COMPARING THE IMMUNOGENICITY OF INFLUENZA VACCINES LAIV AND IIV IN PEDIATRIC PATIENTS DURING THE 2014-2015 SEASON

by

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ABSTRACT

Influenza virus causes a contagious respiratory illness in humans that poses a major public health threat, especially to vulnerable populations such as children and adolescents, who have less mature immune systems. Influenza infections are responsible for over 200,000 hospitalizations and over 3,600 deaths a year. The influenza vaccine is the best form of prevention from influenza illness due to the vaccines ability to elicit an antibody response without causing illness. Currently, there are two types of influenza vaccines available to children and adolescents, the inactivated influenza vaccine (IIV) and the live attenuated influenza vaccine (LAIV). The present study was designed to compare the immunogenicity of the two vaccines in pediatric participant's ages 3 to 17 years. We hypothesized that the LAIV vaccine would produce an increased breath and enhanced antibody response to influenza vaccination compared to the IIV vaccine. Results from this study suggest that IIV was superior compared to LAIV at eliciting immune responses following vaccination. However, potential limitations include the fact that current assays measure immune responses in peripheral blood only and may not reflect mucosal immunity. Further, due to issues with the H1N1 virus strain in the LAIV vaccine, these results need to be confirmed in a season where LAIV vaccine is immunogenic (2015-16).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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1.0   INTRODUCTION

Influenza virus contains a segmented negative-sense RNA genome and belongs to the family *Orthmyxoviridae* [1, 2]. This virus is a zoonotic pathogen that infects both humans and animals. Influenza season for humans generally begins in October and ends in March of the following year, with peaks in infections in December and February [3]. In humans, influenza virus manifests as a highly contagious, respiratory illness that is transmitted person to person by droplets in the air (coughing, sneezing or talking up to six feet away), or by touching a surface or object that is contaminated with virus and then touching their mouth, nose or eyes [4]. It can also be transmitted from birds to humans via a mammalian intermediate host [4]. Influenza virus affects everyone in the population, generally causing mild illness that does not require medical intervention [5]. In children, adults over age 65, and those with certain medical conditions, influenza virus can cause serious illness leading to hospitalization or even death [5]. In the Unites States, this virus is responsible annually for over 200,000 hospitalizations and 3,600 deaths, and is responsible for the most deaths by a vaccine-preventable disease [6-8] .

Influenza virus can be treated with anti-viral medications, but these medications are not often used in a preventative manner. Some antivirals such as Tamiflu can be used prophylactically in instances of an outbreak in the community or if someone in your house is sick and you want to try to prevent transmission. Currently, vaccines are the best prevention method for influenza virus infection and there are two types of vaccines are available: the live attenuated

influenza vaccine (LAIV) and the inactivated influenza vaccine (IIV) [6]. LAIV is administered intranasally and is recommended for people ages 2-49 years, whereas IIV is administered intramuscularly and is recommended for people ages 6 months and older [9]. Currently there is no official preference for LAIV over IIV; although children have generally been administered LAIV due to the presumption that a live attenuated vaccine will result in longer lasting and increased breadth and maturation of the immune response. The Center for Disease Control and Prevention (CDC) stresses the importance of an annual influenza vaccine for everyone over the age of 6 months.

1.1   INFLUENZA VIRUS

Influenza viruses are classified as type A, B or C. Type A viruses are classified according to their surface glycoproteins, hemagglutinin (H or HA) and neuraminidase (N or NA) [1, 3]. Currently there are 18 H (H1-H18) and 11 N (N1-N11) identified subtypes for the influenza A virus [2, 3]. The most common circulating human strains of influenza A in the Unites States are H1N1 and H3N2 [3]. Influenza A viruses are able to cause both epidemics and pandemics. Type B influenza viruses are known to cause seasonal epidemics, and different linages are utilized in the creation of vaccines. Type C viruses only cause mild respiratory issues and are not known to cause epidemics or pandemics. Therefore, type C influenza is not used in preventative medicine such as vaccines [8].

Influenza A and B viruses are named according to their type, geographic origin, strain number, and year of isolation. Influenza A virus is further classified by subtype, and this subtype is how we typically refer to the virus (i.e. H1N1) [3]. An example of a type A virus

strain would be: A/California/7/2009(H1N1) (Figure 1). Influenza B is not divided into subtypes like type A, instead they are classified and referred to by lineage. An example of a Type B virus would be B/Brisbane/60/2008(B/Victoria lineage) [3]. A culmination of different influenza A and B viruses are used in seasonal vaccines to prevent against predicted circulating strains each year. Predictions of future circulating strains are based off of patterns from previous years [10].

A/ California/ 7/ 2009 (H1N1)

Virus Type/Geographic origin/Strain #/Year of Isolation/Virus subtype (A)

Figure 1. Influenza Nomenclature.

This figure depicts how influenza A and B viruses are named. The virus is named according to the 1) virus type (determined by the nuclear material), 2) geographic origin (where the virus was first isolated) 3) specific stain number, and 4) year of isolation (representative of the year the stain first emerged). Influenza A viruses are also identified by their virus subtype using the two surface proteins (Hemagglutinin and Neuraminidase).

Influenza virus variations are due to the virus poor replication skills. Influenza virus replication is inherently error-prone, resulting in mutations that arise as a result of the virus replication cycle. Thus, over time, we have seen an evolution of influenza variants emerge. Influenza virus initiates infection by the attachment of the hemagglutinin protein on the surface of the virus binding to the sialic acid receptors on the surface of human respiratory tract cells. Once attached, the influenza virus is able to enter the cell via endocytosis. Once inside the cell, the virus genome is released, and is transported to the cell nucleus for replication of the RNA genome into a DNA template. The newly created DNA genome segments are transported into the cytoplasm, where they are translated into the proteins needed to create a new progeny virus. The newly produced viral proteins assemble at the cell membrane where they are released from the

cell. This cleavage even involves release of the hemagglutinin and sialic acid receptors by neuraminidase, producing new progeny virions that are free to infect neighboring cells [8].
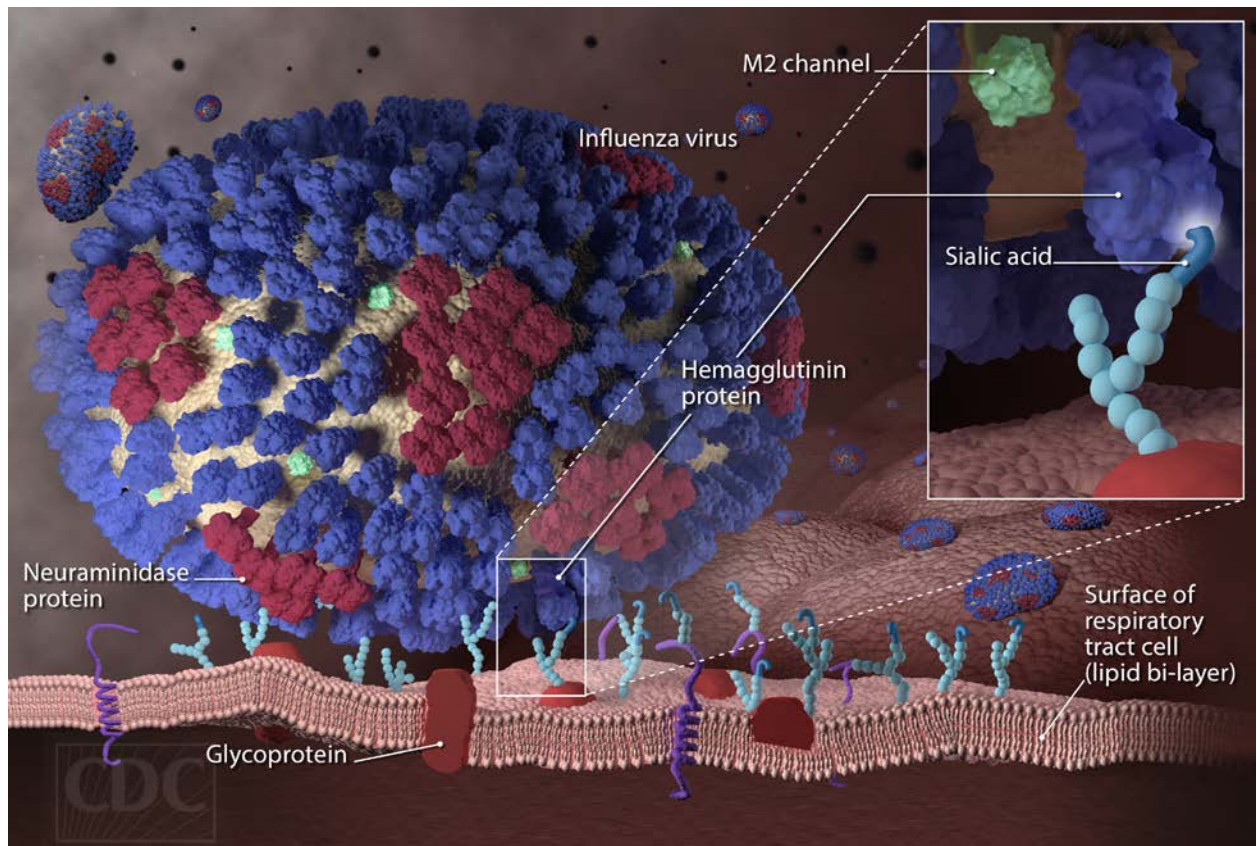
Figure 2. Influenza virus binding to respiratory tract cells[3].

The influenza virus attaches to the respiratory track via the glycoprotein on the surface of the virus. The hemagglutinin binds to the sialic acid on the respiratory track and is then endocytosed into the cell.

1.1.1   Antigenic Shift and Drift

Variations in the H and N of influenza viruses are commonly referred to as antigenic shift and drift, are what cause epidemics and pandemics, respectively. Antigenic drift refers to frequent minor errors in virus replication, known as point mutations, on the H and N surface glycoproteins of the virus. These mutations lead to epidemics in a population, mainly the result

of the naivety of the immune system of the infecting population [2, 10]. Antigenic shift occurs through major changes to the H or N proteins after reassortment of two or more virus genomes leading to new subtypes and is responsible for pandemics. These shifts in surface protein are due to an exchange of genetic segments between different virus strains, and generally involve the recombination of strains from an animal host with one from a human. While recombination events may occur in theory within a human host, those to date that have been responsible for the historical pandemics have been the result of avian and human or avian and avian recombination events. [10]. Influenza pandemics are introductions of new viruses from animal into human species, such as from birds or pigs. Pandemics stretch widely across a geographic region, or even a continent, affecting higher proportions of a population over a short period of time.

Pandemics have been of particular concern due to zoonosis which is the ability of avian influenza strains to jump species and now infect the human population. An influenza A virus originating in birds cannot directly infect human cells due to the lack of the avian-specific receptors. The same is true for influenza A viruses in humans being segments of both viruses [10]. However, through an intermediate host that has receptors for both species, the two virus strains are able to reassort and create a new virus strain. For example, pigs have receptors for both avian and human virus stains in their throats, allowing reassortment of the genetic material to create a new virus that can infect the human population [10].

1.1.2 Epidemiology and Transmission

Influenza has been plaguing the human population for over 6,000 years and it wasn't until the 1930's that humans discovered what was causing these disease [11, 12]. Researchers believe that humans inherited influenza infection following the domestication of animals and the

establishment of organized settlements. The close quarters of the settlements with livestock allowed the influenza virus to easily jump species from birds to humans, through pigs as the intermediate host [11, 12].

The first documented influenza pandemic was in the 1580s [11, 12]. This pandemic spread from lower Asia to Northern Africa. The disbursement of those infected by the virus was speculated to be due in part to troops' migration to fight the Dutch in the Spanish Netherlands. In that period, people did not know that an influenza pandemic was occurring, but were overwhelmed with the number of people falling ill with the disease.

In the 1700s it was believed that influenza outbreaks were caused from miasmas (unpleasant odors) or bad air [11, 12]. Later, it was hypothesized that it was bacteria, not miasmas, that caused influenza outbreaks [13]. There were major influenza epidemics recorded between 1830 and 1848. The 1830-1831 influenza epidemic may have originated in China, then advanced westward out of Russia into Europe in 1833. In 1836-1837, influenza spread southward, and in 1847 it swept through the Mediterranean to Southern France and into the rest of Western Europe [12]. The first pandemic of the modern world occurred in 1889-1890, most likely due to advancements in transportation and trade (travel by train and boat). This pandemic was first recorded in St. Petersburg, Russia and quickly spread throughout the Northern Hemisphere. The virus had a high infection rate (25% of the population) and rarely caused death [12].

