine virus developed for potential use as human vaccines.

Wood JM, Gaines-Das RE, Taylor J, Chakraverty P. 1994. Comparison of influenza serological techniques by international collaborative study. *Vaccine*. 12: 167-174.

Wright PF, Bryant JD, Karzon DT. 1980. Comparison of influenza B/Hong Kong virus infections among infants, children and young adults. *J Infect Dis*. 141: 430-435.

Wright PF, Webster RG. 2001. Ortomyxoviruses. In: Knipe DM, Howley PM, Griffin DE et al., editors. Fields Virology, 4th edn. Lippincott William & Wilkins, 2001;pp.1533-1579.

Wright PF, Neumann G and Kawaoka Y. 2007. Orthomixoviruses. In Fields Virology, DM. Knipe and PM. Howley, eds. (Philadelphia: Lippincott, Williams & Wilkins), pp. 1691-1740.

Zahn M, Pursiful P, Carrico R, Woods C, Troutman A. 2013. Self-immunization with live attenuated influenza vaccine in a mass vaccination clinic. Dis Med Public Health Preparedness. 7(2):2015-2017.

Desidero ringraziare la mia famiglia, tutta, per la vicinanza, il supporto e l'affetto dimostrato in tutti questi anni di studio… con questo "ultimo" lavoro si chiude un ciclo importante della ma vita. Un bacio particolare alla Nonna Alma

Grazie ad Elisa…per tutto!! Soprattutto per riuscire ogni giorno a "sopportarmi e supportarmi" in ogni mia scelta… anche nella più improbabile.

Grazie al Prof. Emanuele Montomoli, per l'opportunità di crescita personale, le esperienze e la fiducia datami in questi tre anni.

Un ringraziamento particolare va a tutti i "Compagni/Amici/Colleghi/Collaboratori/Dottorandi/Dottorande /Tesisti/Tesiste/Stagisti/Stagiste" di VisMederi ed EpidMol (Claudia, Ilaria, Damiano e Serena) che hanno saputo rendere questi tre anni più leggeri e spensierati… grazie per gli aiuti, gli incoraggiamenti e gli scoraggiamenti, la fiducia e la sfiducia, gli sguardi di comprensione e incomprensione, per i sorrisi spensierati, per le cene del "Mitico Staff VisMederi"…grazie a Fabrizio per aver condiviso a pieno questi tre anni….dalla Norvegia all'Inghilterra passando per Acquapendente… per le corse selvagge e per aver sempre la battuta pronta anche nei momenti peggiori…A Ciano (detto Giacomo) amico di una vita ed ai nostri caffè mattutini (è Prutuu)…alle "Tesiste… ora Dottorande" Marta e Francesca per le cene-pizzate spensierate!!!.... Grazie alla "Spanish" per la condivisione dei viaggi mattutini, delle corse e per il suo essere "Spanish" in tutto!!!...Ad Alessandro per gli ottimi consigli in Lab, al gruppo MATS Elena e Giulia (e chiaramente al Meningo)… a Laura per il suo caos entropico "ordinato" della scrivania... A Donata per il suo essere sempre fantasticamente Stonatz ☺(Davveroooo)…a Valerio per la simpatia…A Edoardo per le elocubrazioni scientifiche sempre a portata di foglio… A Licia per la comprensione ed il suo essere sempre tranquillamente tranquilla…A Giulia L. per i silenzi… che valgono più di mille parole, a Chiara C. con cui ho condiviso i miei primi esperimenti di HAI, a Elisa (Menni) …con B..NET sempre nel cuore…a Silvia che ha sempre la risata esplosiva pronta... al gruppo ALIMENTI (Sara, Irene, Claudia e Serena) per tenere sempre sotto controllo l'acqua che beviamo e il cibo che mangiamo…(puzze nel corridoio a parte!!!)… al gruppo "Qualità" Primo ed Emilia per indirizzarci sempre sulla retta via documentale… all'AMMINISTRAZIONE Sara P., Laura B., Giulia A. e Simone (Paletz) per la simpatia, per il riuscire sempre a mettere ordine ai nostri DIS-ordini, per la spensieratezza, l'aiuto quotidiano e gli assoli di chitarra blues!!!