University of Alberta

Influenza Vaccination in Solid Organ Transplant Recipients

by

Aliyah Baluch

A thesis submitted to the Faculty of Graduate Studies and Research

In partial fulfillment of the requirements for the degree of

Master of Science

In

Experimental Medicine

Department of Medicine

©Aliyah Baluch

Spring 2013

Edmonton, Alberta

Permission is hereby granted to the University of Alberta Libraries to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only. Where the thesis is converted to, or otherwise made available in digital form, the University of Alberta will advise potential users of the thesis of these terms.

The author reserves all other publication and other rights in association with the copyright in the thesis and, except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatsoever without the author's prior written permission.

Dedication

First and foremost I would like to dedicate my Masters to Mommy and Baba aka Faye and Mohammed H. Baluch. Without their constant love and support, I would have never been able to follow my professional dreams and come to Alberta.

I would also like to express my appreciation to my Masters' committee (Supervisor: Deepali Kumar, Atul Humar, Patricia Campbell and Simon Urschel). Hamid Liacini and Noureddine Berka at University of Calgary were also immensely helpful in running the samples for HLA alloantibody. I could not count the number of times I asked questions that were patiently answered in order to help me re-direct myself.

Lastly thank-you to my lab and office co-workers: Adrian Egli, Luis Lisboa, George Huang, Moacyr de Silva, Hillary Vanderven, Daire O'Shea, Victoria Richards and Leticia E. Wilson. They have been stalwart supporters of my work and have been there through the high and low points of the research required for my Masters.

Abstract

Immunogenicity of intramuscular influenza vaccine is suboptimal in organ transplant recipients although intradermal vaccine may be superior by targeting dermal dendritic cells to stimulate a response. 229 patients were randomized to IM or high-dose ID vaccine during the 2010-11 season. Pre- and 1 month post-vaccine bloodwork measured serology by hemagglutination inhibition assay (for influenza A/H1N1, A/H3N2 and B strains) and HLA antibody. Seroconversion was defined as a ≥4-fold rise in titer. Median time from transplant was 4.9 years. In 212 evaluable patients (105 IM, 107 ID), seroconversion to at least one antigen was 46.7% & 51.4% respectively (p=0.5). Seroconversion to at least 1 antigen was greater if i) ≥ 6 months post-transplant (53.2% vs. 19.2%; p=0.001) or ii) on <2g of mycophenolate mofetil (60.0% vs. 36.7%; p=0.001). Our study suggests that intradermal vaccine is safe, may be more immunogenic in selected subgroups and does not increase clinically relevant HLA antibody.

Table of Contents

1. Introduction
    A. Influenza molecular structure
    B. Mechanism of infection
    C. Types of influenza
    D. Influenza treatment
    E. Immunization in influenza, general
    F. Formulations of influenza vaccine
    G. Issues with animal studies in influenza vaccine research
    H. Solid Organ Transplant (SOT) Recipients and Influenza
    I. Solid Organ Transplant Recipients, Human Leukocyte Antigen (HLA) and Influenza
2. Objectives of Masters of Science
3. Hypothesis of Masters of Science
4. Methods of Masters of Science
5. Results of Masters of Science
6. Discussion of Masters of Science
7. Bibliography
8. Appendix 1

List of Tables

1. Table 1: Influenza Vaccine Requirements by CPMP
2. Table 2: Recommendations for LAIV versus Inactivated Influenza Vaccine
3. Table 3: Review of Current Literature Referencing Influenza Vaccination, HLA Antibody Production and Risk of Rejection
4. Table 4: Baseline Characteristics of Study Population During the 2010-2011 Influenza Season
5. Table 5: Vaccine Immunogenicity After Seasonal Influenza Vaccination in 2010-2011 as Measured with HIA
6. Table 6: Seroconversion to H1N1, H3N2 and B in Lung-only Patients versus Remaining Transplant Cohort
7. Table 7: Initial Results of Screening 212 Pre- and Post-vaccine Sera for HLA Alloantibody
8. Table 8: HLA Class I Screened Samples with New Alloantibody
9. Table 9: HLA Class II Screened Samples with New Alloantibody

List of Figures

1. Figure 1: Hemagglutination Inhibition Assay
2. Figure 2: Outline of Study Flow
3. Figure 3 (a through g): Reverse Cumulative Curves Detailing Different Subgroup Analyses
4. Figure 4: Seroconversion to 1 antigen, 2 antigens, and 3 antigens versus % Participants
5. Figure 5: Adverse Events within 7 Days
6. Figure 6 (a through f): Detailed Breakdown of Adverse Events Within 7 Days

1. Introduction

Annual outbreaks of influenza not only lead to decreased productivity in healthy individuals but can also lead to significant rates of morbidity and mortality especially in the immunocompromised population [1]. Symptoms of influenza include but are not limited to fever, malaise, myocarditis, gastrointestinal symptoms and respiratory symptoms. Upper respiratory infections can progress to pneumonia and respiratory failure. In Canada, Schanzer et al. have found in any given year there may be up to 20,000 hospitalizations occurring that are related to influenza and that the majority of influenza related deaths are in seniors due to pneumonia [2]. Clinical attack rates in the immunocompetent population range from 11 to 25% [3]. Solid organ transplant (SOT) recipients with influenza have higher rates of mortality requiring a more aggressive treatment paradigm in order to prevent post infectious complications such as increased graft rejection [4]. Immunocomprimised persons have higher pneumonia rates post influenza infection, higher death rates and higher rates of neurological complications post infection especially during the early post-transplant period [4-7]. Lifelong immunosuppression is likely the driving force for the increased risk of influenza infection and its complications.

A. Influenza molecular structure

Influenza is a RNA virus of the *Orthomyxoviridae* family with two predominant and clinically relevant strains: influenza A and influenza B.

The virion is predominantly found as spherical or ovoid in shape. It is 80 to 120 nanometers in diameter [8]. In the laboratory the usual form found is the non-filamentous variety though it is theorized that *in-vivo* human cases are predominantly caused by filamentous virions [9]. Each new virion leaving the host cell is encased in a lipid bilayer removed from the host cell. Antigenic matrix protein lines the inner aspect of the envelope that surrounds the influenza virion. Each influenza A and B virion has 8 segments of single stranded negative sense RNA. The 8 segments encode for 11 different proteins of which only 8 proteins are actually packaged into the newly formed infectious virion. The viral segments range in size from 890 to 2341 base pairs [10]. Influenza C, a minor strain and less clinically relevant, has only 7 segments of single stranded RNA. When the virion is packaged into its ribonucleoprotein form, it forms a helix with nucleoprotein, RNA and 3 polymerase peptides per RNA segment [11-13].

Influenza viral envelope contains predominantly 2 types of glycoprotein: hemagglutinin (HA) and neuraminidase (NA). The 17 major types of HA comprise 80% of the spikes on the exterior of the influenza virion [14]. Though HA is a trimeric protein it forms rod-shaped spikes that allow the virus to attach to a new host cell prior to infection and aid entry into cells. The receptor of HA binds and facilitates membrane fusion with the host cell membrane. There are nine major types of NA that create the remaining 20% of spikes on the outer surface of the influenza virion. NA

spikes are mushroom-shaped with a box-shaped head connected to the lipid membrane by a stalk. NA is involved in the release of newly formed virus particles from the host cell at the final stage of virion production.

The influenza virion includes numerous other proteins that are assembled into the infectious particle. Capsid protein (M1) lies inside the viral envelope and creates a layer beneath the envelope. M1 helps maintain the virion structure, whether it is spherical or filamentous. Signaling between the viral lipid membrane and ribonucleoprotein core is mediated by capsid protein. Another significant membrane protein for the influenza virus is the ion channel protein (M2). It is a multifunction proton-selective ion channel that participates in both virus entry as well as in assembly and budding of new virions. The ribonucleoprotein (RNP) complex inside the influenza virion is a combination of multiple proteins: nucleoprotein, polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA) and nucleocapsid proteins (NC). These proteins all form the influenza polymerase unit that helps mediate the binding and packaging of the viral genome.

Not all of the proteins encoded by influenza RNA are included in the virion of budding new viruses. Non-structural protein 1 (NS 1) is a multi-functional protein that has a major role in allowing the virion to evade the host's immune system but it is not included in the virion. The second protein not included is non-structural protein 2 (NS 2). NS 2 mediates the export of viral RNPs from the cell nucleus during replication. The third

protein not included in newly formed virions is polymerase basic protein 1-F2 (PB1-F2) that is present only in select strains. After being made from the second reading frame of PB1, it becomes involved in the induction of host-cell apoptosis.

B. Mechanism of infection

The HA spikes bind sialic acid moieties on the surface of the host cell, triggering endocytosis of the virus. Further details of the receptor-mediated endocytosis of an infecting influenza virion is unknown although it is theorized that activation is mediated by a membrane receptor linked to intracellular tyrosine kinases causing *de novo* production of clathrin coated pits resulting in the uptake of the attacking virion [8]. Once the virion is in the host cell, HA mediates the fusion of the viral-endosomal membranes and activates its M2 ion channel. The RNP complex dissociates from the M1 protein and migrates to the nucleus to start viral replication.

Replication and transcription of new viral material occurs in the newly infected cell's nucleus using host equipment. After new genomic RNA material and viral proteins are formulated, new RNPs are complexed in the nucleus. M1 and nuclear export proteins (NEP/NS 2) mediate the export of the new RNP complexes to the cytoplasm of the host cell [8]. Both spherical and filamentous forms of the influenza virus use lipid raft domains in the plasma membrane of infected host cells as sites of virus assembly and ultimately budding [15]. Lipid raft domains are cholesterol

and sphingolipid-enriched regions of the plasma membrane which help concentrate proteins within the plasma membrane. HA and NA of influenza are intrinsically associated with lipid raft domains but not M2. HA is superior to NA for the initiation of budding out of new virions but per experimentation if HA is mutated then NA can also initiate budding. M2 is not required for budding initiation but it is required to complete new virus budding. It is theorized that M1 crosslinks the cytoplasmic tails of HA and NA allowing them to be incorporated into the budding virion. M2 localizes to the neck of the budding virion and helps create the positive curvature allowing for the new virion to bud off. NA is thought to be involved in the final step of budding as well by cleaving off of a sialic acid from the cell surface thus allowing the bud to be released from the HA-receptor interaction [8].

C. Types of Influenza

There are 3 main strains of influenza: A, B and C. The significant differences between strains are related to site of reservoir, virulence, and prevalence.

Influenza A is the most virulent strain of the influenza virus. The main reservoir is mammals and birds. Historically numerous epidemics have been linked to specific influenza A strains. Nomenclature for influenza A is the citation of the HA followed by NA components. HA is classified as H1 to H17 and NA is classified as N1 to N9. All possible

combinations of HA and NA can infect birds but only those with H1, H2, H3, H5, H7, H9, N1, N2 and N7 have been found to cause infection in humans. For example, influenza A/H1N1 is the specific strain that was not only typed for the Spanish Flu of 1918 which killed more than 20 million persons but is also the strain of the pandemic influenza virus of 2009. The Asian flu of 1957 was H2N2, the Hong Kong Flu of 1968 was H3N2 and the Avian Flu of 2004 is H5N1. Occasionally viruses are described in further detail with the nomenclature: A/location/isolate number/year. The isolate number is arbitrary but tends to be specific per laboratory.

Influenza B is less virulent than influenza A but still can have serious consequences by instigating local outbreaks. Influenza B mutates approximately 2 to 3 times slower than influenza A. Unlike influenza A that classifies its strains according to HA and NA, there are simply 2 strains of influenza B: Yamagata and Victoria. The reservoir of influenza B is thought to include humans, ferrets and seals. In terms of post-vaccine responses, it is harder to create an adequate response to the influenza B component of the vaccine compared to the influenza A component.

Thirdly, influenza C is an influenza strain that is morphologically and genetically different from influenza A and B. Humans, dogs and pigs are the reservoirs for influenza C. Illness is usually asymptomatic.

Circulating strains of influenza A and B can mutate resulting in either minor or major changes. Exposing a population to a mutated strain allows for an increase in probability of infection as an individual's circulating antibodies produced either after exposure to the previous season's influenza virus or after vaccination will no longer be adequate for full protection. Global surveillance programs of influenza strains catalog the continuous changes around the world in circulating strains and attempt to keep abreast of potential issues for the human population. Mutations occur in influenza resulting in two different scenarios, i.e. antigenic drift and antigenic shift. Antigenic drift is the more common circumstance where mutations occur in a gradual but continuous manner for both influenza A and B strains. Point mutations occur in the amino acid sequence of the virus, resulting in minor changes in the HA and NA moieties. The slight change in the strain may be enough to evade neutralization or at least decrease the degree of neutralization by the previous influenza season's antibodies in the host. Antigenic shift, on the other hand, is a much more complex strategy and can have much more severe complications secondary to the lack of neutralizing antibody that that can recognize the newly combined influenza virion. Antigenic shift can result in epidemics and pandemics via two mechanisms. First, antigenic shift can occur when there is a major mutation of the influenza virion within a single host. Secondly genetic reassortment of influenza A virion components from different hosts can occur leading to a new virus.

The hosts usually involved in genetic reassortment tend to include birds, pigs and humans. A human host's repertoire of neutralizing antibody would be unable to recognize and neutralize the newly reassorted influenza A virus, leading to potentially deadly consequences upon infection. An intermediate host may also be involved in the creation of new influenza A strains. In addition to reassortment issues of influenza strains, the virus itself must become highly transmissible in order to spread within communities. A virus may be virulent by having new genetic material, like the Avian Flu/H5N1, but if it is not easily transmissible from human-to-human then the consequences are less. If H5N1 was to mutate in a manner that would then allow for droplet transmission, there would be definite potential for another pandemic influenza.

D. Influenza treatment

In order to decrease mortality and morbidity risks after exposure to influenza, there are multiple treatment options designed to inhibit different stages of viral infection. Originally M2 inhibitors became available in the 1960s for the treatment of influenza A infection. M2 inhibitors inhibit the influx of hydrogen ions into the virion which is required for the disassembly of the ribonucleoproteins from the virion interior. Without being released from the virion, virion RNA cannot enter the nucleus of the host and start replicating. The M2 gene is very susceptible to mutation. If 1 of 5 amino acids in the transmembrane protein mutates, the virus gains resistance to existing M2 inhibitors resulting in an irreversible change to the attachment

site in the proton channel for M2 inhibitors. The two M2 inhibitors currently on the market are Amantidine and Rimantidine. During the 2004 influenza season, the rate of M2 inhibitor resistance among human influenza A (H3N2) in the United States had risen to 92.3% [16]. Theoretically the effect of M2 inhibitors results in the decrease in the severity of influenza infection by shortening the duration of viral shedding and reduces the frequency of complications such as requiring antibiotics in the immunocompetent ambulatory patients [17]. M2 inhibitors are known to have an increased incidence of neurological toxicity as compared to neuraminidase inhibitors such as oseltamivir [18].

The second treatment option, neuraminidase inhibitors (NAIs), has activity against both influenza A and B. In general NAIs have a lower frequency of side effects while having superior activity against influenza compared to M2 inhibitors. Oseltamivir blocks glycoprotein neuraminidase so the host cells can no longer release new virions. Zanamivir also blocks glycoprotein neuraminidase but requires its dose to be given via inhalation whereas oseltamivir is an oral capsule. Zanamivir additionally may also cause bronchospasm during administration. Oseltamivir resistance has been documented in seasonal A/H1N1 as early as the 2007-2008 influenza season at a rate of 12.3% [19]. Ison et al. described a cohort of stem cell transplant recipients treated with either M2 inhibitors or NAIs had a reduced risk of progression to viral pneumonia post influenza infection and fewer superinfections. Having fewer complications post influenza

infection led to an overall decrease in mortality in the stem cell patients [20]. .

E. Immunization in influenza, general

The goals of an optimal vaccine include: safety in all patient populations and induction of an adequate protective level of antibody against a particular antigen thus leading to decreased viral transmission and/or prevention of disease. Via multiple successive research trials, ideal route, formulation and dose of the antigen are all variables that are optimized during vaccine development.