The 20th century brought about the most devastating influenza pandemics, killing more people worldwide than the Great War (~50 million people) [13]. The most notable pandemic in the US began in 1918 and was the subtype H1N1. The virus was of avian origin and spread to other parts of the world including Europe, and Southern and Eastern Africa and Asia. This

pandemic was especially devastating to the younger population [11, 13]. The pandemic was referred to as the "Spanish flu" because during World War I, the virus spread from France to Spain, at which time it was first reported. At the time, Spain was a neutral country and was able to freely report the outbreak of the influenza infection. Other countries did not have the same freedom of press and were unable to report the pandemic in a timely manner. Although it is speculated that the 1918 influenza pandemic originated in British army camps in Europe, it will forever be known as the Spanish Flu. In the 1930s, while conducting studies on pigs, scientist were able to isolate influenza and discovered the true cause of influenza outbreaks were due to a virus, not a bacteria like previously hypothesized [11, 13]. Two more notable pandemics followed in 1957 (H2N2) and 1968 (H3N2). The H2N2 pandemic, otherwise referred to as the Asian flu pandemic, originated in China in early 1956 and lasted until 1958. It spread from China to Singapore early into 1957, then to Hong Kong and the US by June of that year. Total deaths approached 70,000 in the US, with deaths worldwide estimated to be from 1 to 4 million. Today, this strain is extinct, but the Asian flu strain evolved via antigen shift into H3N2 and caused a milder pandemic from 1968-1969. Both pandemic strains clearly arose from reassortment between human and avian viruses (compared to the 1918 strain, which was entirely avian) [11]. Influenza pandemics of the 20th century exceeded a total death toll of one million worldwide [2].

In March of 2009, the first pandemic of the twenty-first century emerged as an H1N1 virus. Air travel provided the accessibility needed help the virus spread very quickly and WHO recognized this virus as the most widespread pandemic after it was identified on at least two different continents [2]. This pandemic seemed to have the greatest effect on children and young

adults. Researchers speculated that the older population had some immunity due previous exposure to the 1957 epidemic [2].
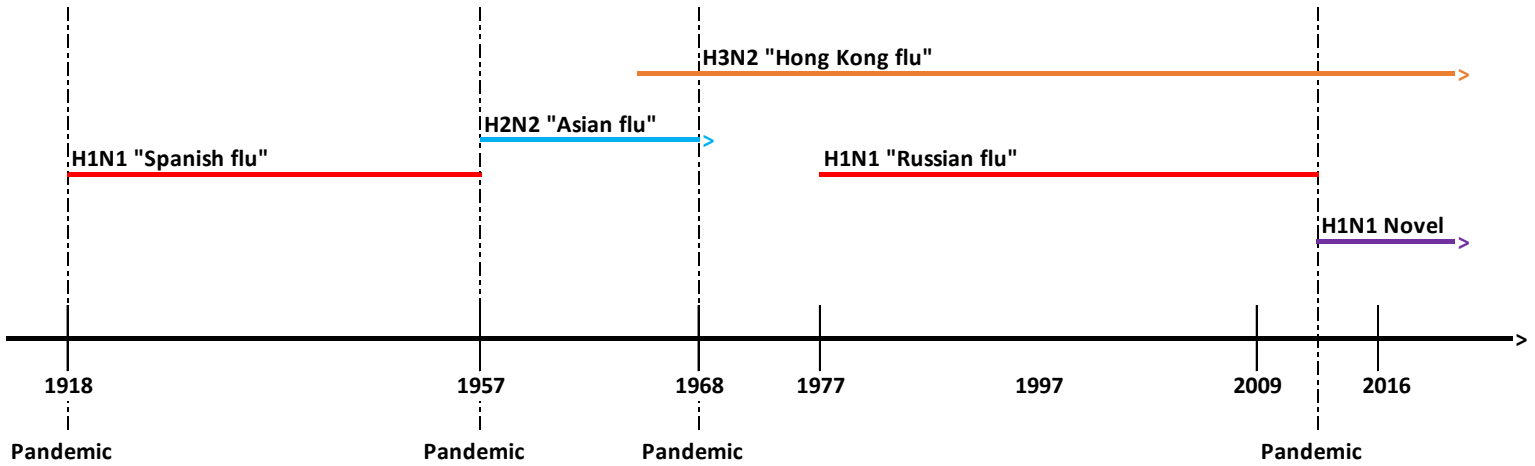
Figure 3. Pandemics of influenza.

Timeline for major flu pandemics from 1918 to 2016. Virus strains with arrows indicate the virus strain is still in circulation.

1.1.3 Pathogenesis

Influenza virus infects many species, including birds, pigs, and humans. In birds, the virus replicates in the digestive tract and is evacuated through the feces [10]. In pigs the virus replicates in the through and is transmitted through close contact such as touching noses and dried mucus [14]. In humans, influenza virus infects the upper and lower respiratory tract via inhalation of infected aerosol droplets or through saliva [10]. Symptoms of influenza include: respiratory tract infection, fever, myalgia, sore throat, and cough, all of which are elicited due to

cytokine responses to infection [10]. The influenza virus has an incubation period of one to two days, and an infected individual is able to transmit one day prior to sign of symptoms and 5 to 7 days after onset of symptoms [5]. Children can remain contagious for up to 10 days after infection [3]. Complications with influenza, such an pneumonia, are due to loss of natural epithelial barriers during influenza infection [10]. Influenza infections generally resolve themselves within a week thanks to the innate and acquired immune responses. However, ~3,600 people annually in the United States do not recover and die from the disease [8].

1.2   TREATMENT AND PREVENTION

1.2.1   Treatment

Antiviral drugs can be given to those already infected with the influenza A or B virus, or can be taken as a prophylactic if a known outbreak of influenza occurs in the community. There are currently two different types of FDA-approved antivirals on the market, neuraminidase (N) inhibitors and matrix 2 (M2) channel Inhibitors [8]. N inhibitors, including zanamivir and oseltamivir, block the neuraminidase enzymes synthesized by the influenza virus, interfering with the viruses' ability to release virions from the cell [10]. Oseltamivir is administered orally and zanamivir is administered via inhalation. If taken within 48 hours there can be a reduction in influenza-related symptoms by approximately one day [10]. Examples of M2 channel inhibitors are amantadine and rimantadine. Once the virus is inside the host cell, these drugs block the transmembrane M2 ion channel proteins that are responsible for the uptake of protons, rendering the virus unable to release its genetic material into the cell for replication. Today, M2 channel

inhibitors are not used much due to the rapid development of resistance; N inhibitors are slightly better but, still seeing resistance [15].

1.2.2 Prevention

Vaccines are the most effective line of prevention against seasonal influenza viruses [9]. Vaccines allow the body to produce specific antibodies and T cell responses so that later exposure to some influenza viruses result in protection from the virus or less severe symptoms through immune memory stimulation [10]. The American Academy of Pediatrics (APA) along with Center for Disease Control and Prevention (CDC) recommend that people ages 6 months or older be vaccinated with the seasonal flu vaccine annually to help reduce the spread of influenza virus [9].

Currently, there are two types of vaccines administered to prevent influenza, the live attenuated influenza vaccine (LAIV) and the inactivated influenza vaccine (IIV) [9]. These vaccines provide protection from the influenza virus by exposing the immune system to a specific influenza strain [10]. When virus infection is first detected by the immune system, innate immune response develops rapidly and controls virus during the early stages of virus infection. This involves recognition of foreign antigen through mechanisms that are not antibody-specific. However, innate immunity also involves the secretion of cytokines that serve to stimulate and recruit cells involved in the adaptive immune response. When immune memory is present (either from prior vaccination or infection) the antigen-specific adaptive response is faster than in patients seeing the antigen for the first time (primary adaptive immunity). Adaptive immune responses involve generation of antigen-specific B cell responses that secrete antibodies to neutralize, prevent virus from infection naïve cells[10]. Alternately, activation of T lymphocytes

10

occurs when antigen is taken up and presented in the form of viral-derived peptides to T lymphocytes by antigen presenting cells. Through the production of cytokines, T helper cells contribute to B cell proliferation and differentiation to plasma cells, activation and proliferation of virus-specific cytotoxic T lymphocytes (CTLs) leads to killing of virus infected target cells[10]. Activation of the adaptive immune response occurs through peptides derived from viral proteins, which are presented on antigen-presenting cells to the T lymphocytes. Helper T cells, through the production of cytokines, contribute to B cell proliferation and differentiation to plasma cells, and to the activation and proliferation of virus-specific cytotoxic T lymphocytes (CTLs).

Both types of vaccines, LAIV which is a live, replicating virus, and IIV, which is a mixture of viral proteins, are capable of eliciting immune memory in patients following vaccination. By eliciting and stimulating immune memory through vaccination, it is presumed that patients, if and when exposed to influenza later in the season, will either not get infected or have a lessened infection and disease course. [10].

The LAIV vaccines contains live virus that cannot grow well in a host, but replicates enough to elicit an immune response [10]. The viruses in this vaccine are made less virulent by heating the virus at nonphysiological temperatures during propagation [10]. This vaccine is administered intranasally and is approved for people 2-49 years of age [9]. LAIV vaccines can contain either three (trivalent) or four (quadrivalent) virus stains. The quadrivalent LAIV vaccine contains two strains of influenza A virus and two types influenza B virus. There is no influenza C virus in vaccines. For example, the quadrivalent LAIV vaccine used in the seasonal 2014-2015 vaccine as part of this study contained: A/California/7/2009 (A/H1N1),

A/Texas/50/2012 (A/H3N2), B/Massachusetts/02/2012 (B/Yamagata lineage), and B/Brisbane/60/2008 (B/Victoria lineage).

IIV vaccines contain viruses that have been "killed" by chemical or physical means. In the case of the quadrivalent IIV vaccine used in this study by Sanofi, the virus is produced and inactivated by a combination of heat and formaldehyde x 2 iterations, followed by purification to remove the chemicals and concentrate the virus. Finally, the viral membrane is disrupted so that all viral proteins are available to the immune system upon vaccination. This preparation is therefore noninfectious and free from harmful reagents. [10]. The IIV vaccine is administered intramuscularly and is approved for anyone over the age of six months [9]. Similar to LAIV, IIV can be trivalent or quadrivalent. LAIV and IIV vaccines have identical virus strains that change seasonally.

The major limitation to the current vaccine strategy is due to antigenic shift and drift, [10]. Each year a new vaccine is created based off a prediction of the circulating strains for the prior year. Beginning in January of each year, the World Health Organization (WHO) and experts from 82 different countries get together and decide what circulating stains should be placed in the vaccine for the upcoming flu season. This decision on strain is made while still in the midst of the current influenza season and 9 months prior to the next vaccination season. Thus is partially relies on prediction of what strains will circulate in the next season. For this reason, there is never a guarantee that the vaccine will be completely matched to the next season strains. [16]. Once decided, the predicted strains are sent to the vaccine manufactures that then make and test the vaccine for distribution to the public. Vaccines are produced and ready to distribute for the influenza season by late August to early October [16]. Clearly, new and faster methods for vaccine production are needed to improve this process.

Vaccines are also analyzed each year for their effectiveness, measuring how well the influenza vaccine protected against the influenza illness. Effectiveness of the influenza vaccines are determined annually by the CDC utilizing randomized control trials [3]. Two main factors contribute to the effectiveness of the influenza vaccine, 1) the characteristics of the person being vaccinated (age and current health conditions) and 2) how closely the vaccine strains match with the circulating viruses. The protective benefit of the influenza vaccine is low in years when the vaccine does not match the circulating stains, however there is still some protection [3]. During influenza seasons where the vaccine is a close match to the circulating viruses, protection averages between 50-60% among the overall population [3].

1.3   PUBLIC HEALTH SIGNIFICANCE

In the United States, seasonal influenza virus infections are the highest cause of death from a vaccine preventable disease [7]. Influenza infections are highly contagious, especially in our vulnerable populations, such as children/adolescents, and are responsible for more than 200,000 hospitalizations and 3,600 deaths each year [6, 7, 10]. In the United States, Direct medical cost associated with influenza virus annually averages ten billion dollars [17]. Overall, Influenza virus-attributed economic costs annually in the United States average 87.1 billion dollars [17]. This overall burden can be greatly reduced through prevention by vaccination. There are currently two types of influenza vaccines on the market; LAIV and IIV. These vaccines can aid in reducing a person's risk for disease and reduce transmission. The financial burden endured due to time lost to work and cost of medical bills can all be reduced with the low cost of the influenza

vaccine. Each year a new influenza vaccine is created predicating circulation virus strains for the next influenza season. Therefore ensuring annual vaccination of all able people ages 6 months is imperative in producing herd immunity to protect those who are unable to be vaccinated.   Knowing which virus strains and vaccine type are most efficient in a certain population is pivotal to the reduction of influenza Infections.

2.0   STATEMENT OF PROJECT

Determination of the most effective seasonal influenza vaccine and providing the vaccine to the population at large can reduce the number of cases of influenza infection and death annually. This in turn reduces the significant health care burden and costs associated with influenza. The goal of this study was to determine whether LAIV would produce an increased breath and antibody response compared to IIV vaccination in children 3-17 years of age. Historically, we can use hemagglutinin antibody inhibition (HAI) assay and microneutralization (MN) assay to measure antibody titers in children pre and post seasonal influenza vaccine inoculation to determine if vaccination elicited an immune response capable of generating immune memory. *Our hypothesis was that LAIV would produce an increased breadth and enhanced antibody response to circulating influenza strains in comparison to IIV in a cohort of children, ages 3-17 years old.* This hypothesis was tested using the following specific aim:

Aim 1: To compare antibody responses elicited by LAIV and IIV vaccination in pediatric patients, ages 3-17 years old. To address this aim, an HAI assay and an MN assay were optimized for use with children's serum samples. The HAI assay can detect antibody titers to virus in human serum. This assay can verify if antibodies have been created [10].   The MN assay measures the highest dilution of antibodies that neutralize virus replication, which is

important in understanding the antigenic structure of the virus and the immune response elicited

[10].