A vaccine-induced response requires a complex interaction between T- and B-cells that leads not only to antibody production after exposure to vaccine antigen but also to the production of memory B-cells. The traditional paradigm is that both T and B-cell lineages have a large antigen specific response upon exposure to a new antigen, for example after vaccination. After clearance of the antigen or infection, there is contraction of the cell populations leading to the maintenance of low volumes of memory B and T-cells. Memory B-cells can be re-activated post exposure to antigens and lead to new plasma cells producing neutralizing antibodies. Memory T-cells can rapidly proliferate as well and aid in killing infected cells as well as secreting inflammatory cytokines [21]. In addition to the production of memory B-cells, there is production of different types of memory T-cell subsets. Effector memory T-cells (T_{EM}:

CCR7-, CD62L-) tend to migrate to peripheral tissues and produce interferon-γ upon stimulation. On the other hand central memory T-cells (T_{CM}: CCR7+, CD62L+) are usually found in the lymph nodes and lack immediate effector function but have greater proliferation potential upon stimulation [21]. Murine models have shown that T_{CM} cells are more protective and efficacious against a viral challenge post vaccination compared to T_{EM}. The transplant population has additional issues with trying to maintain their immunologic memory once it is created secondary to the ongoing need of immunosuppressants [21]. An example of the poor maintenance of immunological memory is found in kidney transplants post hepatitis A vaccination. At 2 years post hepatitis A vaccination 100% of healthy controls still had protective antibody levels whereas only 59% of liver and 26% of kidney transplant recipients have an equal level of antibody [22].

A variety of factors affect the final outcome of whether or not a vaccine meets international requirements of immunogenicity. Usually proteins from the original infectious particle are utilized as antigen in the vaccine to stimulate a response by the host's immune system. The more purified the antigen, the lower the rates of an adequate immunogenic response but if the vaccine utilizes particulate and denatured proteins, then immunogenicity can be increased. Carbohydrates, nucleic acids and other molecules are all potential antigens but they need to be affixed to a protein carrier in order to create an immunogenic response. A superior

response will be elicited from antigens that are large, complex and vary greatly from self proteins as it is easier for T-cells to recognize the peptide:MHC complex leading to the induction of most antibody responses. Adjuvants can be added to vaccines in order to boost the response of the host to the antigen of the vaccine. Slow releasing adjuvants are better than those that are released rapidly into the host's circulation [23].

Immunogenicity of a particular year's seasonal or adjuvanted influenza vaccine is based on post-vaccine antibody responses and if they meet the requirements set by the European Committee for Proprietary Medicinal Products (CPMP) (see **Table 1**). Current testing post influenza vaccine is only a surrogate marker of immunogenicity as it is measuring antibody and is not necessarily synonymous with protection [24]. Antibody titers are denoted in the literature as Geometric Mean Titers (GMTs). Seroprotection is defined as a hemagglutination inhibition assay (HIA) titer greater than 40. Seroconversion is a more stringent requirement where the hemagglutination titers increase 4 folds between pre and post vaccine titers.

Table 1: Influenza Vaccine Requirements by CPMP [25]

Age (years)	**18 to 59**	**60+**
Mean geometric titer increase between pre and post vaccination	>2.5	>2
Percentage of seroprotection subjects 21 days post vaccination	>70%	>60%
Seroconversion rate or significant increase of titer 21 days post vaccination	>40%	>30%

Influenza vaccine, regardless of the final route of vaccination, is manufactured in the same general manner [26]. In embryonated hens' eggs, the allantoic compartment of the egg is accessed as each strain is inoculated separately along with a 0.5 mg solution of neomycin. After incubation, allantoic fluid is collected, clarified and the virus strains are concentrated from the egg. The strains are further purified by zonal centrifugation in a sucrose gradient. The antigens are split with Triton X-100, a type of detergent. The antigens are inactivated with formaldehyde, concentrated and then diluted again in a phosphate buffered saline solution.

Once an immunogenic version of the seasonal influenza vaccine is created via incubation in a chicken egg, there are still various factors either pertaining to the environment or particular host issues that affect a vaccine's end result. For example, the choice of the influenza strains included in any given year's seasonal vaccine is made approximately 6 months before influenza season begins in the Northern Hemisphere based on the circulating strains in the Southern Hemisphere and in the Far East. If there is a good match between the vaccine and circulating virus then the

seasonal influenza vaccine has a higher chance of being efficacious and cost-effective in immunocompetent populations [9].

Although there is a large volume of literature pertaining to immunogenicity of influenza vaccines, seasonal and adjuvanted in immunocompetent persons, there is less related to various immunosuppressed populations. It has been documented that overall the greater the individual's immunosuppression, the less likely they are to create an adequate response. It is well known that the SOT population tends to have lower responses to hepatitis A and diphtheria toxin but creates an adequate response to pneumococcus and tetanus toxoid although there is no consensus yet for responses post influenza vaccine [21]. On the other hand, those with senescent immune systems have lower requirements for a vaccine to meet international standards whilst still requiring higher doses of the vaccine antigen as is seen with Intanza® (an intradermal seasonal influenza vaccine) [26]. Recommendations for influenza vaccine usage in the SOT population are published by the AST Infectious Diseases Community of Practice as well as others [27, 28].

Different routes of vaccination have been explored in order to create better responses while minimizing side effects. Intradermal (ID) vaccines place vaccine antigen in the dermal layer below the epidermis. Langerhans' cells, a member of dendritic cells (DCs), are present in the epidermis, and dermal DCs, a type of conventional DC, are in circulation in the dermis. After injection of viral antigen, a high volume of LCs migrate

from the epidermis to the dermal layer but were interacting and participating in CD8+ humoral processes. Dermal DCs (dDCs) are theorized to be the most likely DC subtype participating in the processing of vaccine antigen to ultimately result in a vaccine response [29, 30]. After the ID vaccine antigen is introduced, the dDCs utilize the high density of lymphatics and blood vessels to quickly leave the dermis and migrate to the closest lymph node. Once in the lymph node, the DCs present antigens to T-cells which then lead to activation and expansion of both T and B-cell populations. The expansion of these populations is integral to create long lasting populations of antigen specific humoral and cellular immunity [31]. The creation of antibody against the surface hemagglutinin of the influenza virus reduces the likelihood of infection and lessens the severity of disease if infection does occur. ID vaccines require approximately 1/5th of the volume of antigen compared to IM vaccines in order to stimulate an equivalent response in immunocompetent persons thus extending the supply of vaccine as may be needed during an epidemic [30, 32]. The deltoid muscle of the arm, the standard site of IM vaccines, has a paucity of dendritic cells (DCs) used to present vaccine antigen compared to the dermis [33]. Intradermal vaccines have been used in a variety of infections other than influenza such as rabies and hepatitis B. Both monovalent and bivalent vaccine preparations have been looked at in the past as options for the influenza vaccine but with varying degrees of positive results [34].

Currently the mainstay of adult influenza seasonal influenza is intramuscular (IM) vaccination as it meets the requirements of the European Committee for Proprietary Medicinal Products CPMP (see **Table 1)**. IM vaccine side effects are generally related to the use of a long needle and the discomfort that is generated. Historically IM vaccine is manufactured in a multi-use bottle requiring the addition of a preservative, thiomersal, as a stabilizing agent. Intranasal influenza vaccination has come forward as an enterprising option for the young in order to create a superior immune response in naïve vaccine recipients. Vaccines passing via the mucosal layer of the nares may create more of a heterovariant cross-protection with the production of mucosal immunoglobulin A though generally the use of direct antigen delivery via oral or nasal routes lead to a poor immune response [35]. In addition intranasal influenza vaccines are live attenuated vaccines and therefore, not applicable to immunocompromised patient populations such as SOT recipients.

Adjuvants may be added to vaccines to boost the level of immunogenicity independent from route of delivery. A boosted response may be particularly advantageous in a naïve population receiving their sentinel vaccination. Adjuvants may be any substance that enhances the response without creating stable linkages with the immunogen, unlike protein carrier molecules. Adjuvants typically increase inflammation and activate the complement cascade. Vaccine adjuvants can expose vaccine

antigen longer at the site of vaccination to antigen presenting cells (APCs) resulting in a more robust response [35]. There are 6 main adjuvants currently used in vaccine technology. Incomplete Freund's adjuvant is a synthetic emulsion, oil-in-water, that not only delays the release of antigen but enhances the uptake of the antigen by surrounding macrophages. Complete Freund's adjuvant is also an oil-in-water adjuvant but is combined with dead mycobacteria. Freund's adjuvant with muramyldipeptide (MDP) is an oil-in-water emulsion with only a single component of mycobacteria, MDP. The mechanisms of action for complete, incomplete Freund, and Freund's adjuvant with MDP are the same. A common adjuvant utilized is alum (aluminum hydroxide gel) by itself or in combination with killed *Bordetella pertussis*. Alum acts as an adjuvant by delaying the release of antigen and creating an enhanced macrophage uptake. Lastly there are adjuvants that are immune stimulatory complexes (ISCOMs) such as matrix of Quil A. It helps induce the production of cytotoxic T-cells [35, 36].

When assessing if an individual patient is a candidate for the annual influenza vaccine, one must consider a variety of issues first. Standard exclusion criteria for the annual influenza vaccine includes a history of severe allergy or anaphylaxis to any of the components in the influenza vaccine - egg, neomycin or formaldehyde - it is recommended not to receive further doses of the vaccine. Immunization should be delayed in persons with recent febrile illnesses. If a patient presents with

a recent history of neurological disorders such as Guillain-Barre syndrome (GBS) then vaccination is recommended to be delayed. Conversely if a person develops GBS within 6 to 8 weeks after a previous influenza vaccine, both the National Advisory Committee on Immunization (NACI) and the Advisory Committee on Immunization Practices (ACIP) recommend never to vaccinate again [37]. NACI recommends vaccination for all pregnant persons as a population with increased risk for complications during influenza infection [37]. Intanza®, intradermal influenza vaccine, currently has no warnings against its use in pregnancy. Generally NACI also states that continued breastfeeding is also safe after influenza vaccination.

F. Formulations of influenza vaccine

There are many ways to take influenza antigens and manipulate them to make safe vaccines in a variety of patients. Each formulation of influenza vaccine attempts to exploit different mechanisms of the immune system to create a superior immunologic response. Influenza vaccines are divided first into live attenuated versus inactivated vaccines [13]. Live attenuated cold-adapted influenza stains (LAIV) are often used in children as their first ever exposure to influenza antigens in order to stimulate a superior response. LAIV themselves may be able to be produced more rapidly compared to inactivated vaccines but unfortunately it also requires a higher level of biosafety containment [35]. Inactivated vaccines can be further categorized as whole virus vaccines, split vaccines and subunit

vaccines. Whole inactivated vaccines are more immunogenic than purified versions but have a higher rate of side effects. The higher incidence of adverse events is theorized to be secondary to the presence of viral RNA being recognized by Toll like receptor 7 (TLR 7) and causing a higher amount of inflammation [38]. Split vaccines are vaccines where the viral antigens have been disrupted by detergents or solvents whereas subunit vaccines are simply purified HA and NA. LAIV and inactivated vaccines are recommended for different age groups based on previous literature showing which age groups get the best benefit from which type of vaccine (see **Table 2**).

Table 2: Recommendations for LAIV versus Inactivated Influenza Vaccine

Type of Influenza Vaccine	Age Group	Misc.	Advantages
Seasonal inactivated vaccines	Age > 6 months		Better in those with previous vaccinations
Seasonal LAIV	Age 2 yrs to 49 yrs	Cannot use in less than 2 yrs secondary to concern for wheezing and hospitalizations; post hoc analysis showed there was no efficacy for those 50 to 64 yrs	Better in those with limited previous vaccination or infection

LAIV has been shown in a meta-analysis of children to have greater efficacy as well as greater durability of response compared to inactivated vaccination [39, 40]. LAIV also was found to protect its recipients from infection but has a more limited increase in hemagglutinin titers [41]. Beyer et al. evaluated the efficacy in all age groups comparing LAIV

versus trivalent inactivated vaccines (TIV) in a meta-analysis. They found that there was no difference in reactogenicity or efficacy between the 2 types of inactivated influenza vaccine. There was also no significant difference in the vaccines' abilities to protect against drifted strains [41].

Research is ongoing into the creation of a universal influenza vaccine that would be impervious to issues generated secondary to antigenic drift and perhaps even to antigenic shift [42]. Using a mouse model, researchers are exploring options with an influenza vaccine whose proteins mimic M1 and NP as they are more conserved between different yearly strains. Ferret models using the M2 and NP influenza vaccine only worked with exposure to a low dose pathogen but failed to remain immune when exposed to high doses of influenza viral exposure. Ideally if M2 and NP provided robust immunity to influenza then a single infection would provide lifetime immunity which is not supported by follow-up serology. Additional issues being addressed as part of improving the influenza vaccine includes research into the increase in use of oil-in-water emulsions to augment response especially in immunocompromised populations. During the original outbreak of pandemic H1N1 in 2009, a second influenza vaccine was manufactured and augmented with adjuvant.

Pandemrix® is a monovalent adjuvanted inactivated split-virus vaccine manufactured by Glaxo-Smith Kline. A single dose of Pandemrix® is 0.5 mL which contains 3.75 μg of antigen (influenza

A/California/7/2009 H1N1 like strain X-179A). It was manufactured in 5 mL multidose vials where each dose contained thiomersal as a preservative. The adjuvant used in Pandemrix® was AS03: including squalene (10.69 mg), DL alpha tocopherol (11.86 mg) and polysorbate 80 (4.86 mg). Following vaccination, false positive serology can occur if one uses ELISA as the testing method for HIV-1, hepatitis C and HTLV-1. Importantly confirmatory testing such as Western blot will be unaffected and therefore negative. These transitory false positive tests may occur secondary to IgM production post influenza vaccination [43].

Intanza® is the intradermal (ID) seasonal influenza vaccine licensed by Sanofi Pasteur as of May 2010. It is a split virion vaccine using inactivated influenza strains. There are 4 clinically relevant non-medical ingredients included in this vaccine: neomycin, formaldehyde, ovalbumin and Triton X-100 (a detergent). Split virion is in reference to the use of Triton X-100 to disrupt the vaccine particles to insure inactivation. Additionally sodium chloride, potassium chloride, disodium phosphate dehydrate and potassium dihydrogen phosphate constitute the buffer solution for a total vaccine volume of 0.1 mL. During drug exploration, Sanofi Pasteur tested 6, 9, 15 and 21 μg of each antigen in a 0.1 mL volume leading to FDA approval for both 9 and 15 μg doses. For adults aged 18 to 59 years, the FDA recommends the 9 μg/strain/0.1mL whereas in adults with age 60 years or greater the FDA recommends 15 μg/strain/0.1 mL version of Intanza® to meet CMPA requirements. The

data reported to FDA was extracted from 4 large clinical trials held in Europe, Australia and New Zealand where Intanza® was compared head-to-head with the standard IM influenza vaccine with 15µg/strain/0.5 mL. Within 7 days of vaccination, the most common side effects experienced with Intanza® were erythema, induration, headaches, myalgia and malaise. Most reactions were self-limited and resolved within 1 to 3 days of vaccination though there were a few cases where local erythema lasted up to 7 days. A higher proportion of those with side effects were within the age group of 18 to 59 compared to those aged greater than or equal to 60 despite the older group receiving a greater amount of antigen in their vaccine. Unusual side effects included lymphadenopathy within 21 days of the vaccine. Infrequent neurological sequelae were diagnosed post vaccination, for example there was a case of neuritis 15 days post vaccination in a patient who received the 15 µg dose as he/she was aged greater than 60. To assess duration of effect, those within the 18 to 59 year old group were found to have seroprotection rates and geometric titers still above pre-vaccination levels at the 12 month follow-up time point. Intanza® uses a 1.5 mm micro-needle that reaches the dermis when applied perpendicularly to the skin, typically in the deltoid of the non-dominant part of the arm. It is not necessary to visualize a wheal at the site of injection in order to assess if the vaccine has been applied correctly. Even if liquid is seen leaving the injection site, per the package insert, it does not change the immunogenicity of the vaccine [26].