3.0   METHODS

This study was sponsored by the Centers for Disease Control and Prevention (CDC) and was initiated after considering preferential recommendation of influenza vaccination using the LAIV over the IIV in children 2 to 8 years of age. The University of Pittsburgh worked in collaboration with the Children's Hospital of Pittsburgh Primary Care Office, UPMC Shadyside Family Health Center, and UPMC Lawrenceville Family Health Center to collect specimen samples.

Following Institutional Review Board (IRB) consent, and receiving protocols from the CDC, all experiments for this study were performed in the biosafety level 2+ laboratory in the Center for Vaccine Research (CVR) at the University Of Pittsburgh. All participants in the study received quadrivalent LAIV or IIV and had blood drawn on three separate time points: Day 0 (prior to vaccination) Day 7, and Day 21 following vaccination. Blood samples were processed for serum, plasma, and PBMC. Processing was performed under a class II biosafety cabinet using vesphene for decontamination. Once all samples where processed they were sent to CDC Contracting Lab, Battelle, Inc., and CDC for analysis.

3.1   STUDY PARTICIPANTS

All participants in this study were recruited from August to December of 2014, from the Children's Hospital of Pittsburgh Primary Care Office, UPMC Shadyside Family Health Center, or UPMC Lawrenceville Family Health Center, Pittsburgh, PA. Recruitment methods included letters, phone calls, in person recruitment, and flyer/poster advertisements. Those contacted by letter were sent inclusion criteria along with study activities and numbers to contact if interested. Anyone who was contacted by phone or in person was read a script explaining the participant's rights upon involvement in the study and questions regarding inclusion and exclusion. These surveys were then sent to the appropriate research assistant for potential enrollment. Children between the ages of 3 and 17 years were recruited for the study and underwent a health evaluation to determine eligibility. If applicable, children were given a unique identification number and segregated into groups based on age (3-8 years vs. 9-17 years). Children were further segregated based on the chosen vaccine type (LAIV vs. IIV).   There were 173 participants recruited, 23 of which did not complete all three visits required for the study. Of the 150 participants that finished the study, there were 60 participants for the 3 to 8 years age group; 20 participants chose to receive quadrivalent IIV and 40 participants chose the quadrivalent LAIV (shown in Table 1). Ninety participants were recruited into the 9-17 year age group; 33 chose to receive quadrivalent IIV and 57 chose the quadrivalent LAIV.

Table 1. Pediatric Patients Recruited for the 2014-2015 Vaccine Study.

	2014-2015 Vaccine	Completed Day 0	Dropouts	Total
3-8 YEAR OLDS	LAIV	40	8	48
	IIV	20	7	27
9-17 YEAR OLDS	LAIV	57	23	80
	IIV	33	0	33
Total Participants:		**150**	**23**	**173**

3.1.1   Inclusion

Eligible participants for the study had to meet several requirements. Participants must be between the ages of 3 to 17 upon enrollment, not turning 18 prior to completion of the required3 visits; willing to receive the seasonal influenza vaccine for the 2014 year at one of the study enrollment sites; and meet all inclusion criteria as listed in the IRB. Participants were given the choice of which quadrivalent vaccine they would like to receive, LAIV or IIV. Participants also had to provide documentation of the vaccine they received the previous year (2013-14 influenza season).

3.1.2   Exclusion

There were also several criteria that excluded children from participating in the study. These included any participant/parent unable or unwilling to complete all required study activities, such as informed consent paperwork; or participants that were known to have a compromised immune system, or that were taking immunosuppressing medication such as steroids. If children had not participated in the 2013-2014 vaccination study, or had already received a vaccine for 2014-2015 season, they were unable to be enrolled in the study. Other

exclusions from the study included not meeting the weight requirement (<17kg), pregnancy, or an allergy to influenza vaccines.

3.2   BLOOD COLLECTION

Once a child was deemed eligible for the study, a blood sample was taken at the enrollment site prior to vaccination (Day 0). Two more blood samples were taken from the participants on Day 7 and Day 21 post vaccination for a total of 3 blood draws per child. The blood samples, labeled with a unique identifier, were then sent to the Center for Vaccine Research (CVR) within four to six hours of each blood draw for further sample extraction. Blood samples were processed for plasma and peripheral blood mononuclear cells (PBMC) from D0, D7, and D21 blood draws. Serum from D0 and D21 blood draws were collected using a special separation tube.

3.3   BLOOD PROCESSING

3.3.1   Peripheral Blood Mononuclear Cells (PBMCs) and Plasma

Pediatric blood samples were sent to the CVR twice daily from different recruitment sites in Pittsburgh for processing. Blood draws from pediatric patients in the study were conducted during normal business hours Monday through Friday. Samples were delivered to the CVR in the morning (twelve o'clock pm) and afternoon (5 o'clock pm) from August 2014 through

January 2015. Each blood tube was checked and documented for time of draw (to ensure the draw had occurred within the last four hours), blood draw time point (day 0, 7, or 21), and participant identification number. If a sample had been drawn from a patient more than four hours prior to processing, centrifugation time of the sample was be increased. After verification of the blood sample information, cryo tubes for serum, plasma, and PBMC were labeled with the following: study name (Pitt Option C), participant ID number, blood draw time point, and date.

In order to ensure the highest level of purity and viability, the CVR followed the standard operating procedure approved by the CDC for all processed blood samples. PBMC and plasma were isolated from whole blood by density gradient centrifugation. To start, the blood samples were received in BD Vacutainer CPT Tubes; these tubes were balanced and centrifuged at 1600xg for 20 minutes with no brake. After centrifugation four layers could be recognized: plasma, PBMC, a gel barrier, and erythrocytes and neutrophils (Figure 4). Three 1mL aliquots of the top plasma layer were pipetted into three labeled cryo tubes. Any extra plasma was pipetted out and discarded into waste consisting of a 50% bleach solution. The cryo tubes containing plasma where then placed in the -80°C freezer for storage.

The PBMC layer was carefully pipetted out and place into a 50 mL conical tube. The conical tubes were then brought to total volume with sterile PBS and centrifuged at 500xg for 15 mins at room temperature. Following certification, the supernatant was discarded and the cell pellet was resuspended with 1 mL PBS. Once the pellet was resuspended, PBS was added to bring the volume of the conical tube up to 50 mL, and the sample was centrifuged again for 15 mins at 500xg. This wash process was repeated two more times. After the third centrifugation, the pellet was resuspended in 10 mL of PBS and 20 µl were used for cell counting. The conical tube was brought to a total volume of 50 mLs with PBS and centrifuged for the last time.

While the sample was in the centrifuge, the 20µl cell sample was counted using a hemocytometer. The 20µl sample was mixed with 0.4% trypan blue solution to make a 1:2 dilution. The cells were then counted using as hemocytometer; all four outer quadrants were counted, the average of the four quadrants was recorded, and that final number represented the number of cells (N) x 10^4/ml in the sample. This number (N x 10^4/ml) was multiplied by the dilution of trypan blue used (2) and the total volume of cells (10 mL) to obtain the total number of recovered PBMCs. This final number was used to calculate the number of aliquots to be frozen, which would contain between 0.5-1.0 x 10^7 cells/ml. After the final centrifugation, the supernatant was removed and the PBMCs were resuspended in freezing media (90% FBS/10% DMSO). Aliquots of 1 mL were pipetted into labeled cryovials (approx. 1-3 per participant per time point) and placed into a Mr. Frosty freezing chamber in a -80°C freezer for slow freezing. Cells were keep in the -80°C freezer for no more than 72 hours, at which time they were transferred to a liquid nitrogen tank for long-term storage. Once all the pediatric blood samples were processed for plasma and PBMC they were catalogued, organized numerically by participant according to the time points, and sent to the CDC or a reference lab for analysis.
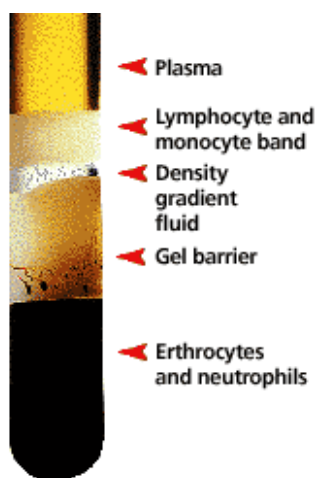
Figure 4. CPT tube separation[18].

Four layers recognized after centrifugation: plasma, PBMC, a gel barrier, and erythrocytes and neutrophils

3.3.2   Serum

An individual blood tube was collected in BD Vacutainer ™ Venous Blood Collection Tubes: Serum Separator Tubes on D0 and D21 for the sole purpose of isolating serum. These tubes had a polymer gel layer to separate serum from red blood cells. Serum samples were also isolated by centrifugation. The serum tubes were centrifuged at 1800rpm for 10 minutes. The top serum layer was removed and aliquoted (500µl each) into four labeled cryovial tubes. Serum aliquots were then placed in the -80°C freezer for storage. Once all the pediatric serum samples were processed, they were catalogued, organized numerically by participant according to the time points, and sent to the CDC or a reference lab for analysis.

3.4   ANALYSIS

3.4.1   Hemagglutination Antibody Inhibition (HAI) Assay

Processed sera were sent to a CDC contracting lab, Battelle, Inc., where they were analyzed using HAI assays. This assay measures the binding ability of surface protein HA on influenza virus to the sialic acid receptors on red blood cells. If an antibody binds to the HA on the influenza virus, then binding of HA to the sialic acid receptors is blocked, and hemagglutination occurs. The assay was performed using day 0 and day 21 sera samples, with erythrocytes from poultry (chicken or turkey).

A round-bottomed 96-well plate was prepared by pipetting 50 µl of PBS to each well rows B-H; Row A had 100µl of virus. 50ul aliquots of virus were added to columns B through H. A 2-fold serial dilution by transferring 50µl of solution from column A to B, changing tips, mixing then removing 50 µl from B and transferring to C, etc. down the plat to column H. To keep total volume the same amongst wells, 50µl was discarded from row H. The 96-well plate was then incubated for 30 minutes at room temperature. A 0.5% Turkey red blood cell (TRBC) solution (50 µl) was added into all wells and the plate was incubated for another 30 minutes. Following incubation, the wells were observed for the appearance of red dots (hemagglutination inhibition) or a diffused reddish color (hemagglutination). Hemagglutination inhibition occurred if antibodies were present in the serum samples. A diffused reddish color indicated that antibodies were not present and hemagglutination was able to occur. According to the CDC, HAI assay data can be used as a correlate of protection, but this does not necessarily mean a person will be protected from influenza infection.

3.4.2   Microneutralization (MN) Assay

Serum samples were also sent to the CDC for analysis by MN assays. This assay was used to measure the breadth of antibody response elicited by influenza vaccination and measured antibody neutralization to virus strains in the vaccines. The neutralization assay measures two-fold dilutions of heat-inactivated serum incubated with virus prior to infection of a cell line.

For this assay, Serum was heat inactivated at 56°C for 30 minutes. A sterile 96 well dilution plate was used for the dilution of sera and incubation of sera + virus. A representation of the sera-virus dilution plate can be seen in Figure 3. For the sera dilutions, 50µl diluent

24

(media) was added to each well rows B-H; Row A had 90μl. 10ul aliquots of sera were added to columns 1 through 11, row A; Column 12 wells were used as virus and cell controls for the study (Figure 3). A 2-fold serial dilution by transferring 50μl of solution from column A to B, changing tips, mixing then removing 50 μl from B and transferring to C, etc. down the plat to column H. To keep total volume the same amongst wells, 50μl was discarded from row H.
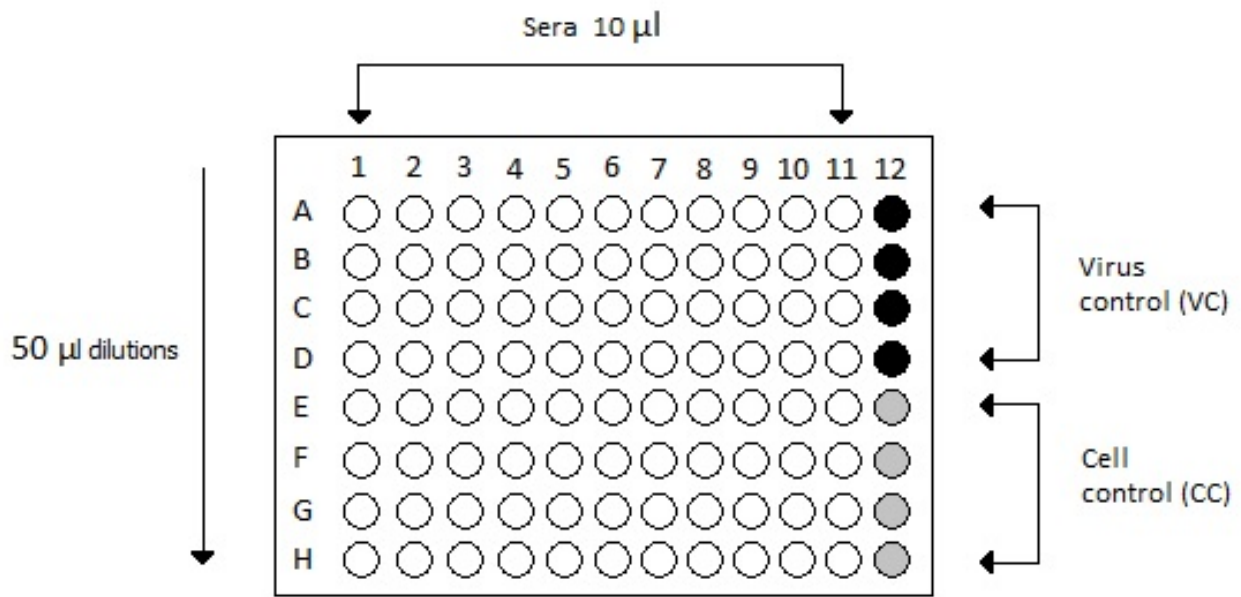
Figure 5. Virus microneutralization assay plate set up.