G. Issues with animal studies in influenza vaccine research

Trying to recreate the complex interaction between the human host immune system and the influenza virus is very difficult. Humans are hosts that can be repeatedly exposed to influenza antigens via serial vaccinations or infections over a lifetime. This is very difficult to recreate in an animal model. The host is effectively already primed prior to the newest influenza vaccine about to be given. Currently ferret animal models can be induced to have an acute respiratory illness after intranasal inoculation of modest doses of human influenza, therefore mimicking infection [44]. There are distinct differences though between the immune responses of ferrets and children under 3 years of age. For example, if a ferret is infected with either H1N1 or H3N2, then they get partial cross-protection from a challenge of a separate hetero-subtypic strain unlike infants [45, 46].

H. Solid Organ Transplant (SOT) Recipients and Influenza

Influenza infection risk increases once a newly transplanted patient returns to his or her own home and is in contact with the community. In North America the influenza season peaks generally in January and February. During the influenza season, SOT recipients are found to have higher rates of hospitalization and higher rates of complications if infected such as the increased risk of disease progression from an upper respiratory illness to pneumonia. In the case of lung transplant recipients,

the transplanted graft is in direct contact with the influenza virus which has been documented to lead to graft dysfunction after influenza infection [47].

The volume of published literature is constantly increasing discussing the wide range of influenza vaccine protocols – seasonal, adjuvanted, booster vaccine either with seasonal IM or ID vaccine – and the response rates in the SOT population along with the factors affecting rates of seroconversion and seroprotection as measured with the hemagglutination inhibition assay (HIA). Certain authors have found that SOT recipients have poorer response as measured by HIA compared to immunocompetent controls yet other authors find comparable levels of HIA production in other SOT cohorts [48-50]. Overall there are few studies discussing cell-mediated immunity post influenza vaccination: a separate aspect of the immune system which is thought to aid in viral clearance during an ongoing influenza infection and may help an individual develop a humoral response post infection [51].

Using HIA as the method of choice for serology measurement, certain cut offs and definitions must be set for seroprotection and seroconversion. As per WHO recommendations and various supporting studies, a titer of greater than 1:40 (or its Japanese equivalent of 1:128) is consistent with seroprotection post influenza vaccination [52-55]. Hirota et al. noted that increased age independently decreased one's ability to create an equivalent increase in antibody post vaccination [54]. Repeated influenza vaccination with inactivated influenza vaccine is also known to

have diminishing returns as the first vaccination gives the greatest amount of protective effect though subsequent vaccinations may decrease the incidence of new influenza-like illnesses [54, 56, 57].

I. Solid Organ Transplant Recipients, Human Leukocyte Antigen (HLA) and Influenza

The HLA system categorizes a number of genes related to the function of the human immune system and how the immune system recognizes self from non-self. Chromosome 6 encodes for the MHC genes involved in HLA recognition. MHC class I and class II proteins are used to present a variety of antigens to CD8+ and CD4+ cells, respectively. MHC class I (A, B and C) complexes are found on all nucleated cells. MHC class 1 molecules are complexed with viral particles that have been broken down in the cytoplasm by proteosomes and then taken to the endoplasmic reticulum to meet the MHC class I moiety. Once complexed, transported to the gogli apparatus and then transported again to the cell membrane, it can present a particular viral antigen to CD8+ cells. Conversely MHC class II (DP, DM, DQ and DR) molecules present antigens that have been engulfed by an antigen presenting cell (APC) or lymphocyte. APCs take up viral proteins via endocytosis and then break down the proteins into antigens. These antigens in an endosome are joined by partially fabricated MHC class II moieties. After the complex of MHC class II and antigen are joined then it is transported in a vesicle to the cell membrane. Once in place the class II MHC-peptide complex is

recognized by CD4+ T-lymphocytes leading to stimulation of T-helper cells and therefore leading towards antibody production by B-cells.

Multiple factors can affect an individual's level of circulating HLA antibodies. In the general population the most likely reason to have HLA antibodies is secondary to heterologous immunity post infection or post vaccination. A history of blood transfusions or previous pregnancy can also lead to the production of *de novo* HLA antibody. Lastly if a person has received a transplanted organ, the graft may became a target of the host's immune response leading to *de novo* HLA antibodies or more specifically donor specific antibodies (DSA). In the circumstance of kidney transplants for example, a failed kidney transplant is not usually removed. The patient remains on low dose immunosuppression to decrease the risk of forming *de novo* DSA and not in order to keep the organ from failing.

HLA is especially important in the field of transplantation as the organ recipient recognizes a donor's graft as non-self via its MHC class I and class II antibodies and attempts to reject through a combination of antibody production and complement fixation. In an attempt to decrease the risk of rejection of any given graft, pre-transplant work-ups of patients include HLA screening and a variety of tests i.e. virtual or real crossmatching. Currently the majority of HLA labs utilize solid-phase assays that identify HLA-specific antibodies to help risk stratify the transplant candidates [58]. Solid-phase HLA assays include both Flow cytometric HLA analysis and Luminex-based HLA analysis which are

considered to have high sensitivity and specificity, allowing them to quickly become the new gold standard for HLA measurement [59, 60]. A positive virtual crossmatch aides in classifying which patients may benefit from recipient IVIG or plasmapheresis prior to transplantation. As more information is published in reference to solid-phase assays for HLA assessment, the transplant community is always looking to improve test quality and analyze the need for the addition of more relevant HLA antigens to current assays. Certain viral infections and drugs are known to downregulate HLA expression temporarily. A sample analyzed on the Luminex platform may unable to detect on screen the patient's HLA alloantibodies. In order not to formulate a false assumption, some recommend testing for HLA antibodies at multiple time points and therefore follow the trend [61, 62].

Results derived from current solid-phase HLA assays may vary greatly between sites for a number of reasons including technologist variations and substrate differences. A well documented example of variability between HLA assays was exemplified with the single antibody specificity testing of HLA-A2 by 8 labs spread over 2 countries using LabScreen Single Antigen Test (One Lambda, Inc, Canoga Park, Ca, USA). The labs reported a mean fluorescent intensity (MFI) that ranged from 2000 to 9000 reinforcing that HLA testing is not an ideal quantitative exam but that its result is more qualitative in nature [58]. Another limitation of HLA testing is the inability to correlate antibody concentrations

to a MFI measurement on a crossmatch. The relationship between the antigen density on a target cell or at the antigen concentration on any given bead in the HLA kit should not be directly compared to its MFI measurements. There is a range of antigen density on a single antigen bead for either class I or II so they cannot be used to conclude such details about the target cell [58].

Virtual crossmatches utilize information gathered from solid-phase HLA assays analyses of both the potential donor and recipient. A positive crossmatch may occur secondary to interactions with non-HLA antibodies or secondary to HLA antibodies not yet identified for use on the solid-phase HLA assays. Not all positive crossmatches though result in graft failure especially as there are now protocols to decrease the theoretical risk of a positive crossmatch. If a positive crossmatch is thought to be secondary to true antibody production in the recipient then he/she may require plasmapheresis and/or IVIG prior to transplantation [58]. Long term graft survival after these protocols is not known at this time and protocols for desensitization do not work equally on all ethnicities [63].

Though routine measurements for *de novo* HLA alloantibody production is not done, if there is concern for graft dysfunction as a cause of new only HLA antibodies or DSA then testing is undertaken and compared to the most recent HLA alloantibody measurement. Transplant recipients are continuously at risk for HLA antibody formation secondary to the constant exposure to the donor graft and fluctuating levels of

immunosuppressants compounded by the increased requirement for blood products especially peri-transplant. Baseline rates of *de novo* HLA antibody production have been measured in the range between 5 and 6% per year in certain SOT populations [64, 65].

An increase in HLA antibody post influenza vaccine is theorized to be secondary to multiple issues occurring singly or in concert. First, the amino acid sequence for influenza hemagglutinin and neuraminidase in the vaccine could include epitopes shared by transplant recipient HLA proteins. Katerinis et al. could not find any similarities using BLAST analysis to support this mechanism [64]. Alternatively certain HLA antigens could be triggered a B-cell response towards the influenza vaccine antigens (bystander activation). This response then would create a cross-reaction with specific HLA molecules leading to an increase in HLA antibody production [51, 65, 66]. Thirdly nonspecific reactions can occur between antibody generated by the influenza vaccine and the HLA beads of the assay. In the cases of adjuvanted vaccines using squalene or tocopherol-based adjuvants (AS03), there might be additional issues affecting the situation. The adjuvant mimics sets of conserved molecules that lead to the enhancement of the innate immune system thus resulting in better stimulation of T- and B-cell responses to the antigen of the vaccine. This augmented response may trigger a higher rate of nonspecific reactions including a greater production of *de novo* HLA antibodies.

Table 3 (see below) is a review of the current literature as of February 2012 detailing articles measuring rates of rejection ± the use of HLA antibody production post influenza vaccination. A mild increase of HLA antibody post influenza vaccination is seen as a trend, especially post adjuvanted vaccine. There is a lack of consensus in the published literature at this point in reference to the risk for rejection post influenza vaccination. More notable is that the majority of papers detailing effects of influenza vaccination in the SOT population failed to have adequate length of follow-up as most trials followed their cohorts for a maximum of 6 months post vaccination, therefore episodes of graft rejection may be underreported [67].

Table 3: Review of Current Literature Referencing Influenza Vaccination, HLA Antibody Production and Risk of Rejection (adapted and updated from Kumar et al. [49])

First author/ year (ref #)	Number of patients/ organ	Mode of vaccine/ adjuvant	Where/ When/ Control group	HLA antibody measurement	Exclusions	Lab and clinical findings	Duration and issues at final follow-up/ conclusion
Blumberg/ 1998 [68]	1993-1994: 9 heart 1994 - 1995: 5 heart	1 dose of seasonal influenza vaccine in 1993-1994 2 doses 3 wks apart in 1994-1995	USA, 1993-1994 and 1994-1995 1993 - 1994: 9 heart transplants without vaccine 1994 - 1995: 5 heart transplants without vaccine	n/a		All patients had heart biopsy between 2 and 7.5 wks post vaccine or enrollment 4 of 14 patients s/p vaccine had biopsies c/w rejection gr 2 to 3A 1 of 14 controls had biopsies c/w rejection gr 2 (p=0.326)	Patients were followed up for a minimum of 2 years post enrollment in the protocol. Influenza vaccine is safe but may be associated with low-level histologic rejection. There was no increased risk for rejection in the booster group. Clinically there were no long-term sequelae of vaccination.
Brakemeier/ 2011 [69]	Single vaccine: 60 kidney Booster vaccine: subset of 19	1 dose of AS03-adjuvanted pH1N1/A vaccine (Pandemrix®) 2nd dose was 3 wks after first	Germany, 2009-2010 season 22 healthy controls	Pre, 3-4 wks post vaccine (if 1 dose) or 2 months post second dose via CDC		3 of 60 patients developed new DSA where 2 of the 3 had biopsy provide acute antibody-mediated rejection post vaccine and 1 pt lost his graft within 10 wks post vaccine secondary to refractory acute humoral rejection	Final HLA measurement was approximately 4 months post vaccination
Broeders/ 2011 [70]	111 kidney	1 dose of AS03-adjuvanted pH1N1/A vaccine (Pandemrix®)	Belgium, 2009-2010 season 21 healthy controls, 53 HD patients	Pre and 4 wks post vaccine via Luminex		HLA class I pre/post: 15% vs. 14% HLA class II pre/post: 14% vs. 14%	Influenza A/H1N1 adjuvanted vaccine was safe Humoral response was lower in kidney transplants than even HD pts

Candon/ 2009 [51]	63 kidney	1 dose of seasonal influenza vaccine	France, 2005-2006 season 19 healthy controls	Pre and 4 wks post vaccine via Luminex		*De novo* DSA with very low MFIs in 3 of 63 (4.8%) post vaccine	No cases of new clinically relevant DSA or change in pre-existing DSA; no cases of rejection at the 3 month follow up
Carroll/ 1974 [71]	25 kidney	1 dose of seasonal influenza vaccine	UK, 1970-1971 season 17 healthy controls	n/a		Serum creatinine was followed pre vaccination, post vaccination at 1 mo, 3 mo and 12 mo	No new cases of rejection post vaccine. 4 pts who had rejection prior to vaccine continued to have issues with rejection post vaccination.
Danziger-Isakov/ 2010 [72]	9 lung 8 kidney	1 dose of seasonal influenza vaccine of corresponding year	USA, 2006-2007 season and 2007-2008 season 30 healthy controls s/p vaccine; 20 healthy controls without vaccine	Pre, post vaccine at 2 wks, 4 wks and 12 wks via FlowPRA Screening		All vaccinated persons had increased cellular response to human alloantigens post vaccination, maximum effect 2 to 4 wks post vaccine, waned by 3 months in most persons	Graft function in transplant patients was followed up to 1 yr post vaccination (FEV_1 in lungs, creatinine in kidneys). There were no episodes of acute rejection
Katerinis/ 2011 [64, 73]	Cohort 1: 92 kidney Cohort 2: 59 kidney	Seasonal influenza vaccine of 2009 + 2 doses of AS03-adjuvanted pH1N1/A vaccine (Pandemrix®)	Switzerland, 2009-2010 season Historical cohort control (recipients from 2008-2009 season)	Cohort 1: Pre, 4-6 wks post 2nd dose of adjuvanted vaccine, 6 months f/u if indicated Cohort 2: Pre, 4-8 wks post 2nd dose of adjuvanted vaccine, 6 months f/u if indicated Testing via Luminex	Cohort 2: excluded if + Class I and II	Cohort 1: 16 of 92 (17.3%) had new anti-HLA antibodies (I, II or both classes) Cohort 2:7 of 59 (11.9%) had new anti-HLA antibodies (I, II or both classes) DSA: + in 13 of 20 pts for initial f/u	Up to 8 months post last vaccine Only 1 patient had an increase or stable level of DSA 19 of 20 patients had decreased levels of DSA 2 pts of the 20 with DSA had an increase in creatinine: first pt's bx with TMA (C4d negative), second pt's bx with acute humoral rejection (C4d positive)

Kimball/ 2000 [74]	29 heart	1 dose of seasonal influenza vaccine	USA, 1998-1999 season No controls	Pre, post vaccine at 3 to 4 wks via PRAStat		Comparing the rate of anti-HLA antibodies prior to vaccination and post was 0 vs. 2 (p NS), both were low level and were not DSA	No clinically relevant antibody was produced post vaccination. The rates of rejection were similar prior to vaccination as to after vaccination at 6 months (9 vs. 4, p NS)
Kumar/ 2011 [75, 76]	60 lung	2 doses of seasonal influenza vaccine 4 wks apart	Canada, 2006-2007 season No controls	Pre, 4 wks post first vaccine, 4 wks post second vaccine via Luminex		21 of 60 with positive HLA screening post vaccine though *de novo* alloantibody was only in 1 patient and it was not DSA	There were no cases of vaccine induced DSA. 5 patients had rejection by the 6 month follow-up without a relationship to HLA
Magnani/ 2005 [77]	Cohort 1: 21 heart Cohort 2: 21 heart	Cohort 1: 1 dose of seasonal influenza vaccine: without adjuvant (Agrippal®) Cohort 2: 1 dose of seasonal influenza vaccine with MF59 adjuvant (Fluad®)	Italy, 1999-2000 season 16 heart transplants without vaccine	n/a		Scheduled cardiac biopsies were done every 4 to 6 months (mean time from vaccination to biopsy was 44 ± 26 days)	During 6 month follow-up in ref to ≥ gr 3A rejection, 1 in Agrippal® group, 1 in Fluad® group and 2 in control group (p=0.30)
Sanchez-Fructuoso/ 2000 [78]	49 kidney	1 dose of seasonal influenza vaccine	Spain, 1996-1997 season 37 healthy controls	n/a		Bloodwork was at – 6 mo, - 3 mo, day 0, + 1 mo, + 3 mo, and + 6 mo	There were no episodes of acute rejection in the vaccine group. Mean creatinine and creatinine clearance improved in vaccine arm (p<0.01)
Schaffer/ 2011 [79]	15 heart	1 dose of AS03-adjuvanted pH1N1/A vaccine (Arepanrix®)	Canada, 2009-2010 season 45 heart transplants without vaccine	n/a		No increase in *de novo* DSA or antibody mediated rejection	Post vaccine 1 of 45 vs. controls with 6 of 15 with acute cellular rejection grade ≥2 (p=0.001)
Scharpe/ 2008 [50]	165 kidney Booster of	1 dose of seasonal influenza vaccine	Belgium, 2003-2004 season	n/a		Renal function was assessed at D0, post 1 wk, 1 month and up	No episodes of acute rejection were found during the 6 month

	vaccine: subgroup of 83	2nd dose was given 3 months after the first	41 healthy controls			to 6 months	follow-up
Schuurmans/ 2011 [80]	Cohort 1: 148 lungs Cohort 2: 115 lungs	Cohort 1: 1 dose of AS03-adjuvanted pH1N1/A vaccine (Pandemrix®) Cohort 2: 2 doses of AS03-adjuvanted pH1N1/A vaccine (Pandemrix®) separated by 3 to 4 wks 131 of 148 hearts also had seasonal influenza vaccine in the same year	Switzerland, 2009-2010 season 20 heart transplant without vaccine	n/a		Clinical follow-up was initially 1 to 2 wks post vaccination; evidence for allograft rejection (i.e. bronchiolitis obliterans via FEV_1) was measured at the 6 month follow-up post vaccine; rate of influenza infection was statistically lower in vaccinated arm (2 of 148 vs. 5 of 20 in controls, $p<0.01$)	During 6 months post vaccine period, 7 potential BOS and 7 BOS (stages 1-2) in vaccinated lungs vs. 1 potential BOS and 5 BOS (stages 1-3) thus there was no increased rate of BOS in those vaccinated

CDC:complement-dependent lymphocytotoxicity

gr: grade

n/a: not applicable

wks: weeks

DSA: donor specific antibody

MFI: mean fluorescence intensity

FEV_1: forced expiratory volume in 1 sec

2. Objectives of Masters of Science[1]

The primary objective of the study was to assess if high-dose intradermal influenza vaccine lead to a superior immunologic response compared to standard intramuscular influenza vaccine in solid organ transplant (SOT) recipients. Serology was used as a surrogate marker for immunological response as per the standard in influenza research. The secondary objective was to assess the risk of *de novo* HLA alloantibody production after either high-dose intradermal influenza vaccination or standard intramuscular influenza vaccination. Lastly detailed information was to be collected in reference to the general safety of the novel use of high-dose intradermal influenza vaccines within the solid organ transplant population.