Heat-inactive serum was added to the top row of a 96 well plate (A1-A11) and A 2-fold serial dilution of the serum was then conducted (i.e. A1toB1; B1 to B2; etc. down to G1 to H1)

Virus (50 μl) was then added to row A (wells 1-11). Column 12 was reserved for controls and the same controls were used for each plate in the assay. After the serum dilutions were completed, 50μl diluted virus (at TCID50 to achieve MOI = 0.1) was then added to control wells. Serum-virus mixtures were incubated for 1h at 37C.

For these assays, we utilized a special cell line, Madin-Darby Canine Kidney-Sialic acid over expression (MDCK-SIAT1) cells because some of the virus strains required increased levels of sialic acid receptors for the ability to infect in vitro. These cells (100μl) were added to each well of the plate and the plate was incubated for 18-20 hours at 37 °C. The titers for the assay were the reciprocal of the highest dilution of serum that yielded 50%.

4.0   RESULTS

4.1   AIM: TO COMPARE ANTIBODY RESPONSES ELICITED BY LAIV AND IIV VACCINATION IN PEDIATRIC PAITIENTS, AGES 3-17 YEARS OLD.

4.1.1   HAI Assay Results

HAI data was analyzed for antibodies to vaccine virus strains: influenza A viruses (A/California/7/2009 (H1N1) and A/Texas/50/2012 (H3N2)) and influenza B viruses (B/Brisbane and B/Massachusetts). Titer levels were measured for D0 and D21 from processed serum samples. Below are tables displaying the results of the HAI assay conducted (Tables 2-5). For this study, the titers were defined as the reciprocal of the last dilution of serum that completely inhibited hemagglutination.

An increase in titer of 4-fold or higher between D0 and D21 sera was considered to be a seroconversion; smaller increases in antibody response were not considered to achieve a level consistent with seroconversion in the assay. Further, a reciprocal titer of 80 was considered to be non-reactive as this was at or below the cutoff for the assay. Patients that demonstrated positive antibody responses at D0 but failed to achieve the 4-fold increase in response at D21 were considered to be seroprevalent.

27

The 3-8 year old cohort receiving the IIV vaccine demonstrated an increased antibody response in fifteen out of the twenty participants, seroconverting to one or more of the virus strains measured in the assay (Table 2). Interestingly, two of the patients seroconverted to all four virus strains tested. Fourteen of the twenty patients were seroprevalent, demonstrating a wide range of reactivity at D0, but not showing a 4-fold increase in antibody titer at D21. None of the pediatric participants that received IIV were non-reactive, with all patients demonstrating antibody responses to at least one or more influenza strains tested.

Table 2**. Hemagglutination inhibition (HAI) assay data for the 3-8 year old cohort vaccinated with IIV.**

AGE GROUP	2014-15 Vaccine	H3N2			H1N1			B/Brisbane			B/Massachusetts		
		DAY 0	Day 21	Fold	Day 0	Day 21	Fold	Day 0	Day 21	Fold	Day 0	Day 21	Fold
3-8 yo	IIV	5	20	4.0	5	20	4.0	40	80	2.0	80	160	2.0
3-8 yo	IIV	80	80	1.0	20	160	8.0	10	453	45.3	160	640	4.0
3-8 yo	IIV	113	160	1.4	453	640	1.4	57	80	1.4	40	80	2.0
3-8 yo	IIV	40	160	4.0	1280	1810	1.4	40	320	8.0	160	320	2.0
3-8 yo	IIV	40	160	4.0	80	226	2.8	80	320	4.0	80	160	2.0
3-8 yo	IIV	320	320	1.0	5	40	8.0	14	160	11.3	40	320	8.0
3-8 yo	IIV	10	320	32.0	20	160	8.0	160	160	1.0	80	226	2.8
3-8 yo	IIV	10	453	45.3	5	640	128.0	5	10	2.0	5	113	22.6
3-8 yo	IIV	80	640	8.0	80	640	8.0	5	57	11.3	28	640	22.6
3-8 yo	IIV	453	640	1.4	113	320	2.8	10	40	4.0	57	160	2.8
3-8 yo	IIV	640	640	1.0	80	160	2.0	160	320	2.0	80	320	4.0
3-8 yo	IIV	320	640	2.0	80	226	2.8	10	160	16.0	160	453	2.8
3-8 yo	IIV	160	640	4.0	5	40	8.0	5	80	16.0	160	640	4.0
3-8 yo	IIV	80	640	8.0	5	80	16.0	160	320	2.0	320	640	2.0
3-8 yo	IIV	20	640	32.0	10	2560	256.0	5	40	8.0	10	320	32.0
3-8 yo	IIV	320	640	2.0	226	640	2.8	14	80	5.7	226	640	2.8
3-8 yo	IIV	160	640	4.0	160	640	4.0	57	640	11.3	80	640	8.0
3-8 yo	IIV	640	905	1.4	320	640	2.0	226	640	2.8	320	640	2.0
3-8 yo	IIV	320	1280	4.0	160	320	2.0	80	640	8.0	640	2560	4.0
3-8 yo	IIV	40	2560	64.0	10	160	16.0	80	320	4.0	80	320	4.0
3-8 yo	IIV	92	408	4.4	44	264	6.1	30	155	5.2	84	361	4.3

The virus used in the assay is listed across the top row. Reciprocal endpoint antibody titers for D0 (pre-vaccination) D21 and the fold change between the two time points are listed for each virus strain. The bottom row represents the average for each column (highlighted in red numbers). Seroconverters (green) are defined as those vaccines with at least a 4-fold increase in antibody titer from day 0 to day 21; seroprevalent (yellow) patients are defined as those vaccines with positive antibody titers at day 0 and less than 4-fold increase in titer at day 21; non-reactors are defined as those vaccines whose antibody titers were below the cutoff of the assay (< 1:80)

In contrast, 3-8 year old patients receiving the LAIV vaccine demonstrated very few seroconversions (shown in Table 3). Only 7 of the 40 vaccines had a 4-fold increase or higher antibody response, while 8 of the 40 vaccines were nonreactive. The majority of patients (26 of the 40) were seroprevalent, together, this data showed a poor antibody response to the LAIV vaccine compared to the IIV vaccine in the 3-8 year old cohort

Table 3. Hemagglutination inhibition (HAI) assays data for the 3-8 year old cohort administered LAIV.

AGE GROUP	2014-15 Vaccine	H3N2			H1N1			B/Brisbane			B/Massachusetts		
		DAY 0	Day 21	Fold	Day 0	Day 21	Fold	Day 0	Day 21	Fold	Day 0	Day 21	Fold
3-8 yo	LAIV	20	20	1.0	80	113	1.4	40	57	1.4	40	40	1.0
3-8 yo	LAIV	10	20	2.0	5	5	1.0	5	40	8.0	57	320	5.7
3-8 yo	LAIV	28	40	1.4	80	80	1.0	10	20	2.0	20	40	2.0
3-8 yo	LAIV	40	40	1.0	320	320	1.0	320	320	1.0	226	320	1.4
3-8 yo	LAIV	40	57	1.4	20	20	1.0	5	40	8.0	40	80	2.0
3-8 yo	LAIV	160	160	1.0	80	80	1.0	160	160	1.0	160	160	1.0
3-8 yo	LAIV	160	160	1.0	40	40	1.0	10	20	2.0	10	40	4.0
3-8 yo	LAIV	160	160	1.0	5	5	1.0	40	40	1.0	160	160	1.0
3-8 yo	LAIV	320	320	1.0	160	160	1.0	160	160	1.0	160	160	1.0
3-8 yo	LAIV	320	320	1.0	40	40	1.0	80	160	2.0	160	160	1.0
3-8 yo	LAIV	320	320	1.0	40	40	1.0	80	80	1.0	320	320	1.0
3-8 yo	LAIV	320	320	1.0	160	226	1.4	320	226	0.7	160	226	1.4
3-8 yo	LAIV	57	40	0.7	5	5	1.0	5	5	1.0	40	40	1.0
3-8 yo	LAIV	40	40	1.0	80	80	1.0	10	20	2.0	40	80	2.0
3-8 yo	LAIV	40	40	1.0	28	20	0.7	40	40	1.0	28	28	1.0
3-8 yo	LAIV	80	80	1.0	320	320	1.0	160	160	1.0	7	20	2.8
3-8 yo	LAIV	160	80	0.5	40	40	1.0	10	28	2.8	40	80	2.0
3-8 yo	LAIV	113	113	1.0	57	40	0.7	80	80	1.0	28	80	2.8
3-8 yo	LAIV	160	160	1.0	80	80	1.0	40	80	2.0	40	113	2.8
3-8 yo	LAIV	160	160	1.0	160	160	1.0	160	160	1.0	57	160	2.8
3-8 yo	LAIV	160	160	1.0	40	40	1.0	80	80	1.0	80	80	1.0
3-8 yo	LAIV	320	320	1.0	320	320	1.0	640	640	1.0	320	320	1.0
3-8 yo	LAIV	5	5	1.0	5	5	1.0	5	5	1.0	5	40	8.0
3-8 yo	LAIV	20	20	1.0	40	40	1.0	40	160	4.0	5	80	16.0
3-8 yo	LAIV	10	20	2.0	20	20	1.0	5	160	32.0	5	40	8.0
3-8 yo	LAIV	20	28	1.4	80	80	1.0	5	80	16.0	40	113	2.8
3-8 yo	LAIV	40	40	1.0	10	20	2.0	20	14	0.7	80	80	1.0
3-8 yo	LAIV	40	40	1.0	160	80	0.5	57	40	0.7	10	10	1.0
3-8 yo	LAIV	40	80	2.0	7	40	5.7	113	113	1.0	320	80	0.3
3-8 yo	LAIV	160	160	1.0	80	80	1.0	160	160	1.0	320	320	1.0
3-8 yo	LAIV	160	160	1.0	14	20	1.4	160	160	1.0	7	14	2.0
3-8 yo	LAIV	160	160	1.0	160	160	1.0	80	80	1.0	113	160	1.4
3-8 yo	LAIV	320	160	0.5	320	320	1.0	10	113	11.3	453	640	1.4
3-8 yo	LAIV	320	320	1.0	80	80	1.0	160	160	1.0	160	160	1.0
3-8 yo	LAIV	320	320	1.0	320	320	1.0	40	40	1.0	80	320	4.0
3-8 yo	LAIV	320	320	1.0	20	20	1.0	80	320	4.0	20	113	5.7
3-8 yo	LAIV	320	320	1.0	160	160	1.0	20	40	2.0	5	20	4.0
3-8 yo	LAIV	1280	640	0.5	20	20	1.0	5	113	22.6	5	640	128.0
3-8 yo	LAIV	640	905	1.4	226	160	0.7	10	160	16.0	5	113	22.6
3-8 yo	LAIV	100	103	1	53	55	1	38	74	2	44	98	2

The virus used in the assay is listed across the top. Reciprocal endpoint antibody titers for D0 (pre-vaccination) D21 and the fold change between the two time points are listed for each virus strain. The bottom row represents the average for each column (highlighted in red numbers). Seroconverters (green) are defined as those vaccines with at

least a 4-fold increase in antibody titer from day 0 to day 21; seroprevalent (yellow) patients are defined as those vaccines with positive antibody titers at day 0 and less than 4-fold increase in titer at day 21; non-reactors are defined as those vaccines whose antibody titers were below the cutoff of the assay (< 1:80)

Next we evaluated the 9-17 year old cohort that received the IIV vaccine. The results in the older children were similar to those observed in the younger children (Table 4). In general, children in the 9-17 year old cohort who received the IIV vaccine had a good antibody response to at least one of the four strains of virus in the vaccine. Out of the 43 participants who received IIV, there were 16 seroconverters participants, 39 seroprevalent and 17 non-reactive patients across one or more virus strain.