3. Hypothesis of Masters of Science

High-dose intradermal influenza vaccination should lead to superior immunological responses compared to standard intramuscular influenza vaccine in SOT recipients. In addition, high-dose intradermal influenza vaccination will not lead to an upregulation in *de novo* or pre-existing HLA antibody. High-dose intradermal influenza vaccine is hypothesized to be safe for use within the SOT populations.

4. Methods of Masters of Science

Patient population and study design

Our study was conducted at the University of Alberta Hospital in Edmonton, Alberta, Canada after receiving institutional research ethics board approval. Clinically

[1] A version of this chapter has been submitted for publication. Baluch et al. 2012.

stable outpatient adult organ transplant recipients were screened using the study's predetermined inclusion and exclusion criteria starting in August 2010. Patients were included if they were at least 3 months post transplant and had not yet received the 2010-11 influenza vaccine. Once included in the trial and they had provided written informed consent, SOT recipients were randomized to receive their influenza vaccines from October until December 2010. The vaccines used during the 2010-2011 influenza season were either the standard IM vaccine (Vaxigrip®) or the high-dose ID vaccine (Intanza®). The study was registered at www.clinicaltrials.gov number NCT01180699.

At enrollment, patients received either standard dose IM or high-dose ID seasonal influenza vaccine in a 1:1 ratio. Randomization was done using a computer generated schedule, in blocks of four to ensure equal numbers in each interventional arm. Once the patient agreed to participate, the treatment assignment was provided by the study coordinating office. As the vaccines appeared grossly different and were administered via different means, the patient and study team member were unblinded at the time of vaccine administration. Both vaccines contained the same three influenza antigens: influenza A/California/7/2009 (H1N1)-like virus, influenza A/Perth/16/2009 (H3N2)-like virus and influenza B/Brisbane/60/2008 (Victoria lineage). The IM vaccine, Vaxigrip® (Sanofi-Pasteur, Canada), contained 15μg antigen of each strain in the standard 0.5mL volume. The split virion vaccine was injected into the deltoid muscle of the non-dominant arm as per standard practice. The ID vaccine, Intanza® (Sanofi-Pasteur, Canada), is also a split virion vaccine. The version available as of 2011 in Canada was the pre-filled syringe containing 9 μg antigen per strain in 0.1 mL volume. In order to reach a cumulative dose of 18 μg of antigen, two doses in quick succession

were given in the deltoid area of the non-dominant arm of the recipient. Sera were collected pre- and 1 month post vaccination for 1) strain-specific serologic testing and 2) HLA alloantibody measurements. Detailed information was collected on all participants including but not limited to demographics, type of transplant, and immunosuppression data at the time of vaccination.

Adverse events data was collected by those blinded to vaccine allocation. Additionally, the outcomes assessor was masked to treatment allocation (observer blinded). Safety data were collected at the following post-immunization times: 24 hours, 48 hours, 7 days, 1 month, and 6 months. Phone calls were done at 24 hours, 48 hours, 7 days and at 1 month in order to assess the adverse events in real time. The 6 month follow-up was provided by a blend of phone calls and chart review. All adverse effects were graded as none, mild (no interference in daily activities), moderate (some interference in daily activities) and severe (patient unable to participate in activities of daily living). Local adverse events included issues such as erythema, induration, tenderness and pruritis at the site of vaccination. Systemic effects were fever, fatigue greater than baseline, nausea and vomiting. Patients were followed for a total of 6 months from vaccination for the development of influenza infection and biopsy-proven allograft rejection.

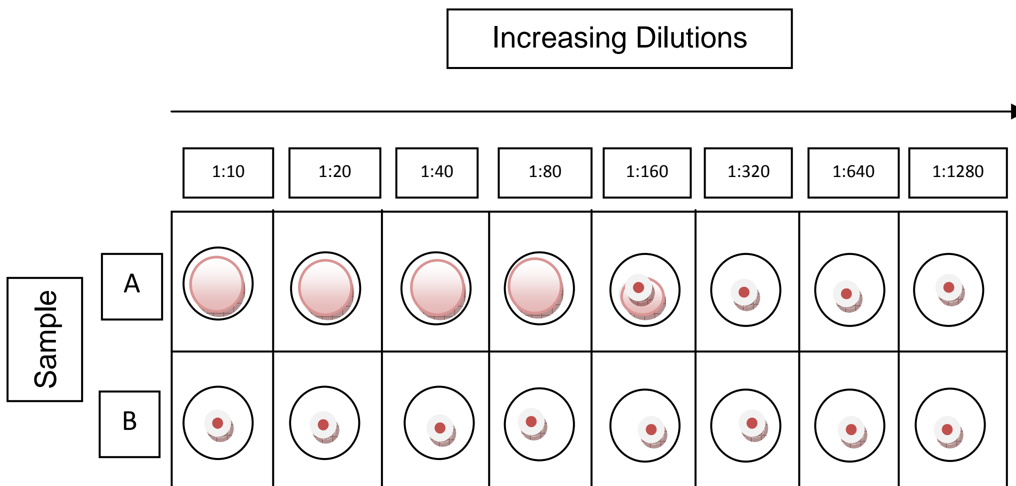
Laboratory Methods: Hemagglutination Inhibition Assay

Sera were collected pre- and 1 month post-vaccine in 212 SOT vaccine recipients out of 229 and were stored at -80°C until the day of analysis. The mean follow-up bloodwork was collected 36.48 days post vaccination (95% CI 31 to 40.75).

The samples underwent serologic testing for each of the three strains in the 2010-2011 seasonal influenza vaccine using the standard technique of hemagglutination inhibition assay (HIA) at the Health Protection Agency, U.K. via a previously described method [81]. The laboratory staff performing the assay was blinded to vaccine allocation. During the HIA process, the sera of the patients were incubated with a receptor-destroying enzyme to remove any nonspecific inhibitors of hemagglutination. Serially diluted sera were then incubated with influenza virus (containing 4 hemagglutination units of virus) followed by addition of 0.5% turkey red blood cells. Titers were determined by doubling dilutions of antibody where the initial dilution was 1:10 and the final dilution was 1:1280. Antibody concentrations that were below the lower limit of detection (<10) were assigned a concentration of 5 for the purposes of analysis (see **Figure 4** as an illustration of the theory behind HIA measurements). All sera were tested in duplicate and their results were required to be within 1 dilution of each other as per lab policy.

Figure 1: Hemagglutination Inhibition Assay

HIA is traditionally run in a 96-well plate. Sample A inhibits hemagglutination until the 1:160 titer so the HIA titer is 1:160. The sample in B has no detectable antibody as there is agglutination in all wells so the titer would be less than 1:10.



Laboratory Method: Human Leukocyte Antigen (HLA) Antibody

212 patients' pre- and post-vaccination sera underwent HLA screening with LIFECODES LifeScreen Deluxe (Gen-Probe®) per package insert at the HLA reference lab at University of Calgary, Calgary, Canada. The initial screen was designed to detect IgG antibodies to both HLA Class I and Class II antigens using coated micro beads. In brief, 5µl of HLA bead mix was incubated with 12.5µl of patient sera in the dark for 30 minutes on a rotating platform at room temperature. After 4 washes, a secondary antibody, goat anti-Human IgG conjugated to phycoerythrin, was added and incubated for an additional 30 minutes as per protocol. After the last wash, samples were read on

the Luminex Fluoroanalyzer. The mean fluorescent intensity (MFI) of each bead was compared to the signal intensity of the negative control beads within the same preparation in order to calculate if a particular bead was positive or negative for bound alloantibody.

If the samples pre-vaccine screened negative for HLA antibody but became post-vaccine positive, they were set aside for additional testing with single antigen bead (SAB) testing via Luminex Single Antigen Bead Assay (OneLambda®) using a similar methodology as described above. A positive SAB specimen was categorized as positive if it had a MFI > 1000 and included a change in MFI of greater than 20% from pre- to post-vaccine.

Methods: Definitions and Statistics

The following variables were used to assess vaccine immunogenicity: seroprotection to an individual strain was defined as a post-vaccination titer of greater than or equal to 1:40 and seroconversion was defined as a ≥ 4-fold rise in titer from baseline. Seroconversion factor (SCF) was derived by dividing the post immunization titer by the pre-vaccine titer. Geometric mean fold rise (GMFR) was calculated as the geometric mean of seroconversion factor. Our sample size was based on historical data showing a vaccine response after intramuscular vaccination of 60% and a hypothesized increase in immunogenicity of 20% after the integration of high-dose ID vaccine. Using an alpha of 0.05 and an increase in vaccine immunogenicity from 60% to 80%, power was calculated at 90%. Demographics were analyzed using descriptive statistics. Our primary endpoint comparing intramuscular (IM) versus intradermal (ID) vaccination was defined as seroconversion to at least 1 of 3 influenza vaccine antigens.

Associations between factors affecting vaccine response and statistical analysis was performed using IBM SPSS version 19.0 (Chicago, IL) and GraphPad Prism version 4.0 (La Jolla, CA). Univariate analyses were performed to determine the most significant factors affecting seroconversion to at least one vaccine antigen. These factors included patient age, time from transplant, type of organ transplanted, and immunosuppression. The multivariate model was constructed using variables that had a p-value < 0.2 on univariate analysis or those that are known to be clinically important in affecting vaccine response as per the literature. Multivariate analysis was performed using stepwise conditional regression. Statistical significance was defined as a $p < 0.05$.

5. Results of Masters of Science

Patient Population

From October 2010 to December 2010, we enrolled 229 organ transplant recipients (115 IM, 114 ID). Baseline characteristics of the cohort were similar between intervention groups at the start of the trial and are detailed in Table 4. The four most common types of transplant were kidney (n=94, 41.0%), lung (n=74, 32.3%), liver (n=26, 11.4%), and heart (n=18, 7.9%). The overall median time from transplant to vaccination was 4.9 (0.2 - 32.4) years. Maintenance immunosuppression was similar in terms of drug choice between the IM and high-dose ID groups as well as the number of patients that received anti-thymocyte globulin in the 6 months prior to enrolment.

Table 4: Baseline Characteristics of Study Population During the 2010-2011 Influenza Season

Characteristics	Intramuscular (IM) N=115 (%)	Intradermal (ID) N=114 (%)	P value via chi-square	Total N=229 (%)
Age in years, median (range)	55.4 (19.7 to 76.6)	53.7 (21.4 to 76.9)	0.286	54.3 (19.7 to 76.9)
Gender (Male/Female)	79/36	84/30	0.405	163/66
Time from Transplant to 2010/2011 Influenza Vaccination				
Median (Range in years)	4.5 (0.3 to 29.3)	5.4 (0.2 to 32.4)	0.625	4.9 (0.2 to 32.4)
Type of Transplant				
Lung	36 (31.3%)	38 (33.3%)	0.743	74 (32.3%)
Kidney	51 (44.3%)	43 (37.7%)	0.308	94 (41.0%)
Heart	7 (6.1%)	11 (9.6%)	0.317	18 (7.9%)
Liver	13 (11.3%)	13 (11.4%)	0.981	26 (11.4%)
Combination	8 (7.0%)	9 (7.9%)	0.787	17 (7.4%)
Retransplant	3 (2.5%)	8 (7.0%)	0.119	11 (4.9%)
Documented pandemic H1N1 infection in 2009/ 2010	2 (1.7%)	1 (0.9%)	1.000	3 (1.3)
Use of ATG in prior 6 months	4 (3.5%)	5 (4.4%)	0.748	9 (3.9%)
Maintenance immunosuppression				
Prednisone	83 (72.2%)	85 (74.6%)	0.683	168 (73.4%)
Tacrolimus	86 (74.8%)	85 (74.6%)	0.969	171 (74.7%)

Cyclosporine	26 (22.6%)	18 (15.8%)	0.190	44 (19.2%)
MMF/MPA	76 (66.1%)	86 (75.4%)	0.120	162 (70.7%)
Azathioprine	18 (15.7%)	14(12.3%)	0.462	32 (14.0%)
Sirolimus	12 (10.4%)	16 (14.0%)	0.406	28 (12.2%)

ATG: antithymocyte globulin

Vaccine Immunogenicity

Of the 229 enrolled patients, 17 did not have both pre- and post-vaccination sera and thus were excluded from the immunogenicity analysis (see **Figure 2**) leaving serologic measurements to be conducted on 212 patients (105 IM, 107 ID). Overall, no significant differences in immunogenicity between the two cohorts were seen (**Table 5**). Post-immunization seroprotection to H1N1, H3N2 and B strains was 70.5%, 63.8%, and 52.4% respectively in the IM group, and 71.0%, 70.1%, 63.6% in the ID group (p=0.93, 0.33, and 0.10). The seroconversion rate to influenza antigen was low regardless of vaccine type (ranging from 17 to 38% in both arms of the cohort). Seroconversion to at least one antigen was 46.7% and 51.4% in the IM and ID groups respectively (p=0.49). There were no significant differences between seroconversion to at least 2 or 3 antigens when comparing IM versus ID vaccination.