Table 4. Hemagglutination inhibition (HAI) assays data for the 9-17 year old cohort vaccinated with IIV.

AGE GROUP	2014-15 Vaccine	H3N2			H1N1			B/Brisbane			B/Massachusetts		
		DAY 0	Day 21	Fold	Day 0	Day 21	Fold	Day 0	Day 21	Fold	Day 0	Day 21	Fold
9-17 y	IIV	80	80	1.0	160	320	2.0	113	160	1.4	40	113	2.8
9-17 y	IIV	20	80	4.0	80	160	2.0	20	160	8.0	80	160	2.0
9-17 y	IIV	28	80	2.8	20	80	4.0	10	40	4.0	20	320	16.0
9-17 y	IIV	80	113	1.4	40	160	4.0	40	113	2.8	80	160	2.0
9-17 y	IIV	80	160	2.0	160	160	1.0	80	320	4.0	80	320	4.0
9-17 y	IIV	80	160	2.0	40	160	4.0	40	113	2.8	20	40	2.0
9-17 y	IIV	80	160	2.0	160	320	2.0	20	80	4.0	160	320	2.0
9-17 y	IIV	40	160	4.0	80	160	2.0	40	160	4.0	80	160	2.0
9-17 y	IIV	80	160	2.0	20	113	5.7	10	453	45.3	80	226	2.8
9-17 y	IIV	160	226	1.4	80	80	1.0	80	80	1.0	80	80	1.0
9-17 y	IIV	80	226	2.8	160	640	4.0	80	160	2.0	80	160	2.0
9-17 y	IIV	160	320	2.0	320	320	1.0	113	160	1.4	57	80	1.4
9-17 y	IIV	160	320	2.0	160	640	4.0	226	453	2.0	80	320	4.0
9-17 y	IIV	320	320	1.0	80	113	1.4	14	40	2.8	160	160	1.0
9-17 y	IIV	320	320	1.0	226	160	0.7	57	160	2.8	640	640	1.0
9-17 y	IIV	80	320	4.0	160	320	2.0	40	80	2.0	320	320	1.0
9-17 y	IIV	320	320	1.0	20	113	5.7	80	160	2.0	80	113	1.4
9-17 y	IIV	320	320	1.0	57	80	1.4	5	40	8.0	320	640	2.0
9-17 y	IIV	160	320	2.0	160	320	2.0	10	80	8.0	160	640	4.0
9-17 y	IIV	320	320	1.0	160	320	2.0	80	160	2.0	320	320	1.0
9-17 y	IIV	320	320	1.0	160	226	1.4	80	80	1.0	160	160	1.0
9-17 y	IIV	320	453	1.4	57	113	2.0	160	160	1.0	640	1280	2.0
9-17 y	IIV	320	640	2.0	160	160	1.0	80	80	1.0	160	226	1.4
9-17 y	IIV	640	640	1.0	160	160	1.0	80	80	1.0	226	320	1.4
9-17 y	IIV	640	640	1.0	160	160	1.0	160	160	1.0	640	640	1.0
9-17 y	IIV	640	905	1.4	226	320	1.4	20	640	32.0	640	1280	2.0
9-17 y	IIV	320	640	2.0	160	640	4.0	160	320	2.0	160	320	2.0
9-17 y	IIV	40	113	2.8	160	640	4.0	10	40	4.0	28	226	8.0
9-17 y	IIV	80	160	2.0	10	2560	256.0	40	320	8.0	10	640	64.0
9-17 y	IIV	160	320	2.0	320	320	1.0	20	320	16.0	28	320	11.3
9-17 y	IIV	40	320	8.0	160	320	2.0	7	160	22.6	113	320	2.8
9-17 y	IIV	80	640	8.0	80	160	2.0	20	160	8.0	80	1280	16.0
9-17 y	IIV	80	640	8.0	160	640	4.0	80	640	8.0	57	160	2.8
9-17 y	IIV	137	271	2	101	236	2	41	144	4	107	273	3

The virus used in the assay is listed across the top. Reciprocal endpoint antibody titers for D0 (pre-vaccination) D21 and the fold change between the two time points are listed for each virus strain. The bottom row represents the average for each column (highlighted in red numbers). Seroconverters (green) are defined as those vaccines with at least a 4-fold increase in antibody titer from day 0 to day 21; seroprevalent (yellow) patients are defined as those vaccines with positive antibody titers at day 0 and less than 4-fold increase in titer at day 21; non-reactors are defined as those vaccines whose antibody titers were below the cutoff of the assay ($< 1:80$)

The LAIV vaccine also failed to elicit a good antibody response in the 9-17 year old cohort receiving LAIV. The majority of children receiving LAIV (45 out of 57) were seroprevalent, 7 out of 57 seroconverted to one of the viruses, and 9 out of 57 patients were non-reactive. As displayed in Table 6, average fold change in antibody response was poor. The 3-8 year old cohort were seroprevalent for the influenza B virus only where the 9-17 are were non-reactive across all strains.

Table 5. Hemagglutination inhibition (HAI) assays for the 9-17 year old cohort vaccinated with LAIV.

AGE GROUP	2014-15 Vaccine	H3 DAY 0	H3 Day 21	H3 Fold	H1 Day 0	H1 Day 21	H1 Fold	B/Brisbane Day 0	B/Brisbane Day 21	B/Brisbane Fold	B/Massachusetts Day 0	B/Massachusetts Day 21	B/Massachusetts Fold
9-17 y	LAIV	20	40	2.0	160	160	1.0	57	80	1.4	160	160	1.0
9-17 y	LAIV	80	80	1.0	160	160	1.0	20	28	1.4	320	453	1.4
9-17 y	LAIV	80	80	1.0	160	160	1.0	20	40	2.0	160	320	2.0
9-17 y	LAIV	80	80	1.0	160	160	1.0	80	80	1.0	160	160	1.0
9-17 y	LAIV	160	160	1.0	160	160	1.0	40	40	1.0	40	40	1.0
9-17 y	LAIV	160	160	1.0	320	320	1.0	160	160	1.0	226	226	1.0
9-17 y	LAIV	160	160	1.0	320	226	0.7	80	80	1.0	1280	640	0.5
9-17 y	LAIV	226	226	1.0	5	5	1.0	10	10	1.0	14	20	1.4
9-17 y	LAIV	80	320	4.0	40	28	0.7	10	160	16.0	226	320	1.4
9-17 y	LAIV	160	320	2.0	80	80	1.0	20	20	1.0	640	640	1.0
9-17 y	LAIV	320	320	1.0	5	5	1.0	20	20	1.0	160	160	1.0
9-17 y	LAIV	320	320	1.0	226	160	0.7	160	160	1.0	226	160	0.7
9-17 y	LAIV	320	320	1.0	7	5	0.7	40	40	1.0	113	80	0.7
9-17 y	LAIV	226	320	1.4	5	10	2.0	14	14	1.0	160	320	2.0
9-17 y	LAIV	320	320	1.0	160	160	1.0	40	80	2.0	80	320	4.0
9-17 y	LAIV	320	320	1.0	160	160	1.0	28	40	1.4	320	80	0.3
9-17 y	LAIV	320	320	1.0	320	320	1.0	40	40	1.0	40	160	4.0
9-17 y	LAIV	320	320	1.0	160	160	1.0	80	80	1.0	20	40	2.0
9-17 y	LAIV	640	453	0.7	80	80	1.0	80	80	1.0	160	160	1.0
9-17 y	LAIV	640	640	1.0	160	160	1.0	20	20	1.0	160	160	1.0
9-17 y	LAIV	20	20	1.0	80	80	1.0	40	40	1.0	113	80	0.7
9-17 y	LAIV	40	40	1.0	5	5	1.0	10	20	2.0	20	28	1.4
9-17 y	LAIV	40	40	1.0	10	10	1.0	20	20	1.0	160	226	1.4
9-17 y	LAIV	57	57	1.0	20	20	1.0	5	5	1.0	80	80	1.0
9-17 y	LAIV	160	160	1.0	160	80	0.5	80	80	1.0	160	113	0.7
9-17 y	LAIV	320	160	0.5	5	5	1.0	40	40	1.0	57	57	1.0
9-17 y	LAIV	160	160	1.0	226	320	1.4	40	40	1.0	40	40	1.0
9-17 y	LAIV	160	160	1.0	40	40	1.0	10	14	1.4	160	160	1.0
9-17 y	LAIV	320	226	0.7	320	320	1.0	320	226	0.7	640	640	1.0
9-17 y	LAIV	453	453	1.0	160	160	1.0	40	80	2.0	40	80	2.0
9-17 y	LAIV	320	640	2.0	20	40	2.0	160	320	2.0	160	320	2.0
9-17 y	LAIV	160	640	4.0	80	80	1.0	40	57	1.4	57	57	1.0
9-17 y	LAIV	320	2560	8.0	80	160	2.0	57	80	1.4	226	320	1.4
9-17 y	LAIV	28	14	0.5	80	113	1.4	80	80	1.0	320	320	1.0
9-17 y	LAIV	10	14	1.4	14	20	1.4	80	80	1.0	160	160	1.0
9-17 y	LAIV	20	20	1.0	80	80	1.0	160	160	1.0	320	320	1.0
9-17 y	LAIV	20	20	1.0	57	40	0.7	20	20	1.0	40	57	1.4
9-17 y	LAIV	20	20	1.0	5	10	2.0	5	10	2.0	28	40	1.4
9-17 y	LAIV	40	40	1.0	10	20	2.0	5	10	2.0	5	10	2.0
9-17 y	LAIV	40	40	1.0	20	20	1.0	5	5	1.0	5	5	1.0
9-17 y	LAIV	40	40	1.0	640	640	1.0	20	20	1.0	226	226	1.0
9-17 y	LAIV	40	57	1.4	40	160	4.0	80	80	1.0	40	40	1.0
9-17 y	LAIV	113	113	1.0	20	20	1.0	5	5	1.0	5	10	2.0
9-17 y	LAIV	160	160	1.0	20	20	1.0	20	40	2.0	10	40	4.0
9-17 y	LAIV	160	160	1.0	80	80	1.0	80	80	1.0	160	160	1.0
9-17 y	LAIV	160	160	1.0	320	320	1.0	160	113	0.7	20	20	1.0
9-17 y	LAIV	160	160	1.0	40	40	1.0	20	40	2.0	40	80	2.0
9-17 y	LAIV	80	160	2.0	10	20	2.0	5	57	11.3	5	40	8.0
9-17 y	LAIV	160	160	1.0	5	20	4.0	10	80	8.0	20	160	8.0
9-17 y	LAIV	320	320	1.0	80	80	1.0	5	10	2.0	5	10	2.0
9-17 y	LAIV	320	320	1.0	80	80	1.0	40	40	1.0	10	57	5.7
9-17 y	LAIV	320	320	1.0	7	7	1.0	20	20	1.0	20	20	1.0
9-17 y	LAIV	320	320	1.0	453	453	1.0	40	80	2.0	10	320	32.0
9-17 y	LAIV	320	320	1.0	80	80	1.0	160	160	1.0	160	160	1.0
9-17 y	LAIV	453	453	1.0	80	80	1.0	80	80	1.0	320	320	1.0
9-17 y	LAIV	130	146	1	54	59	1	32	43	1	72	102	1

The virus used in the assay is listed across the top. Reciprocal endpoint antibody titers for D0 (pre-vaccination) D21 and the fold change between the two time points are listed for each virus strain. The bottom row represents the average for each column (highlighted in red numbers). Seroconverters (green) are defined as those vaccines with at least a 4-fold increase in antibody titer from day 0 to day 21; seroprevalent (yellow) patients are defined as those

vaccines with positive antibody titers at day 0 and less than 4-fold increase in titer at day 21; non-reactors are defined as those vaccines whose antibody titers were below the cutoff of the assay (< 1:80)

Overall, the 3-8 year old cohort vaccinated with IIV, had the best antibody response. Average fold increase for this cohort was 4-fold or higher across all four strains (Table 6). The 9-17 year old cohort vaccinated with IIV had an average 4-fold or higher increase in the influenza B virus (B/Brisbane) while all responses to all other strains were less than 4-fold (table 6). For the cohorts who received LAIV, the 3-8 year olds on average demonstrated seroprevalance to both influenza B viruses only; average antibody reactivity to influenza A viruses in the 3-8 year old group and all viruses tested in the 9-17 year old group was non-reactive.

Table 6. Average fold change in HAI titers demonstrated more robust antibody responses in younger children (3-8 yr cohort) receiving IIV vaccination.