Figure 2: Outline of Study Flow

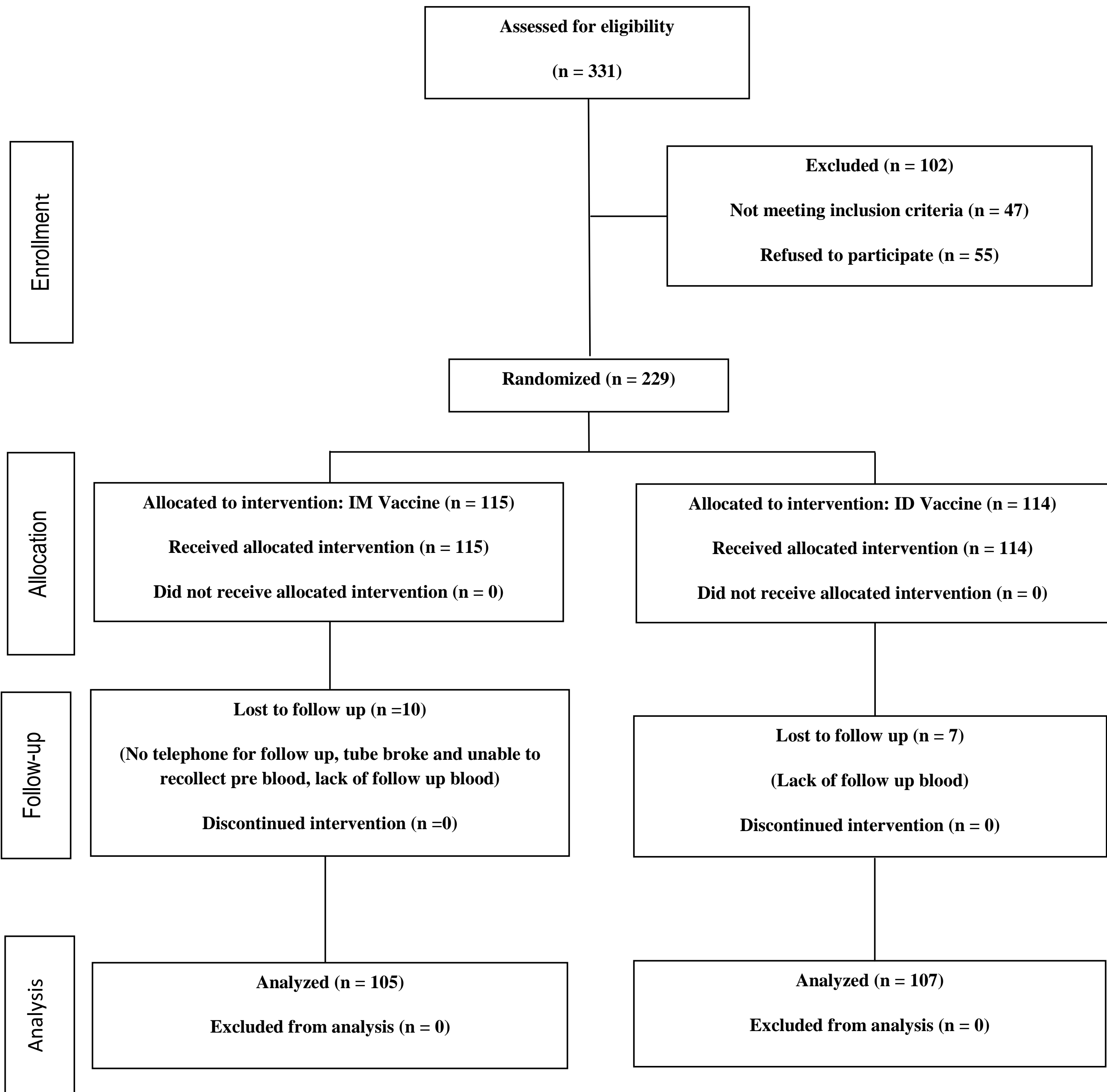


Table 5: Vaccine Immunogenicity After Seasonal Influenza Vaccination in 2010-2011 as Measured with HIA

Variable	Intramuscular Group (n=105)	Intradermal Group (n=107)	P-value via chi-square
GMT (95% CI)			
A/H1N1			
Before vaccination	22.1 (16.7 – 29.3)	24.0 (18.2 – 31.6)	
Post vaccination	62.2 (45.7 – 84.9)	68.9 (50.4 – 94.3)	0.611
A/H3N2			
Before vaccination	19.7 (15.9 – 24.6)	21.9 (17.0 – 26.7)	
Post vaccination	43.6 (33.5 – 56.7)	51.1 (40.2 – 64.8)	0.484
B			
Before vaccination	17.0 (13.4 – 21.5)	18.6 (14.4 – 24.0)	
Post vaccination	29.1 (22.3 – 38.0)	41.31 (31.8 – 53.6)	0.074
Seroprotection Rate (%)			
A/H1N1	74 (70.5%)	76 (71.0%)	0.930
A/H3N2	67 (63.8%)	75 (70.1%)	0.331
B	55 (52.4%)	68 (63.6%)	0.099
Seroconversion Rate (%)			
A/H1N1	36 (34.3%)	40 (37.4%)	0.638
A/H3N2	32 (30.5%)	31 (29.0%)	0.811
B	18 (17.1%)	23 (21.5%)	0.422
Geometric Mean Seroconversion Factor			
A/H1N1	2.8191	2.8746	0.639
A/H3N2	2.1936	2.3467	0.811
B	1.7183	2.2184	0.424

GMT: Geometric Mean Titer

In terms of the entire cohort, baseline seroprotection (prior to vaccination) to A/H1N1, A/H3N2, and B was present in 42.9%, 42.0%, and 36.3% respectively (no significant difference in rates of baseline seroprotection in the IM arm versus high-dose ID). Excluding those with seroprotection at baseline, rates of seroconversion after IM and high-dose ID vaccination to A/H1N1 were 45.8% and 48.4% (p=0.77), to A/H3N2 it was 32.8% and 40.4% (p=0.39), and to influenza B it was 21.7% and 31.8% (p=0.19). Geometric mean titers (GMTs) after high-dose ID vaccination trended to be higher against influenza B (p=0.074). Figure 3 (a through g) depicts the reverse cumulative curves for the various groups and Figure 4 displays the percentage of participants who seroconverted to only 1, 2, or 3 antigens.

Figure 3: Reverse Cumulative Curve Detailing Different Subgroup Analyses

(X-axis: HIA titers in increasing manner and y-axis: the percentage of the cohort with the particular HIA titer)

Figure 3a: All 3 strains presented together

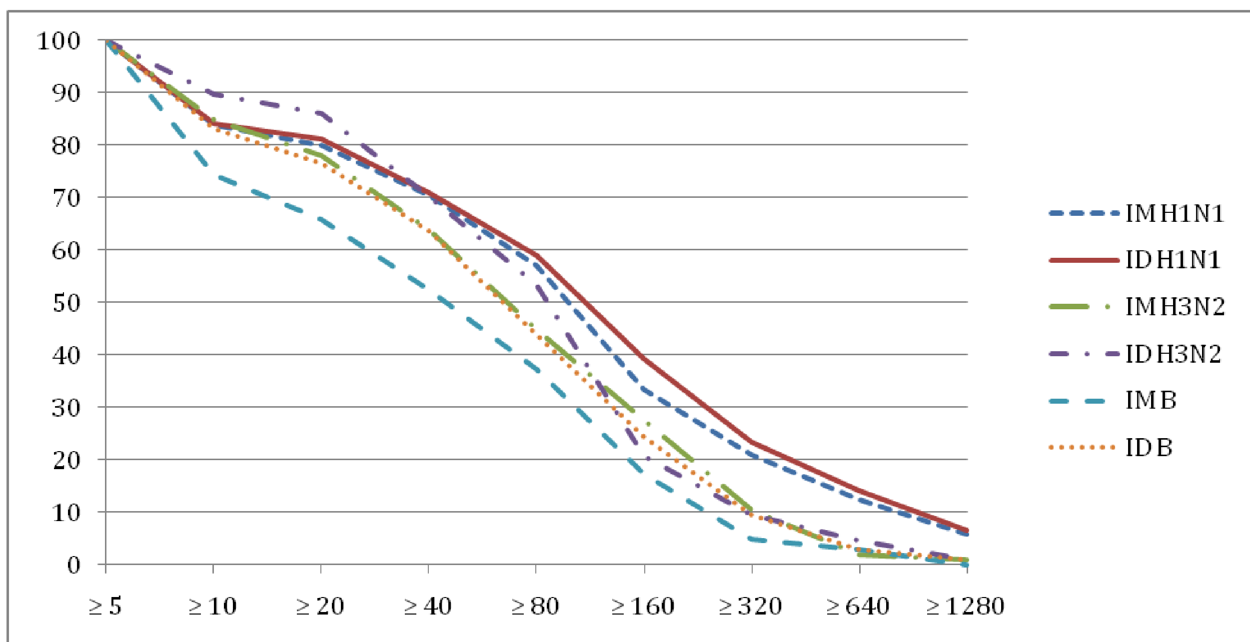


Figure 3b: influenza A/H1N1

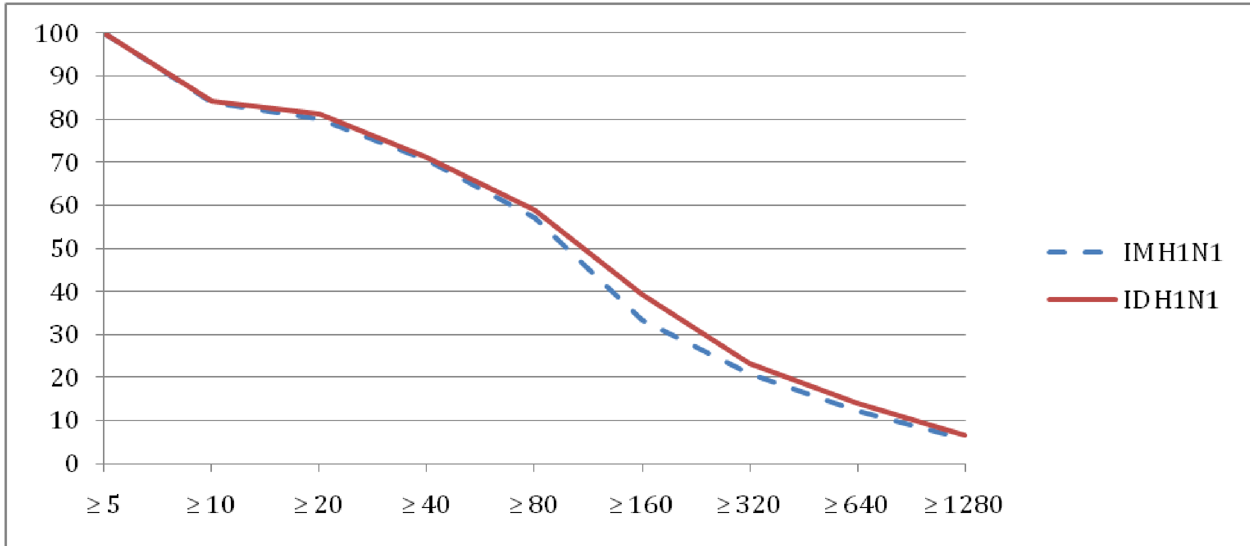


Figure 3c: influenza A/H3N2

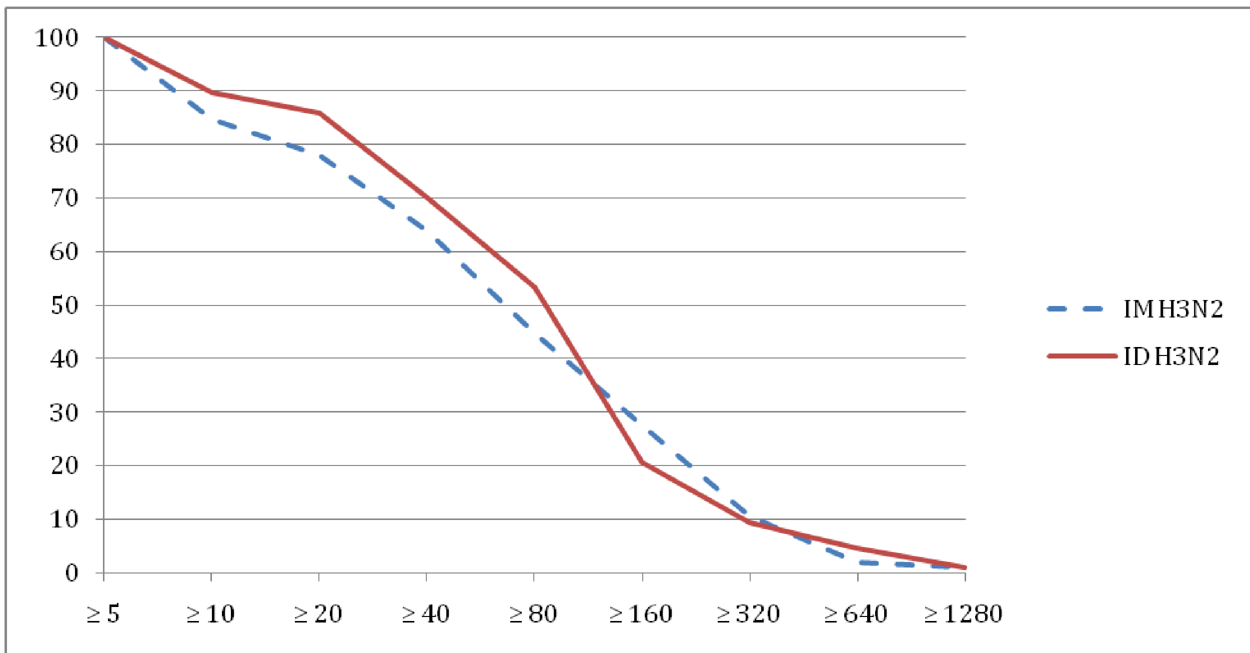


Figure 3d: influenza B

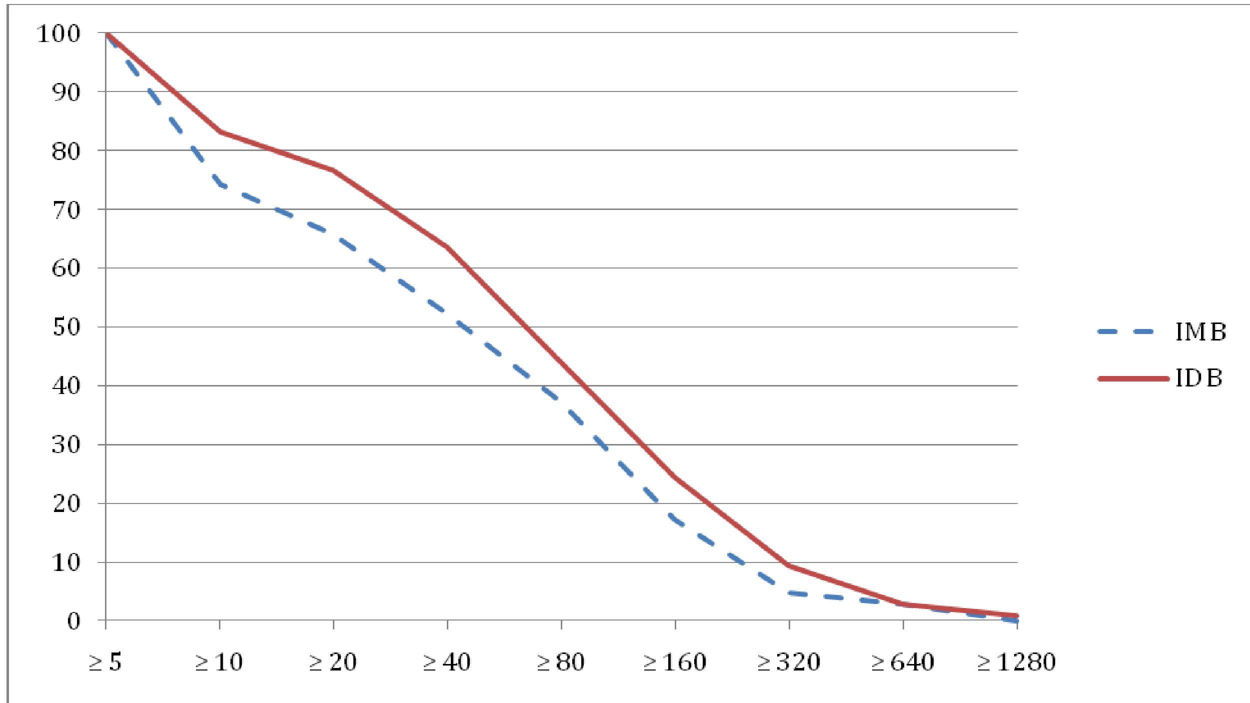


Figure 3e: MMF <2 gms vs. ≥ 2gms (where MMF <2 gms includes those not on MMF)

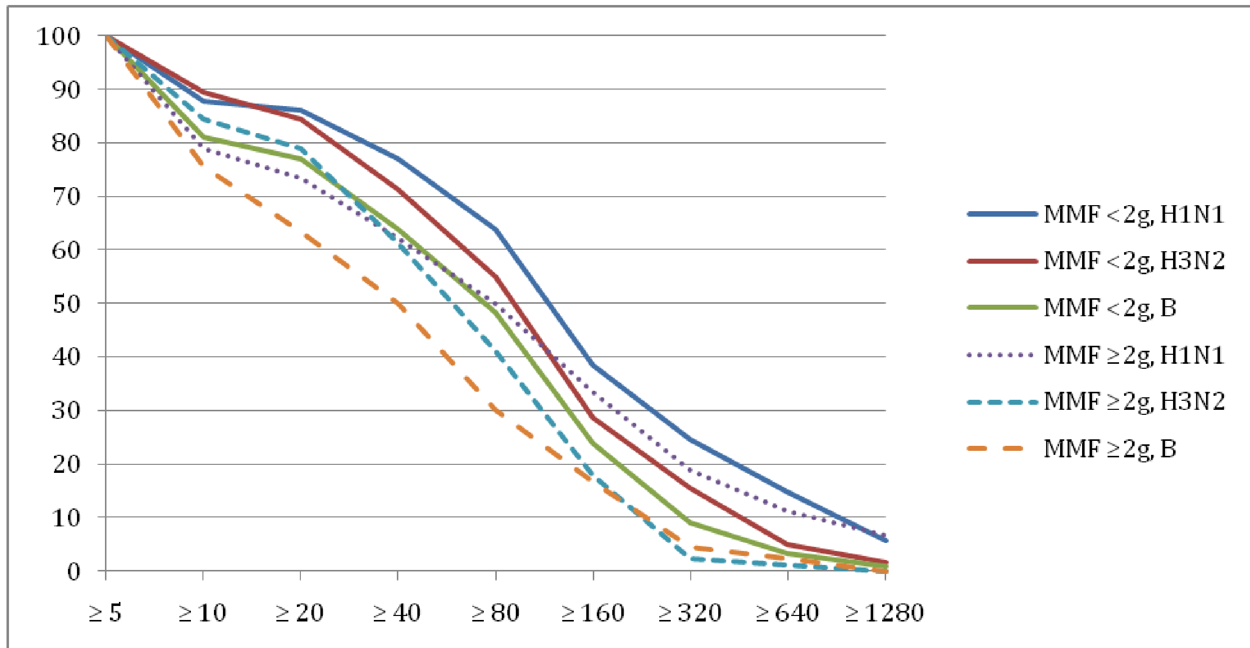


Figure 3f: Time from transplant until immunization

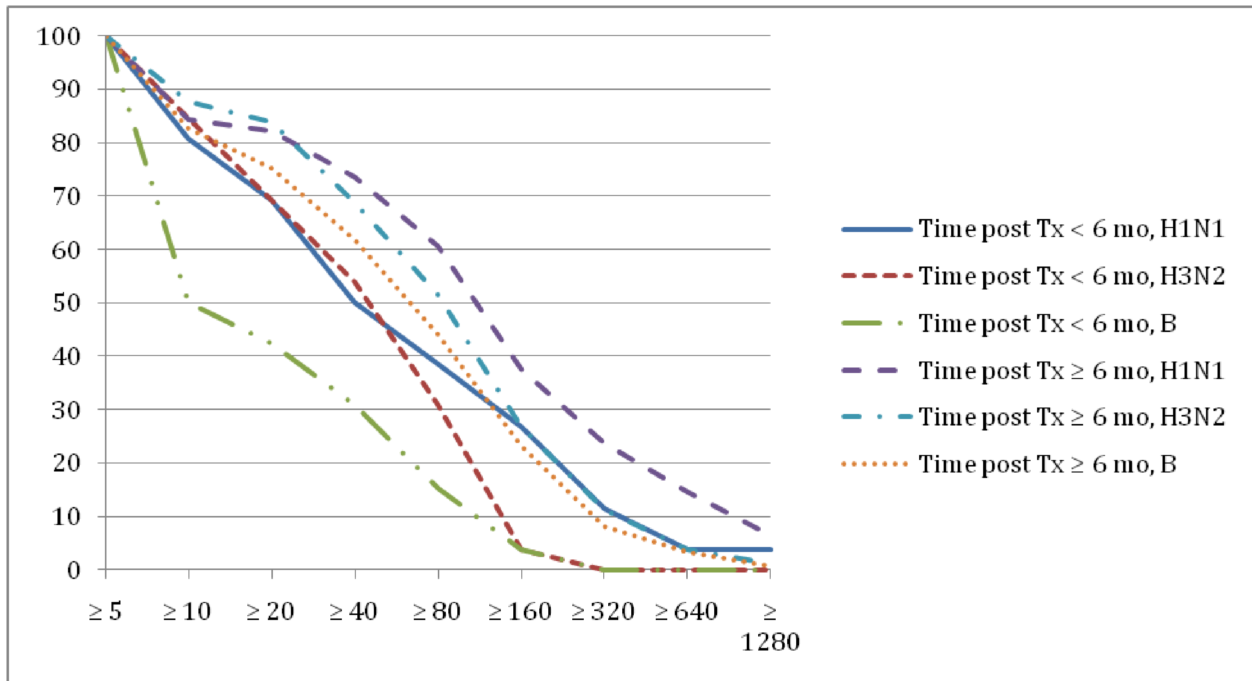


Figure 3g: Lung vs. Non Lung

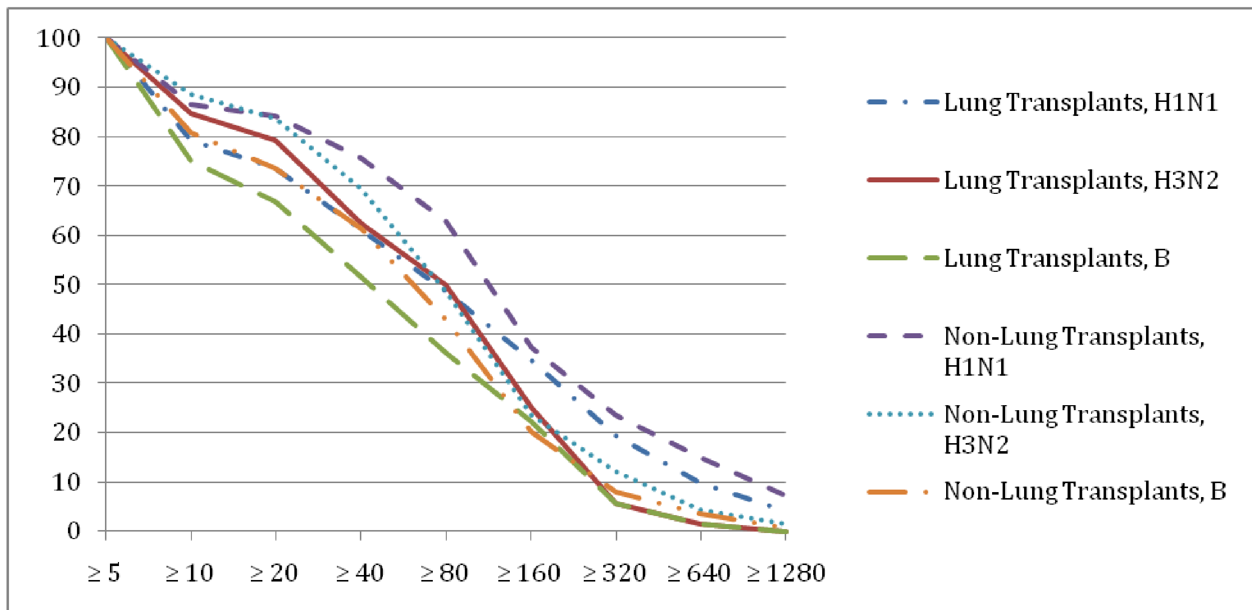
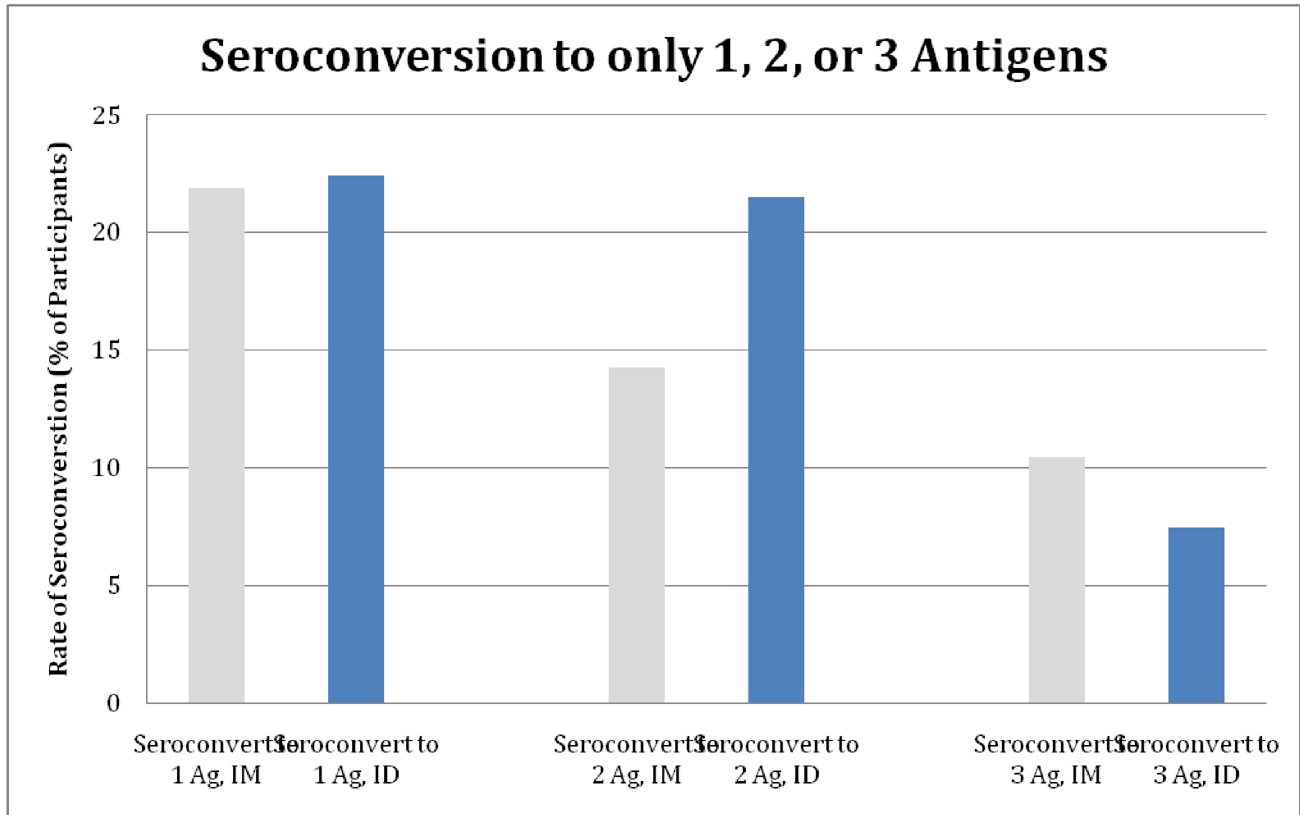


Figure 4: Seroconversion to 1 antigen, 2 antigens, and 3 antigens versus % Participants



Influenza infection rates overall were low in this large cohort of SOT recipients post vaccination during the 2010-2011 season in Edmonton, Canada. Three patients developed microbiologically-documented influenza A infections between 1 and 6 months after immunization. Of the serotyped samples (n=2), they were both influenza A/H3. The third patient contracted influenza while in China for work. All three participants were double lung transplant recipients whom had been transplanted more than 1 year prior to vaccination. Their disease processes were Talcosis, cystic fibrosis and emphysema. Two of the three patients had achieved seroprotective titers to A/H3N2 after immunization though none had achieved levels consistent with seroprotection against A/H1N1. The gentleman with emphysema who had

seroprotection titers (1:80) post vaccination for H3N2 still went on to have microbiologically proven influenza A. There was no protocol to screen SOT recipients of the influenza vaccine on a regular basis. The need to screen for influenza infection was based on the patient's primary transplant physician.

Factors Affecting Vaccine Response

Background literature detailing post-vaccine responses in SOT transplant populations support that there is a difference in serological response per organ and level of immunosuppression after either infection or vaccination for influenza [51, 82-84]. Thus, we underwent subgroup analysis in our cohort comparing individual organ groups or combinations of organs. Lung transplant recipients had the lowest rates of seroconversion to influenza A/H1N1 when compared to the non-lung group (26.4% vs. 40.7%; p=0.05). Seroprotection to A/H1N1 post-immunization was 61.1% in lung transplant vs. 75.7% for non-lung transplants (p=0.04). Conversely we analyzed humoral immunity among the non-lung transplants (n=140, comprising primarily of liver and kidney recipients) and noted that the recipients of the high-dose ID vaccine had significantly greater geometric mean titers (p=0.031), seroprotection rates (p=0.027), and seroconversion factors (p=0.008) to influenza B. Additional rates of seroconversion to the trivalent vaccine of 2010-2011 in both lung transplants and the remaining cohort are detailed in Table 6.

Table 6: Seroconversion to H1N1, H3N2 and B in Lung-only Patients versus Remaining Transplant Cohort

	Lung-only Transplants (n=72)	Remaining Cohort (n=140)	P-value via chi-square
H1N1			
Seroconversion	19 of 72 (26.4%)	57 of 140 (40.7%)	0.0492
Seroconversion after ID	9 of 36 (25.0%)	31 of 71 (43.7%)	0.0899
Seroconversion after IM	10 of 36 (27.8%)	26 of 69 (37.7%)	0.3882
H3N2			
Seroconversion	19 of 72 (26.4%)	44 of 140 (31.4%)	0.5264
Seroconversion after ID	7 of 36 (19.4%)	24 of 71 (33.8%)	0.1757
Seroconversion after IM	12 of 36 (33.3%)	20 of 69 (30.0%)	0.6609
B			
Seroconversion	10 of 72 (13.9%)	30 of 140 (21.4%)	0.2003
Seroconversion after ID	4 of 36 (11.1%)	19 of 71 (26.8%)	0.0820
Seroconversion after IM	7 of 36 (19.4%)	11 of 69 (15.9%)	0.7857

Further analysis was done looking at the rates of seroconversion versus various other factors previously mentioned in the literature. First, seroconversion to at least one vaccine antigen was lower if immunization was performed when patients were less than 6 months post transplant (19.2% vs. 53.2%, p=0.001 in the overall cohort). (Technically the comparison was the rate of seroconversion between those 3 to 6 months post transplant versus those 6 months and greater post transplant due to the previously mentioned inclusion/exclusion criteria of the cohort.) Also GMTs for all three vaccine strains were significantly lower in those vaccinated at less than 6 months post transplant compared to those vaccinated more than 6 months post transplant (A/H1N1, p=0.044; A/H3N2, p=0.015; B, p<0.001). During further subgroup analysis, those who were prescribed ≥ 2 g of mycophenolate mofetil (MMF) at the time of vaccination had significantly lower GMTs and lower rates of seroconversion to influenza A/H1N1 and

influenza B (p=0.001 and p<0.001 respectively). Seroconversion to at least 1 antigen was 36.7% in those taking ≥ 2g MMF vs. 60.0% in those receiving < 2g to 0g MMF (p=0.001). If specifically comparing those on ≥ 2g MMF versus those < 2g to the lowest possible MMF dose, seroconversion to at least 1 antigen is 36.7% vs. 53.5% respectively. Lastly, analysis of serologic responses comparing transplant recipients less than 60 years of age versus greater than or equal to 60 years showed no significant association with vaccine responses.

For multivariate analysis the model included vaccine type (IM versus high-dose ID), time from transplant (< 6 months versus ≥ 6months), organ type (lung versus non-lung), MMF dose < 2g versus ≥ 2g, and age < 60 versus ≥ 60 years. In this analysis, the use of MMF < 2g was significantly associated with increased seroconversion to at least 1 antigen (OR 2.59, 95% CI 1.47 to 4.57, p<0.001). Lastly, the timing of vaccination more than 6 months was independently associated with greater rates of seroconversion to at least 1 antigen (OR 4.78, 95% CI 1.73 to 13.21, p=0.001).