	2014-15 vaccine	A/H3N2 Avg. Fold	A/H1N1 Avg. Fold	B/Brisbane Avg. Fold	B/Mass Avg. Fold
3-8 yr	LAIV	1	1	2	2
	IIV	4.4	6.1	5.2	4.3
9-17 yr	LAIV	1	1	1	1
	IIV	2	2	4	3

Seroconverters (green) are defined as those vaccines with at least a 4-fold increase in antibody titer from day 0 to day 21; seroprevalent (yellow) patients are defined as those vaccines with positive antibody titers at day 0 and less than 4-fold increase in titer at day 21; non-reactors (no shading) are defined as those vaccines whose antibody titers were below the cutoff of the assay and/or whose change from D0 to D21 was ≤1.

4.1.2 HAI Assay Statistics

Statistical analysis of the HAI data was conducted using a paired, two tailed t-test. We reviewed the P values for the 3-8 year old and 9-17 year old cohort antibody levels, comparing

day 0 to day 21, across all virus strains. Those vaccinated with IIV demonstrated a significant increase in antibody response across all strains, for both cohorts, had (Table 7). In contrast, neither the 3-8 year old nor 9-17 year old cohort vaccinated with LAIV had a significant increase in HAI antibody titer to the influenza A viruses (H3N2 & H1N1); interestingly, significant HAI antibody titers to the influenza B viruses (B/Brisbane & B/Massachusetts) were observed for both cohorts. Thus, the data demonstrated that, using a paired t test to directly compare the D0 and D21 antibody titers, the IIV vaccine was capable of eliciting a significant antibody response to all vaccine strains in all vaccine recipients in an age-independent manner. In contrast, the LAIV vaccine only elicited significant antibody responses to the B virus strains, but this antibody response was also in an age-independent manner.

Table 7. Significant Antibody responses elicited by IIV demonstrated increased breadth to all vaccine strains.

Virus strain[a]	Vaccine[b]	Age Group[c]	Mean of diff[d]	Degrees of freedom[e]	P value[f]	# of pairs[g]
H3N2						
	LAIV	3-8	-13.72	38	0.4629	39
		9-17	58.18	54	0.1754	
	IIV	3-8	416.4	19	0.0035	20
		9-17	129.3	32	0.0001	33
H1N1						
	LAIV	3-8	-0.5897	38	0.8657	39
		9-17	2.309	54	0.5810	55
	IIV	3-8	350.3	19	0.0104	20
		9-17	207.3	32	0.0125	33
B/Brisbane						
	LAIV	3-8	28.44	38	0.0051	39
		9-17	10.6	54	0.0314	55
	IIV	3-8	185.1	19	0.0002	20
		9-17	129	32	0.0001	33
B/Massachusetts						
	LAIV	3-8	54.97	38	0.0084	39
		9-17	16.15	54	0.3218	55
	IIV	3-8	359.3	19	0.0008	20
		9-17	199.5	32	0.0001	33

[a]Virus Strain tested in HAI assay
[b]Vaccine received
[c]Age Group of cohort
[d]Mean of Difference in HAI Titer D21-D0
[e]Degrees of Freedom
[f]*P* value as determined by paired, two-tailed, t test
[g]Number of D0D21 pairs analyzed

4.1.3   MN Assay Results

Influenza A (H3N2) virus was the predominant virus in circulation for the 2014-2015 season [19]. Therefore, to evaluate the functional relevance (i.e., ability of antibody to neutralize influenza replication) and the breadth of antibody elicited by vaccination to the circulating strains, we analyzed the MN titer levels using day 0 and day 21 serum samples, testing against three A (H3N2) viruses: A/ Texas/50/2012 (vaccine-matched strain), A/Switzerland/9715293/2013 and A/Nebraska/04/2014 (variant circulating strains) [20]. The data and vaccine groups were further stratified by the vaccine administered in the prior year.

Geometric mean titers in 3-8 year olds vaccinated with IIV demonstrated increases to all three H3N2 strains tested. In contrast, children in the 3-8 year old cohort vaccinated with LAIV in 2014-15 failed to demonstrate increased in GMT at D21 compared to D0 to any H3 strain tested (Figure 6). When looking at the contribution of 2013-14 vaccination history on the 2014-15 MN antibody response, no conclusive correlation was possible, since only the patients receiving IIV in 2014-15 also received IIV in 2013-14. However, data suggests that the IIV vaccine was better at priming and boosting the MN antibody response (Figure 6). Further analyses will be necessary to tease this apart.
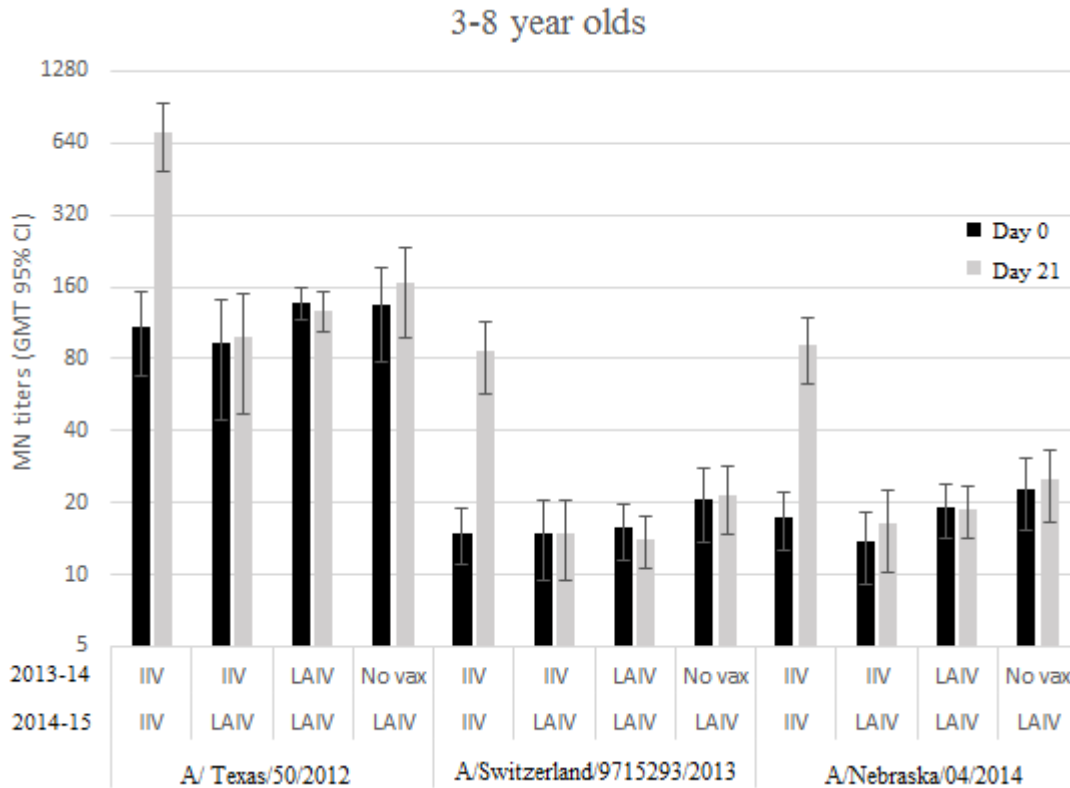
Figure 6. Geometric Mean titers demonstrated broad neutralization against vaccine and variant H3N2 virus strains in 3-8 year olds vaccinated with IIV [20].

Reciprocal Log 2 geometric mean antibody titers (GMT) as measured by microneutralization assay to the vaccine (H3N2 (A/ Texas/50/2012) and variant (A/Switzerland/9715293/2013 and A/Nebraska/04/2014) H3N2 virus strains on day 0 (pre vaccination) and day 21; the x-axis lists both the 2014-15 vaccine as well as the reported 2013-14 vaccine type administered to the 3-8 year old cohort.

Similar to data observed in the 3-8 year old cohort, GMT in 9-17 year olds vaccinated with IIV displayed increases to all three H3N2 strains tested. Similarly, vaccination of the older cohort with LAIV in 2014-15 failed to demonstrate appreciable increases in GMT at D21 compared to D0 to any H3 strain tested. Interestingly, when looking at the contribution of 2013-14 vaccination history on the 2014-15 MN antibody response, LAIV vaccination in the prior year (2013-14) was associated with higher antibody titers at D21 in 2014-15 IIV vaccine recipients compared to those receiving IIV in 2013-14. While these differences were not significant, the

data suggests that LAIV vaccination may serve to prime a better immune memory so that IIV

vaccination would result in a more robust neutralizing antibody response.
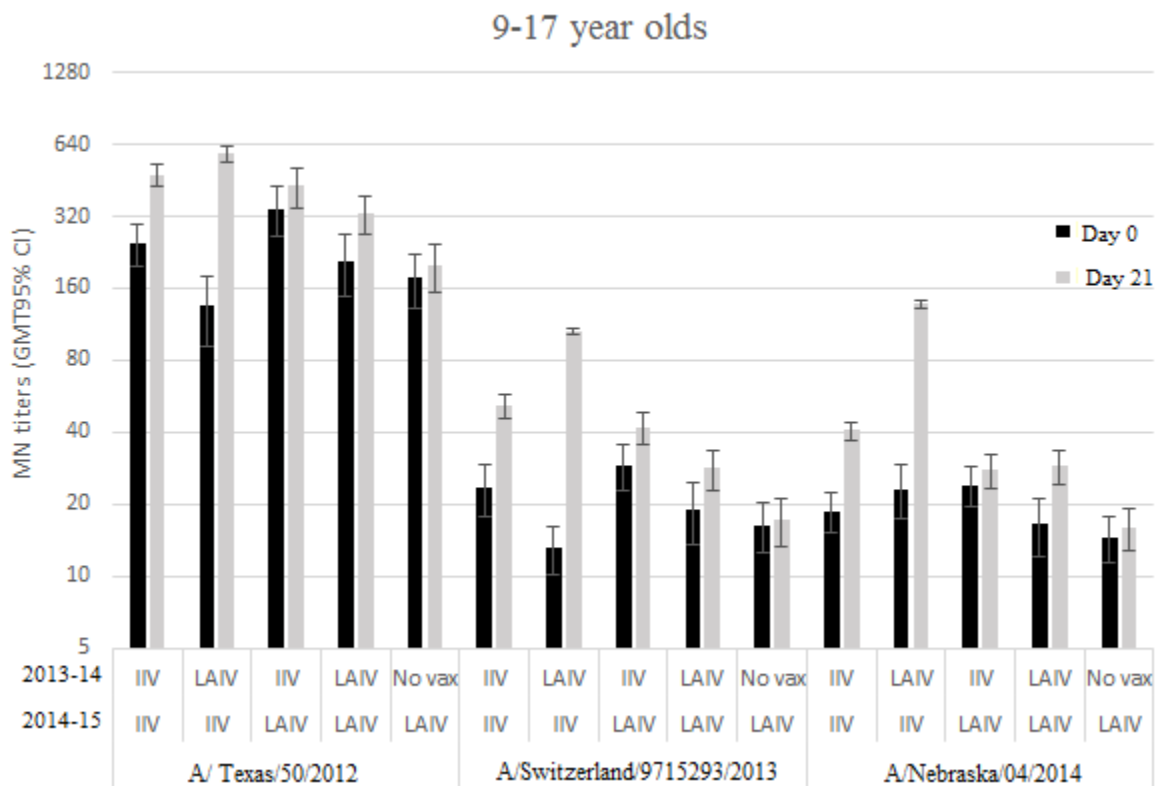
Figure 7. Geometric Mean titers demonstrated broad neutralization against vaccine and variant H3N2 virus strains in 9-17 year olds vaccinated with IIV, with LAIV vaccination in prior year providing better immune memory. [20].

Reciprocal Log 2 geometric mean antibody titers (GMT) as measured by microneutralization assay to the vaccine (H3N2 (A/ Texas/50/2012) and variant (A/Switzerland/9715293/2013 and A/Nebraska/04/2014) H3N2 virus strains on day 0 (pre vaccination) and day 21; the x-axis lists both the 2014-15 vaccine as well as the reported 2013-14 vaccine type administered to the 3-8 year old cohort.

5.0   DISCUSSION

Results from the HAI and MN assays demonstrated that patients in both the younger and older cohorts had increased antibody titers (HAI) and increased breadth of neutralization (MN) to at least one or more viruses present in the vaccine, in patients immunized with IIV.   In contrast, the quadrivalent LAIV vaccine failed to elicit antibody titers above the DO levels in either cohort. At first glance these results seemed to suggest that IIV was superior as a vaccine when compared to LAIV. However there are several important issues that must be addressed with these findings.

One of the items addressed was participant's vaccination history for the previous influenza season (2013-2014). After careful review of this data, it seems that past vaccination played at least a partial role in the quantitative level of antibody response elicited by the participants in particular age groups. This may be in part due to a booster effect (i.e., generation of immune memory) from the previous vaccine.

In the 3-8 year old group receiving the IIV vaccine for both the 2013-14 and 2014-15 seasons, increased HAI titers were observed with the majority of the vaccines being seroconverters (Table 9). When the 3-8 year old group had no vaccination in the prior year, they also demonstrated increased seroconversion when vaccinated with LAIV, but only to B strains. Vaccination in both years, they also demonstrated increased HAI titers but not to the same quantitative level (more seroprevalent/yellow vs. seroconverter/green) (Table 9).   Thus, it

appears that IIV vaccination was able to elicit a higher quantitative level of antibodies in younger children. Interestingly, when the 9-17 year old cohort was vaccinated with LAIV in 2013-14, they had higher levels of antibodies compared to IIV/IIV recipients. This suggests that LAIV may serve to stimulate better immune memory and warrants further evaluation, as there was no comparable group to evaluate in the 3-8 year old cohort.

Table 8. Hemagglutination antibody inhibition (HAI) titers show that prior vaccination (2013-14) with IIV results in increased seroconversion in 3-8 year olds vaccinated with IIV again in subsequent year (2014-15)

AGE Group	2013-14 vaccine	2014-15 vaccine	H3N2			H1N1			B/BRISSBANE			B/ Massachusetts		
			Day 0	Day 21	Fold	Day 0	Day 21	Fold	Day 0	Day 21	Fold	Day 0	Day 21	Fold
3-8 yo	IIV	IIV	80	80	1.0	20	160	8.0	10	453	45.3	160	640	4.0
3-8 yo	IIV	IIV	113	160	1.4	453	640	1.4	57	80	1.4	40	80	2.0
3-8 yo	IIV	IIV	40	160	4.0	1280	1810	1.4	40	320	8.0	160	320	2.0
3-8 yo	IIV	IIV	40	160	4.0	80	226	2.8	80	320	4.0	80	160	2.0
3-8 yo	IIV	IIV	320	320	1.0	5	40	8.0	14	160	11.3	40	320	8.0
3-8 yo	IIV	IIV	10	320	32.0	20	160	8.0	160	160	1.0	80	226	2.8
3-8 yo	IIV	IIV	10	453	45.3	5	640	128.0	5	10	2.0	5	113	22.6
3-8 yo	IIV	IIV	80	640	8.0	80	640	8.0	5	57	11.3	28	640	22.6
3-8 yo	IIV	IIV	453	640	1.4	113	320	2.8	10	40	4.0	57	160	2.8
3-8 yo	IIV	IIV	640	640	1.0	80	160	2.0	160	320	2.0	80	320	4.0
3-8 yo	IIV	IIV	320	640	2.0	80	226	2.8	10	160	16.0	160	453	2.8
3-8 yo	IIV	IIV	160	640	4.0	5	40	8.0	5	80	16.0	160	640	4.0
3-8 yo	IIV	IIV	80	640	8.0	5	80	16.0	160	320	2.0	320	640	2.0
3-8 yo	IIV	IIV	20	640	32.0	10	2560	256.0	5	40	8.0	10	320	32.0
3-8 yo	IIV	IIV	320	640	2.0	226	640	2.8	14	80	5.7	226	640	2.8
3-8 yo	IIV	IIV	160	640	4.0	160	640	4.0	57	640	11.3	80	640	8.0
3-8 yo	IIV	IIV	640	905	1.4	320	640	2.0	226	640	2.8	320	640	2.0
3-8 yo	IIV	IIV	320	1280	4.0	160	320	2.0	80	640	8.0	640	2560	4.0
3-8 yo	IIV	IIV	40	2560	64.0	10	160	16.0	80	320	4.0	80	320	4.0
3-8 yo	IIV	IIV	107	478	4.5	44	264	6.1	30	155	5.2	84	361	4.3

When looking at quantitate levels of antibody by HAI, it seems that IIV, especially in the younger cohort (3-8year olds) being administered consecutively played a role in the overall increase (Table 9). For those administered LAIV consecutively, we observed a poor antibody response (Table 8 and 9). This could be due to LAIV vaccine not producing a booster effect like IIV seem to elicit or the issues that arose with the LAIV vaccine for the 2014-2015 season (see below).

Table 9. Hemagglutination antibody inhibition (HAI) titers show that prior vaccination has no effect on LAIV vaccination in 3-8 year olds while no vaccination provide LAIV-induced immune memory against B strains.

AGE Group	2013-14 vaccine	2014-15 vaccine	H3N2			H1N1			B/BRISSBANE			B/ Massachusetts		
			Day 0	Day 21	Fold	Day 0	Day 21	Fold	Day 0	Day 21	Fold	Day 0	Day 21	Fold
3-8 yo	IIV	LAIV	10	20	2.0	5	5	1.0	5	40	8.0	57	320	5.7
3-8 yo	IIV	LAIV	28	40	1.4	80	80	1.0	10	20	2.0	20	40	2.0
3-8 yo	IIV	LAIV	40	40	1.0	320	320	1.0	320	320	1.0	226	320	1.4
3-8 yo	IIV	LAIV	40	57	1.4	20	20	1.0	5	40	8.0	40	80	2.0
3-8 yo	IIV	LAIV	160	160	1.0	80	80	1.0	160	160	1.0	160	160	1.0
3-8 yo	IIV	LAIV	160	160	1.0	40	40	1.0	10	20	2.0	10	40	4.0
3-8 yo	IIV	LAIV	160	160	1.0	5	5	1.0	40	40	1.0	160	160	1.0
3-8 yo	IIV	LAIV	320	320	1.0	160	160	1.0	160	160	1.0	160	160	1.0
3-8 yo	IIV	LAIV	320	320	1.0	40	40	1.0	80	160	2.0	160	160	1.0
3-8 yo	IIV	LAIV	320	320	1.0	40	40	1.0	80	80	1.0	320	320	1.0
3-8 yo	IIV	LAIV	320	320	1.0	160	226	1.4	320	226	0.7	160	226	1.4
3-8 yo	IIV	LAIV	106	120	1.1	48	50	1.1	45	76	1.7	85	131	1.5
3-8 yo	LAIV	LAIV	57	40	0.7	5	5	1.0	5	5	1.0	40	40	1.0
3-8 yo	LAIV	LAIV	40	40	1.0	80	80	1.0	10	20	2.0	40	80	2.0
3-8 yo	LAIV	LAIV	40	40	1.0	28	20	0.7	40	40	1.0	28	28	1.0
3-8 yo	LAIV	LAIV	80	80	1.0	320	320	1.0	160	160	1.0	7	20	2.8
3-8 yo	LAIV	LAIV	160	80	0.5	40	40	1.0	10	28	2.8	40	80	2.0
3-8 yo	LAIV	LAIV	113	113	1.0	57	40	0.7	80	80	1.0	28	80	2.8
3-8 yo	LAIV	LAIV	160	160	1.0	80	80	1.0	40	80	2.0	40	113	2.8
3-8 yo	LAIV	LAIV	160	160	1.0	160	160	1.0	160	160	1.0	57	160	2.8
3-8 yo	LAIV	LAIV	160	160	1.0	40	40	1.0	80	80	1.0	80	80	1.0
3-8 yo	LAIV	LAIV	320	320	1.0	320	320	1.0	640	640	1.0	320	320	1.0
3-8 yo	LAIV	LAIV	106	95	0.9	65	61	0.9	49	63	1.3	43	75	1.7
3-8 yo	No vax	LAIV	5	5	1.0	5	5	1.0	5	5	1.0	5	40	8.0
3-8 yo	No vax	LAIV	20	20	1.0	40	40	1.0	40	160	4.0	5	80	16.0
3-8 yo	No vax	LAIV	10	20	2.0	20	20	1.0	5	160	32.0	5	40	8.0
3-8 yo	No vax	LAIV	20	28	1.4	80	80	1.0	5	80	16.0	40	113	2.8
3-8 yo	No vax	LAIV	40	40	1.0	10	20	2.0	20	14	0.7	80	80	1.0
3-8 yo	No vax	LAIV	40	40	1.0	160	80	0.5	57	40	0.7	10	10	1.0
3-8 yo	No vax	LAIV	40	80	2.0	7	40	5.7	113	113	1.0	320	80	0.3
3-8 yo	No vax	LAIV	160	160	1.0	80	80	1.0	160	160	1.0	320	320	1.0
3-8 yo	No vax	LAIV	160	160	1.0	14	20	1.4	160	160	1.0	7	14	2.0
3-8 yo	No vax	LAIV	160	160	1.0	160	160	1.0	80	80	1.0	113	160	1.4
3-8 yo	No vax	LAIV	320	160	0.5	320	320	1.0	10	113	11.3	453	640	1.4
3-8 yo	No vax	LAIV	320	320	1.0	80	80	1.0	160	160	1.0	80	320	4.0
3-8 yo	No vax	LAIV	320	320	1.0	320	320	1.0	40	40	1.0	20	113	5.7
3-8 yo	No vax	LAIV	320	320	1.0	20	20	1.0	80	320	4.0	20	113	5.7
3-8 yo	No vax	LAIV	320	320	1.0	160	160	1.0	20	40	2.0	5	20	4.0
3-8 yo	No vax	LAIV	1280	640	0.5	20	20	1.0	5	113	22.6	5	640	128.0
3-8 yo	No vax	LAIV	640	905	1.4	226	160	0.7	10	160	16.0	5	113	22.6
3-8 yo	No vax	LAIV	102	106	1.0	50	55	1.1	29	80	2.8	28	94	3.3

Table 10. Hemagglutination inhibition (HAI) assays data show vaccination in the 2013-2014 and 2014-2015 vaccine season for the 9-17 year olds.

AGE Group	2013-14 vaccine	2014-15 vaccine	H3N2 Day 0	Day 21	Fold	H1N1 Day 0	Day 21	Fold	B/BRISSBANE Day 0	Day 21	Fold	B/Massachusetts Day 0	Day 21	Fold
9-17 y	IIV	IIV	20	80	4.0	80	160	2.0	20	160	8.0	80	160	2.0
9-17 y	IIV	IIV	28	80	2.8	20	80	4.0	10	40	4.0	20	320	16.0
9-17 y	IIV	IIV	80	113	1.4	40	160	4.0	40	113	2.8	80	160	2.0
9-17 y	IIV	IIV	80	160	2.0	160	160	1.0	80	320	4.0	80	320	4.0
9-17 y	IIV	IIV	80	160	2.0	40	160	4.0	40	113	2.8	20	40	2.0
9-17 y	IIV	IIV	80	160	2.0	160	320	2.0	20	80	4.0	160	320	2.0
9-17 y	IIV	IIV	40	160	4.0	80	160	2.0	40	160	4.0	80	160	2.0
9-17 y	IIV	IIV	80	160	2.0	20	113	5.7	10	453	45.3	80	226	2.8
9-17 y	IIV	IIV	160	226	1.4	80	80	1.0	80	80	1.0	80	80	1.0
9-17 y	IIV	IIV	80	226	2.8	160	640	4.0	80	160	2.0	80	160	2.0
9-17 y	IIV	IIV	160	320	2.0	320	320	1.0	113	160	1.4	57	80	1.4
9-17 y	IIV	IIV	160	320	2.0	160	640	4.0	226	453	2.0	80	320	4.0
9-17 y	IIV	IIV	320	320	1.0	80	113	1.4	14	40	2.8	160	160	1.0
9-17 y	IIV	IIV	320	320	1.0	226	160	0.7	57	160	2.8	640	640	1.0
9-17 y	IIV	IIV	80	320	4.0	160	320	2.0	40	80	2.0	320	320	1.0
9-17 y	IIV	IIV	320	320	1.0	20	113	5.7	80	160	2.0	80	113	1.4
9-17 y	IIV	IIV	320	320	1.0	57	80	1.4	5	40	8.0	320	640	2.0
9-17 y	IIV	IIV	160	320	2.0	160	320	2.0	10	80	8.0	160	640	4.0
9-17 y	IIV	IIV	320	320	1.0	160	320	2.0	80	160	2.0	320	320	1.0
9-17 y	IIV	IIV	320	320	1.0	160	226	1.4	80	80	1.0	160	160	1.0
9-17 y	IIV	IIV	320	453	1.4	57	113	2.0	160	160	1.0	640	1280	2.0
9-17 y	IIV	IIV	320	640	2.0	160	160	1.0	80	80	1.0	160	226	1.4
9-17 y	IIV	IIV	640	640	1.0	160	160	1.0	80	80	1.0	226	320	1.4
9-17 y	IIV	IIV	640	640	1.0	160	160	1.0	160	160	1.0	640	640	1.0
9-17 y	IIV	IIV	640	905	1.4	226	320	1.4	20	640	32.0	640	1280	2.0
9-17 y	IIV	IIV	320	640	2.0	160	640	4.0	160	320	2.0	160	320	2.0
9-17 y	IIV	IIV	162	276	1.7	99	195	2.0	46	133	2.9	140	262	1.9
9-17 y	LAIV	IIV	40	113	2.8	160	640	4.0	10	40	4.0	28	226	8.0
9-17 y	LAIV	IIV	80	160	2.0	10	2560	256.0	40	320	8.0	10	640	64.0
9-17 y	LAIV	IIV	160	320	2.0	320	320	1.0	20	320	16.0	28	320	11.3
9-17 y	LAIV	IIV	40	320	8.0	160	320	2.0	7	160	22.6	113	320	2.8
9-17 y	LAIV	IIV	80	640	8.0	80	160	2.0	20	160	8.0	80	1280	16.0
9-17 y	LAIV	IIV	80	640	8.0	160	640	4.0	80	640	8.0	57	160	2.8
9-17 y	LAIV	IIV	71	302	4.2	101	508	5.0	21	202	9.5	40	381	9.5

42

Table 11. Hemagglutination antibody inhibition (HAI) titers show that no or IIV vaccination in 2013-14 resulted in increased seroprevalence following LAIV vaccination in 2014-15.