HLA alloantibody, DSA and Vaccine Safety

We screened 212 pre- and 1 month post-vaccine sera for both class I and class II HLA antibody. The screened samples were assigned to 1 of 4 categories in reference to HLA class I and class II. The categories were 1) pre-vaccine screen negative/ post-vaccine screen negative, 2) pre-vaccine screen negative/ post-vaccine screen positive, 3) pre-vaccine screen positive/post-vaccine screen negative and 4) pre-vaccine screen positive/post-vaccine screen positive (see **Table 7**). Of these, 10 patients from Class I and 15 patients from Class II converted their status from negative screen pre-vaccine to

positive post-vaccine and are detailed in Tables 8 and 9 (there was 1 common patient between both classes so from here onwards the total will be referred to as 24). HLA antibody screen was positive for class I antibody in 10/24, class II antibody in 15/24, and both classes in 1/24 patients. One patient had biopsy-proven rejection during the 6 month follow-up. The patient was a cardiac transplant recipient with a history of rejection prior to influenza vaccination and fluctuated between biopsy proven 1R to no rejection without a correlating increase in HLA alloantibody or donor specific antigen (DSA). Further specificity testing in the 24 patients was done utilizing single antigen bead methodology. 3 of 24 (12.5%) patients met our initial criteria for a positive test as their MFIs were greater than 1,000 post-vaccine and the increase from pre- to post-vaccine was more than 20%. The three patients were recipients of kidney, kidney-pancreas, and liver transplants. Two of the recipients had received IM vaccine and one had received ID. Of these three, two transplant recipients had increases in non-donor specific antibody whereas the third had increases in previously measured DSA. There were no episodes of biopsy proven rejection within the 3 cases and after review by two independent HLA experts, the changes were deemed not clinically significant. There was no significant change as measured with graft function in the three patients between the pre- and 1 month post-vaccine time points (i.e. GFR and creatinine in the kidney recipients and liver enzymes in the liver recipient).

Table 7: Initial Results of Screening 212 Pre- and Post-vaccine Sera for HLA Alloantibody

	HLA Class I (%)	**HLA Class II (%)**	**Total**
Pre-/Post-	150 (70.8%)	104 (49.1%)	254
Pre-/Post+	10 (4.7%)	15 (7.0%)	25
Pre+/Post-	11 (5.2%)	19 (9.0%)	30
Pre+/Post+	41(19.3%)	74 (34.9%)	115
Total	212	212	424

Table 8: HLA Class I Screened Samples with New Alloantibody

Characteristics	**N=24**
Gender (male/female)	17/7
Median Age (years; range)	52.3 (29 - 72)
Time from Transplant to Vaccination (years; range)	3.14 (0.26 to 23.35)
Organ (%)	
Kidney	13 (54.2%)

Lung	6 (25.0%)
Liver	3 (12.5%)
Heart	2 (8.3%)
HLA	
Class I	10 (41.7%)
Class II	14 (58.3%)
Class I and II	1 (4.2%)
Vaccine Type	
Intramuscular	12 (50%)
High-dose Intradermal	12 (50%)

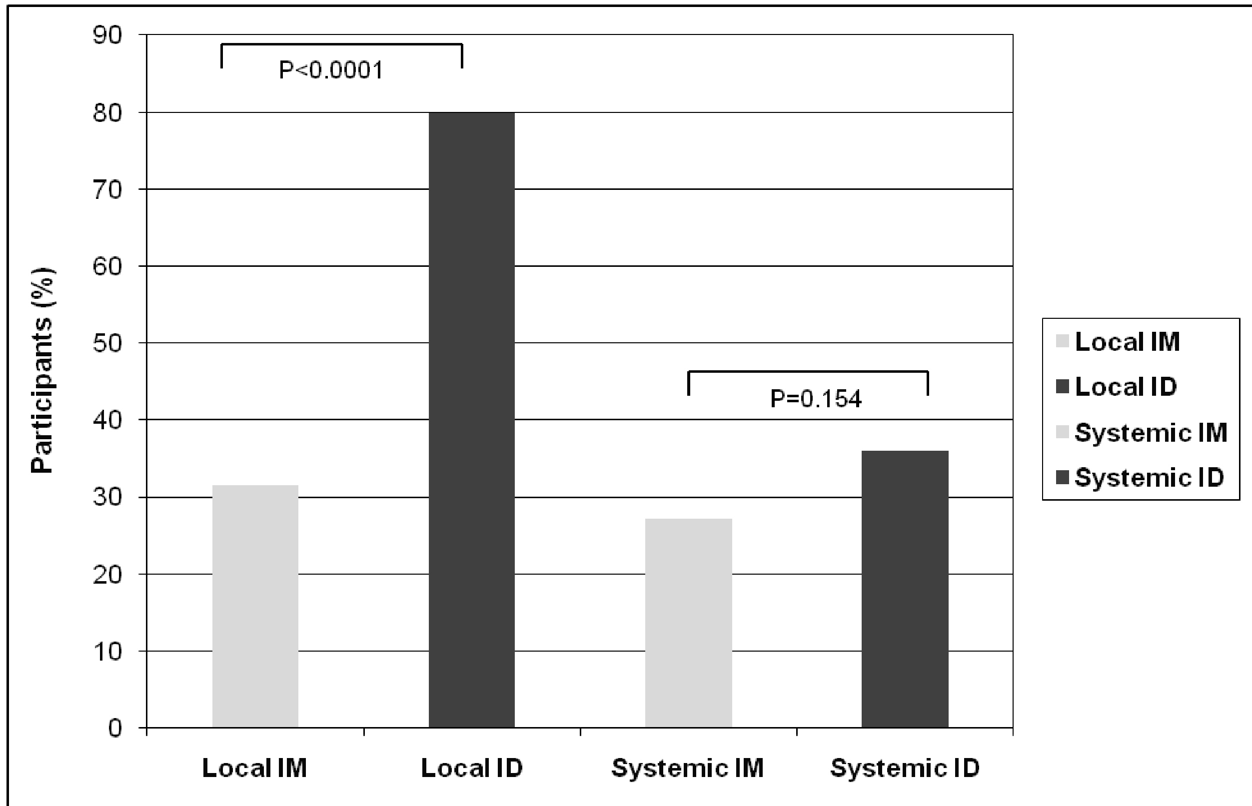
Organ **(n=10)**	Lung 2 (20%) Kidney 7 (70%) Liver 1 (10%)
Gender (M/F)	8/2
Median age in yrs (range)	55.64 (41 to 72)
Time from Transplant to Vaccine in yrs	3.14 (0.27 to 13.41)
Vaccine type	IM 6 (60%) ID 4 (40%)
Previous influenza infection	none

Table 9: HLA Class II Screened Samples with New Alloantibody

Organ **(n=15)**	Heart 2 (13.3%) Lung 4 (26.7%) Kidney 7 (46.7%) Liver 2 (13.2%)
Gender (M/F)	9/6
Median age in yrs (range)	49.46 (29 to 69)
Time from Transplant to Vaccine in yrs	1.64 (0.26 to 23.35)
Vaccine type	IM 7 (46.7%) ID 8 (53.3%)
Previous influenza infection	none

Vaccine safety was assessed at regular intervals up to 6 months post vaccination in 228/229 patients. A single patient was unavailable for telephone consultation for follow-up of adverse events. A cumulative view of adverse events within 7 days post-immunization is presented in Figure 5 and then further detailed in Figure 6 (a-f). There was no significant difference in rates of systemic adverse effects between standard IM and high-dose ID vaccination but there was a significantly higher rate of local adverse events after high-dose ID vaccine ($p<0.001$). There was a single participant who complained of inability to perform daily activities due to his level of pruritus post ID vaccination. There were no hospitalizations as a direct result of immunization. At 6 months post vaccination, 3 patients had died (2 secondary to cardiovascular events and one due to the recurrence of the patient's primary liver disease), all in the ID group.