AGE Group	2013-14 vaccine	2014-15 vaccine	H3N2			H1N1			B/BRISSBANE			B/ Massachusetts		
			Day 0	Day 21	Fold	Day 0	Day 21	Fold	Day 0	Day 21	Fold	Day 0	Day 21	Fold
9-17 y	IIV	LAIV	80	80	1.0	160	160	1.0	20	28	1.4	320	453	1.4
9-17 y	IIV	LAIV	80	80	1.0	160	160	1.0	20	40	2.0	160	320	2.0
9-17 y	IIV	LAIV	80	80	1.0	160	160	1.0	80	80	1.0	160	160	1.0
9-17 y	IIV	LAIV	160	160	1.0	160	160	1.0	40	40	1.0	40	40	1.0
9-17 y	IIV	LAIV	160	160	1.0	320	320	1.0	160	160	1.0	226	226	1.0
9-17 y	IIV	LAIV	160	160	1.0	320	226	0.7	80	80	1.0	1280	640	0.5
9-17 y	IIV	LAIV	226	226	1.0	5	5	1.0	10	10	1.0	14	20	1.4
9-17 y	IIV	LAIV	80	320	4.0	40	28	0.7	10	160	16.0	226	320	1.4
9-17 y	IIV	LAIV	160	320	2.0	80	80	1.0	20	20	1.0	640	640	1.0
9-17 y	IIV	LAIV	320	320	1.0	5	5	1.0	20	20	1.0	160	160	1.0
9-17 y	IIV	LAIV	320	320	1.0	226	160	0.7	160	160	1.0	226	160	0.7
9-17 y	IIV	LAIV	320	320	1.0	7	5	0.7	40	40	1.0	113	80	0.7
9-17 y	IIV	LAIV	226	320	1.4	5	10	2.0	14	14	1.0	160	320	2.0
9-17 y	IIV	LAIV	320	320	1.0	160	160	1.0	40	80	2.0	80	320	4.0
9-17 y	IIV	LAIV	320	320	1.0	160	160	1.0	28	40	1.4	320	80	0.3
9-17 y	IIV	LAIV	320	320	1.0	320	320	1.0	40	40	1.0	40	160	4.0
9-17 y	IIV	LAIV	320	320	1.0	160	160	1.0	80	80	1.0	20	40	2.0
9-17 y	IIV	LAIV	640	453	0.7	80	80	1.0	80	80	1.0	160	160	1.0
9-17 y	IIV	LAIV	640	640	1.0	160	160	1.0	20	20	1.0	160	160	1.0
9-17 y	IIV	LAIV	214	190	1.1	77	190	1.0	36	190	1.3	141	190	1.2
9-17 y	LAIV	LAIV	20	20	1.0	80	80	1.0	40	40	1.0	113	80	0.7
9-17 y	LAIV	LAIV	40	40	1.0	5	5	1.0	10	20	2.0	20	28	1.4
9-17 y	LAIV	LAIV	40	40	1.0	10	10	1.0	20	20	1.0	160	226	1.4
9-17 y	LAIV	LAIV	57	57	1.0	20	20	1.0	5	5	1.0	80	80	1.0
9-17 y	LAIV	LAIV	160	160	1.0	160	80	0.5	80	80	1.0	160	113	0.7
9-17 y	LAIV	LAIV	320	160	0.5	5	5	1.0	40	40	1.0	57	57	1.0
9-17 y	LAIV	LAIV	160	160	1.0	226	320	1.4	40	40	1.0	40	40	1.0
9-17 y	LAIV	LAIV	160	160	1.0	40	40	1.0	10	14	1.4	160	160	1.0
9-17 y	LAIV	LAIV	320	226	0.7	320	320	1.0	320	226	0.7	640	640	1.0
9-17 y	LAIV	LAIV	453	453	1.0	160	160	1.0	40	80	2.0	40	80	2.0
9-17 y	LAIV	LAIV	320	640	2.0	20	40	2.0	160	320	2.0	160	320	2.0
9-17 y	LAIV	LAIV	160	640	4.0	80	80	1.0	40	57	1.4	57	57	1.0
9-17 y	LAIV	LAIV	320	2560	8.0	80	160	2.0	57	80	1.4	226	320	1.4
9-17 y	LAIV	LAIV	136	173	1.3	46	50	1.1	37	46	1.2	99	113	1.1
9-17 y	No vax	LAIV	28	14	0.5	80	113	1.4	80	80	1.0	320	320	1.0
9-17 y	No vax	LAIV	10	14	1.4	14	20	1.4	80	80	1.0	160	160	1.0
9-17 y	No vax	LAIV	20	20	1.0	80	80	1.0	160	160	1.0	320	320	1.0
9-17 y	No vax	LAIV	20	20	1.0	57	40	0.7	20	20	1.0	40	57	1.4
9-17 y	No vax	LAIV	20	20	1.0	5	10	2.0	5	10	2.0	28	40	1.4
9-17 y	No vax	LAIV	40	40	1.0	10	20	2.0	5	10	2.0	5	10	2.0
9-17 y	No vax	LAIV	40	40	1.0	20	20	1.0	5	5	1.0	5	5	1.0
9-17 y	No vax	LAIV	40	40	1.0	640	640	1.0	20	20	1.0	226	226	1.0
9-17 y	No vax	LAIV	40	57	1.4	40	160	4.0	80	80	1.0	40	40	1.0
9-17 y	No vax	LAIV	113	113	1.0	20	20	1.0	5	5	1.0	5	10	2.0
9-17 y	No vax	LAIV	160	160	1.0	20	20	1.0	20	40	2.0	10	40	4.0
9-17 y	No vax	LAIV	160	160	1.0	80	80	1.0	80	80	1.0	160	160	1.0
9-17 y	No vax	LAIV	160	160	1.0	320	320	1.0	160	113	0.7	20	20	1.0
9-17 y	No vax	LAIV	160	160	1.0	40	40	1.0	20	40	2.0	40	80	2.0
9-17 y	No vax	LAIV	80	160	2.0	10	20	2.0	5	57	11.3	5	40	8.0
9-17 y	No vax	LAIV	160	160	1.0	5	20	4.0	10	80	8.0	20	160	8.0
9-17 y	No vax	LAIV	320	320	1.0	80	80	1.0	5	10	2.0	5	10	2.0
9-17 y	No vax	LAIV	320	320	1.0	80	80	1.0	40	40	1.0	10	57	5.7
9-17 y	No vax	LAIV	320	320	1.0	7	7	1.0	20	20	1.0	20	20	1.0
9-17 y	No vax	LAIV	320	320	1.0	453	453	1.0	40	80	2.0	10	320	32.0
9-17 y	No vax	LAIV	320	320	1.0	80	80	1.0	160	160	1.0	160	160	1.0
9-17 y	No vax	LAIV	453	453	1.0	80	80	1.0	80	80	1.0	320	320	1.0
9-17 y	No vax	LAIV	89	92	1.0	41	52	1.3	26	38	1.5	32	61	1.9

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When reviewing this past vaccination history in the MN data we see similar results to that of the HAI regarding prior vaccination. Many of the patients had preexisting neutralizing antibodies to A/Texas/50/2012 and many reached post vaccination titers (MN titers ≥ 40 and ≥ 110) against circulating H3N2 viruses(Figure 8)[20]. Pre- vaccination titers to A/Texas/50/2012 (vaccine- matched strain) were a stronger predictor of seroconversion to circulating strains, even more so than vaccination history and age of patient. However, it is difficult to prove that the higher baseline titers of A/Texas/50/2012(vaccine-matched strain) are not directly related to the age of the patient and prior vaccine history[20]. From the MN data we can see that those in the 9-17 year old cohort had higher baseline titers then those in the 3-8 year old cohort. This would strongly suggest that due to the naivety of the younger 3-8 year old cohort's immune system, they have not yet produced antibodies specific to the virus strains like the 9-17 year old cohort, whose past exposure generates a larger antibody repertoire. When looking at past vaccination history and its impact on baseline levels, we see there are differences, by age cohort and prior vaccination history that contribute to elicitation of higher antibody responses to current vaccination vs. higher baseline titers.

The 3-8 year old cohort, those with a past vaccination of IIV for 2013-14 season and IIV for the 2014-2015 season had highest overall antibody responses. The 9-17 year old cohort saw highest antibody response with those administered LAIV in the 2013-14 season and IIV for the 2014-15 season. This shows that past vaccination history along with age of patient does have impact on the antibody response, and may need to be taken into account when developing future indicators or predicators of vaccine efficacy.

Late into the influenza season, it became apparent that two of the strains predicted for 2014-2015 influenza vaccines [A/Texas/50/2012(H3N2) and B/Massachusetts/2/2012] did not

correlate with variant influenza strains circulating in the 2014-2015 season. Although this discrepancy did not affect our study, it did decrease the effectiveness of the vaccine to protect from the circulating strains. Shortly after this information surfaced, MedImmune, the sole producer of the LAIV vaccine, released an article addressing a production issue associated with the 2014-2015 vaccine. They concluded that the stability of the HA stalk in one of the predicted viruses, H1N1, was unstable and demonstrated free HA protein and much precipitated vaccine falling out of solution [21]. It was hypothesized that during transportation, the vaccine was exposed to high temperatures (>80°F) over an extended period of time; given the temperature sensitivity of the virus, this resulted in virus instability and protein precipitation. Based on this issue, MedImmune chose to replace the H1N1 virus strain with a more heat tolerant virus for the 2015-16 vaccine season. However, the LAIV issue clearly has implications for the results of the present study comparing IIV an LAIV for 2014-15 season. The assays assessing evaluation antibody responses elicited by LAIV vaccination demonstrated little to no response against the H1N1 stain. It will be necessary to evaluate an additional vaccine year prior to making any firm conclusions about LAIV given these unexpected complications.

Another issue affecting our results could be due to the sensitivity of the assays conducted. HAI assays are known to not be the best predictors of antibody responses to LAIV vaccines. Because the vaccines are administered intramuscularly (IIV), the use of peripheral blood serum/cells was acceptable and assays were developed for ease of use and sample collection. However, with the knowledge that influenza is predominantly replicating in a mucosal site, and the introduction of a mucosally-administered vaccine (LAIV), serum may not be the most effective way to analyze antibody responses to a mucosal infection and/or vaccine. Further, due

to the nasal route of administration, LAIV has a high retention rate in the nasal cavity and provides rapid delivery across the mucus membranes of the respiratory tract[22].

The intramuscular administration of IIV produces variable rates of absorption into the blood stream, but can be sustained over extended periods of time [22]. This may make IIV more sensitive to antibody responses in serum samples compared to LAIV simply based on the different routes of vaccine administration. It is important to consider the likelihood that LAIV is eliciting immune responses in mucosal sites (i.e., lungs) that are not being measure by the peripheral blood samples used in HAI and MN assays described here. Additional assays to evaluate mucosal samples (i.e., nasal washes) are warranted and may be more effective in evaluating immunogenicity in individuals vaccinated via the mucosal route. Furthermore, consideration should be given to measurement of nasal wash samples in current assays for patients receiving LAIV vaccine to test the usefulness of these assays in correlating with mucosal responses.

Overall, protection elicited by the 2014-2015 influenza vaccines was low. The vaccines for that season were only 23% effective [23]. On average, vaccine efficacy during a season where the vaccine closely matches the circulating influenza virus strains ranges between 50 and 60% [23]. The pediatric participants who received the IIV, especially those in the 3-8 year old cohort, had a better magnitude and breadth of antibody responses against the seasonal circulating influenza viruses then the LAIV cohort. This increased antibody response from day 0 to 21 was not expected due to two of the strains in the vaccine not matching the circulating seasonal strains, as well as the technical issues encountered with LAIV. These data lead us to believe that past vaccination may play a role in protection for years to come, plainly shows the need for more effective influenza vaccines and careful evaluation of same.

6.0   FUTURE DIRECTIONS

These studies highlight the need for better vaccine strategies and for better assays to evaluate vaccine immunogenicity and predictors of efficacy. For the most part, the LAIV remains in the nasal cavity once administered, so perhaps analyzing nasal washes rather than serum would be a better detection method for antibody response in LAIV. This study has been repeated for the 2015-2016 vaccination season to include a comparison of LAIV to IIV that was unable to take place in the current study. Many of the same Participants from the 2014-15 study were recruited in 2015-16. This is helpful given the importance of prior vaccination found in the present study. It will be interesting to evaluate antibody responses in vaccines without known complications. Finally, the roles of B and T cell responses are being evaluated in both 2014-15 and 2015-16 vaccine cohorts. Together these studies will aid in determining which vaccine elicits the best antibody response in pediatric participants.

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