Figure 5: Adverse Events within 7 Days



Symptom ranking

Mild: does not interfere with normal activities

Moderate: some interference with normal activities

Severe: prevent subjects from engaging in normal daily activities

Figure 6: Detailed Breakdown of Adverse Events within 7 Days

Figure 6a: Erythema

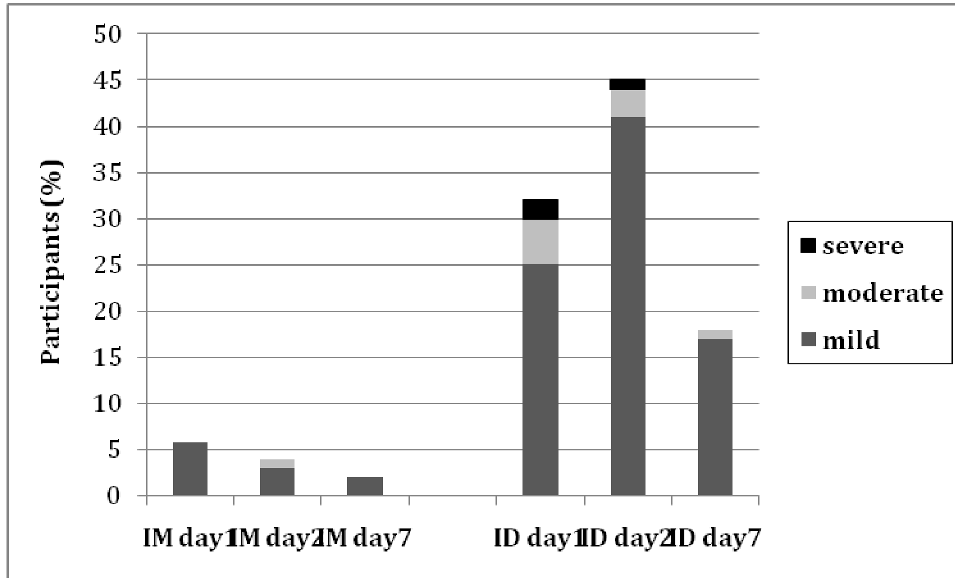


Figure 6b: Induration

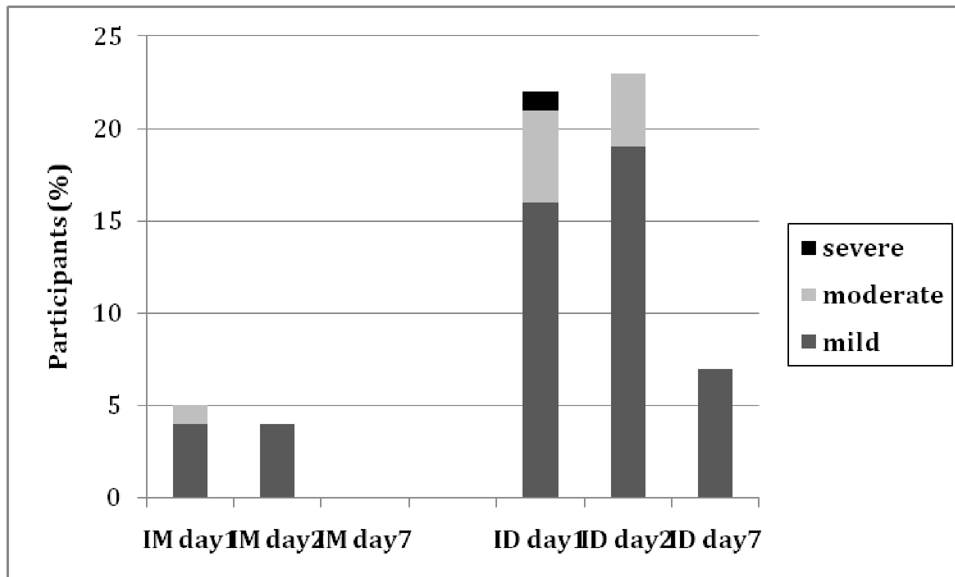


Figure 6c: Tenderness

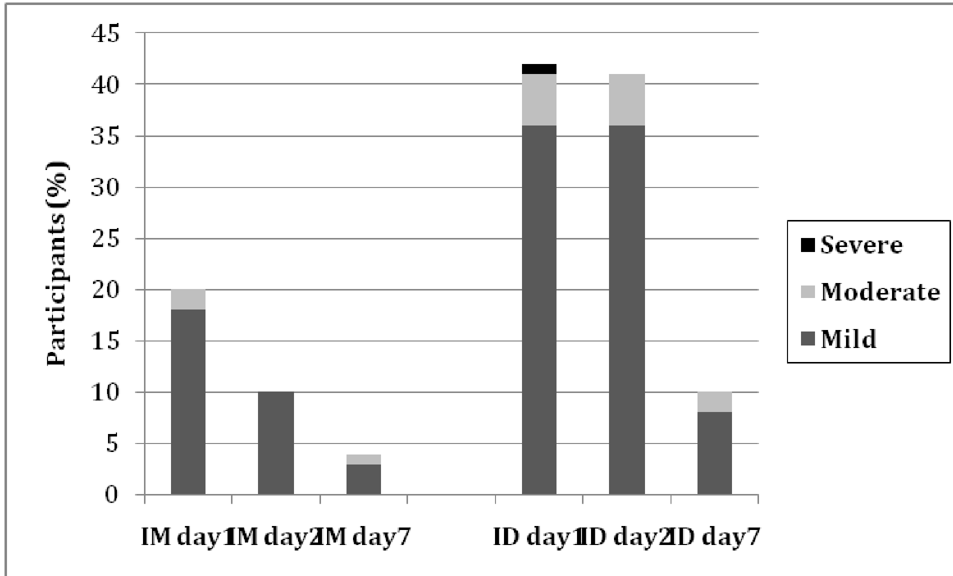


Figure 6d: Pruritus

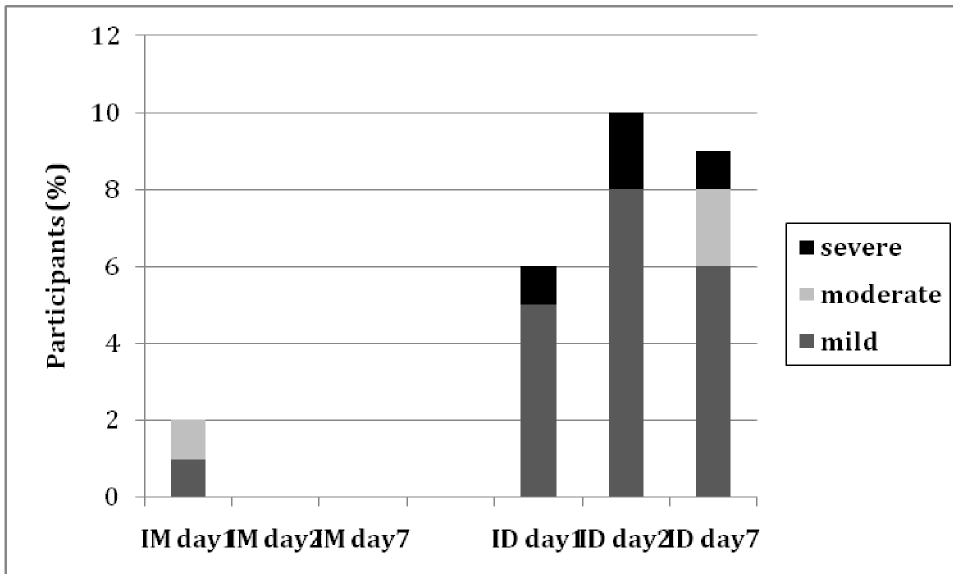


Figure 6e: Gastrointestinal symptoms

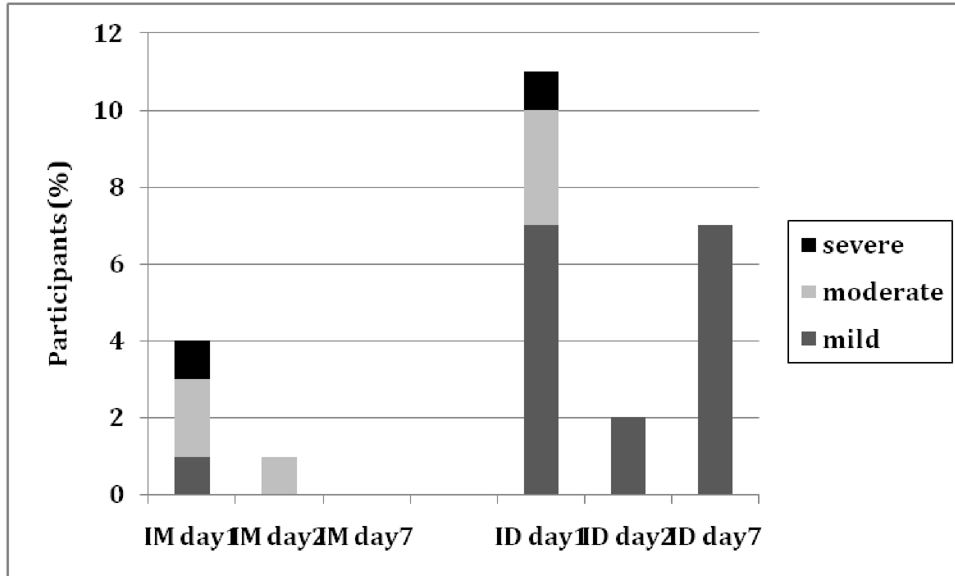
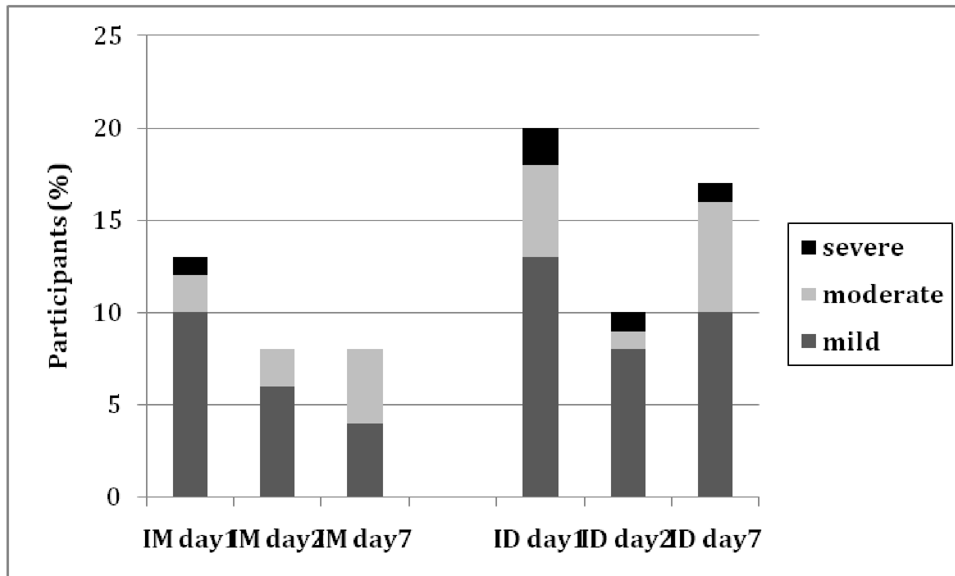


Figure 6f: Fatigue



5. Discussion of Masters of Science

We performed a large randomized trial using a novel strategy of high-dose intradermal vs. standard dose intramuscular vaccine in organ transplant recipients. We found that in the overall cohort, seroconversion to vaccine was low and there were no significant differences in vaccine response (defined as seroconversion to at least one vaccine antigen) between the two vaccine groups (46.7% in IM vs. 54.1% in ID). This is comparable to findings in the transplant literature that show seroconversion ranges from 15-95% with intramuscular vaccine. In the intradermal group arm of the trial, seroconversion to vaccine strains ranged 21.5%-37.4%. This is much lower than rates of seroconversion after 15 μg intradermal vaccine in the immunocompetent population age 18-59 which ranges from 56 to 79% [85-87]. Lung transplant recipients had the poorest responses to the seasonal influenza vaccine, irrespective of vaccine allocation or strain type. If this group was excluded, however, high-dose ID vaccine provided significantly greater GMTs and seroconversion factors to the influenza B strain. In addition, although not significantly different, the high-dose ID vaccine led to greater responses to the two A strains. Vaccine safety in terms of HLA alloantibody as well as adverse effects was also evaluated in our study for both standard IM and high-dose intradermal influenza vaccination in SOT recipients. There were only 3 cases that initially met our *a priori* requirements of HLA alloantibody positivity post vaccination in this trial as defined by MFI > 1000 post-vaccine and a change of more than 20%. None of these patients had significant change in their respective graft function and the post-vaccine antibody measurements were at relatively low levels. In terms of adverse events, there were similar rates of systemic reactions but greater local reactions with

high-dose ID than IM vaccine. Local reactions are well known to occur post ID vaccine and are within the reported range of 29% for pruritus and up to 71.9% for erythema for immunocompetent adults age ≥ 60 years receiving 15 μg dose [26]. Our rates were lower than reported for this group. Perhaps the same mechanism that creates a blunted response to the seasonal influenza vaccine as measured by HIA allows for lower incidence rates of adverse effects from the vaccine as well.

Influenza vaccine immunogenicity is measured via the surrogate marker of post vaccine production of antibody. Production of neutralizing and receptor-blocking antibodies against HA is the primary means of protection to influenza exposure, while antibodies against NA seem to play a secondary role [35]. During the measurement of antibodies, first cross-reactive antibodies are removed such that there are only the antibodies binding to the immunogen that remain in the solution. Antibody production relies on the number of circulating B-cells, the rate of antibody synthesis and the persistence of antibody after production by B-cells. There are potential complicating issues during the assay of post vaccination antibody production: affinity and avidity. Affinity describes the strength of binding of the antibody and antigen in terms of a single antigen binding site whereas avidity describes the total binding strength of a molecule with more than one binding site. Overall the higher the affinity of the antibody for antigen then clinically less antibody is required to eliminate antigen. In the example of antibody production post influenza vaccination, hemagglutination is the standard method. It measures the amount of antibody in a particular patient's serum that binds to surface antigen of the influenza viral strain in question. The assay measures both immunoglobulins IgM and IgG [88]. As early as 2 weeks post influenza vaccination,

antibody measurements can be taken in order to calculate rates of seroprotection and seroconversion rates [89].

There is a small but significant body of literature that disagrees with the assumption that HIA is the optimal means of measuring response to influenza vaccine. Cell mediated immunity (CMI), either measured via ELISPOT or flow cytometric measurement of interferon-γ from peripheral blood mononuclear cells (PBMCs), would be more indicative of the production of a protective response post-vaccine [51, 90]. CMI participates in the clearance of the influenza viral infection during an ongoing infection and is known to give its support for the development of humoral immunity. Historically there has been a lack of correlations between cellular and humoral post-vaccine responses [51, 91, 92]. Ideally when assessing immunogenicity post vaccination, we should analyze both humoral and cellular components of the response. PBMCs were collected during the course of the trial (20 SOT recipients received IM and 20 received high-dose ID) but they will need to be analyzed at a later date.

A literature review of published studies in 2012 revealed 5 main papers stating that transplant recipients had post-vaccine rates of either seroprotection and/or seroconversion that were equivalent to immunocompetent controls. All 5 papers describe cohorts of kidney transplants, both pediatric and adult, that are generally more than 6 months post transplant with stable kidney function. Scharpe et al. described 161 renal transplants (compared to 41 healthy controls) who were vaccinated in 2003 with the trivalent seasonal influenza vaccine [50]. Post-vaccine seroprotection in the renal transplants was 92.7% (A/H1N1), 78.7% (A/H3N2) and 82.9% (B) which is above the required 70% per CPMP. In Iran, 40 kidney transplants and their sex- and age-matched

controls were given the seasonal influenza vaccine in 2006. Sera were measured pre-vaccine and 1 month post-vaccine. Seroconversion post-vaccine in the kidney transplants was 50% (A/H1N1 in MMF arm), 79.2% (A/H1N1 in Azathioprine arm), 50% (A/H3N2 in MMF arm), 50% (A/H3N2 in Azathioprine arm), 56.3% (B in MMF arm) and 62.5% (B in Azathioprine arm). Concomitant healthy controls had seroconversion rates of 75%, 65% and 53% for A/H1N1, A/H3N2 and B respectively [48]. All of the strains met the >40% requirement for seroconversion in both arms of the Iranian cohort. In Greece Grekas et al. described 40 renal transplant recipients who were followed post IM influenza vaccination. At 1 month follow-up, the renal transplants' seroconversion rates again met the requirements of greater than 40% with 42.5% to A/H3N2, 58% to A/H1N1 and 48% to B [93]. Briggs et al. also analyzed a small cohort of 13 kidney transplants compared to 16 controls where both groups met guideline minimums for HIA increases post-vaccination [94]. It was noted by the authors though that the majority of their kidney transplant recipients were on alternate-day or low-dose steroid therapy thereby describing a population with a relatively low net state of immunosuppression. In the pediatric kidney population, Edvardsson et al. followed 47 transplant recipients versus 7 pediatric controls with bronchopulmonary dysplasia with 91% seroconversion versus 71% in controls. In addition the authors noted if a transplant recipient did not seroconvert then they tended to have higher pre-vaccine geometric mean titers (compared to those who did seroconvert) [95].

Conversely, several sources in the literature describe a poorer serologic outcome post-vaccination with seasonal influenza in SOT recipients. One of the first papers questioning the degree of post-vaccine response compared cyclosporine-treated kidney

transplants to azathioprine-treated kidney transplants found cyclosporine-treated recipients had significantly lower rates of seroprotection and seroconversion compared to azathioprine-treated patients. Both immunosuppressed groups had lower response rates compared to healthy controls [96]. In a small study analyzing a variety of chronic kidney patients including transplant recipients, Cavdar et al. measured adequate seroprotection levels post–vaccination but rates of seroconversion were below 40%. A/H3N2, A/H1N1 and B seroconverted in only 11%, 35% and 29% of the 17 kidney transplanted patients. Healthy controls used during the same season also had a low rate of seroconversion of 50%, 50%, and 10%, respectively, so there might have been other confounding issues with the vaccine [97]. A larger cohort of kidney patients, 49 persons, compared to 37 healthy controls during the 1996-1997 influenza season measured seroprotection rates at 1 month at 46% versus 69% with a p-value of 0.06 though the incidence of influenza-like illness were similar between the groups [78]. Smith et al. enrolled 38 kidney transplants with an a priori subgroup analysis comparing mycophenolate mofetil (MMF) versus azathioprine integrated into the study. The MMF-treated kidney transplants had a significantly smaller percentage of persons who seroconverted to all 3 antigens when compared to both the azathioprine arm as well as the controls [98]. In a tacrolimus-based regimen kidney cohort versus healthy controls, there was still a 69% decreased odds of seroconversion (p-value of 0.001) [99]. Blumberg et al. vaccinated a heterogeneous group of solid organ transplant (SOT) patients during the 1993-1994 season whose vaccine included strains A/H3N2, A/H1N1 and B and compared responses versus healthy controls. The SOT recipients post-vaccine rate of seroconversion ranged from 16% to 26% whereas the controls' rate of

seroconversion ranged from 72% to 86% [83]. Influenza vaccine responses in a group of naturally tolerant kidney recipients represented a mixed picture as measured with post-vaccination HIA. 3 of 5 of the cohort had comparable responses to the control group [90].

Since the pandemic of 2009, recent publications have looked specifically at adjuvanted influenza vaccination in the SOT population during the pandemic of H1N1 in 2009. 111 kidney transplants were vaccinated in Belgium with Pandemrix® (AS03-adjuvanted vaccine) and compared to 21 simultaneous controls. The GMTs were statistically lower in the transplant cohort as were the rates of seroconversion [70]. AS03-adjuvanted vaccine was given to 47 heart transplant recipients that were more than 6 months post transplant. Seroconversion at 20 ± 2 days post vaccination was 32% but there were no occurrences of influenza-like illness by the 4 month follow-up time point [100]. It is important to note though that 4 months is a short follow-up period of time considering a dose of influenza vaccine is designed to protect the recipient from the time of vaccination (as early as October) to as late as March i.e. 6 months on average. A separate study in Germany followed 60 kidney transplants post Pandemrix® vaccination and found a rate of only 32.7% seroconversion compared to 86.4% in healthy controls [69]. When comparing the rates of seroconversion between a SOT cohort of liver and kidney recipients versus healthy controls as well as HIV patients with CD4 count greater than 200, an interesting trend was found. The HIV and healthy controls had similar rates of seroconversion after the first and second dose of Pandemrix® but the SOT recipients had rates much lower at 52%. The second dose of vaccine only aided in improving responses in the HIV component of the cohort and not

in the SOT section [101]. Arepanrix® was the AS03 adjuvanted pandemic influenza vaccine licensed in Canada in 2009. Three of 5 pediatric heart transplants that were vaccinated at a median time of 9 weeks post transplant still were able to develop protective titers alluding to the fact that there are important differences between the pediatric and adult transplant populations on multiple levels [102].

Various efforts have been attempted in order to boost the response of SOT recipients after seasonal influenza vaccination. Scharpe et al. attempted boosting with a second dose of seasonal influenza vaccine but without improvement. Importantly though that despite a second exposure to influenza vaccine antigen, there was no change in allograft function or increase in rejection rates in a cohort of kidney recipients [50]. Manuel et al. attempted a second vaccine using Pandemrix® but without improvement in a heterogeneous solid organ cohort [101]. A relatively large cohort of lung transplants, n=60, were given the 2006-2007 IM seasonal influenza vaccine followed by the same strains via low-dose intradermal (ID) vaccination 4 weeks later. Though rates for seroconversion were 52% for A/H1N1, 50% for A/H3N2 and 42% for B at 4 weeks post IM vaccine, seroconversion rates (comparing to initial pre-vaccine titers) dropped to 46%, 49% and 37% at 8 weeks (and therefore post ID booster vaccine) [76]. Overall it appears from the literature that boosting with either another IM or ID influenza vaccine does not increase rates of seroconversion in SOT recipients. Pediatric liver transplants were given 2 doses of seasonal IM influenza vaccine and they too did not have a statistically significant increase in either rates of seroprotection or seroconversion [92].

Specific baseline issues of a target population, i.e. SOT recipients, can affect the rates of seroconversion post influenza vaccination. Firstly, if there is a positive titer for the strains in the vaccine at baseline there is usually a muted rate of seroconversion post vaccination. In 1972 healthy volunteers who were seronegative prior to vaccination were vaccinated: they went on to have lower rates of seroconversion and lower rates of infection during duration of follow-up post vaccination compared to their healthy counterparts who had pre-vaccine HI titers [88]. Other trends seen in immunocompetent persons is 1) if a patient is repeatedly vaccinated against influenza A/H1N1 over a 6 month period there is no significant increase in antibodies and 2) the greatest increase in antibodies was after the first attempt [56]. Scharpe et al. showed that for all 3 strains of the seasonal influenza vaccine used, seroconversion rates were strongly and inversely related to seroprotection rate at baseline though Blumberg et al. only was able to depict the inverse correlations with the A strains of their seasonal influenza vaccine [50, 83]. Unfortunately due to most influenza trials in SOT recipients being small and in a heterogeneous population, teasing out the finer issues of what level of pre-vaccination antibody makes a difference in post vaccine response as well as the differences between generating a response to influenza A versus B has not been done at this time.

Four separate cohorts of kidney transplants found that mycophenolate mofetil (MMF) was an independent factor predicting poor rates of seroconversion after influenza vaccination. MMF is a reversible inhibitor of inosine monophosphate dehydrogenase in purine synthesis (needed by both B- and T-cells). MMF is the prodrug of mycophenolic acid prescribed as part of triple immunosuppressant therapy

for SOT recipients. Physiologically it is understandable that MMF would decrease the ability of the humoral immune system to create antibodies post influenza vaccination but on the other hand all immunosuppressants used by SOT recipients affect the immune system yet not all of them have such a significant effect on antibody production. Smith et al. followed 38 kidney transplant recipients and compared serological responses in those taking azathioprine versus MMF. Both groups had lower rates of seroconversion compared to healthy controls but lowest in the MMF group. None of the patients taking MMF responded to all of the 3 antigens after their seasonal influenza vaccination. There was no additional decrease in the rate of seroconversion depending on the dose of MMF, i.e. 2 or 3 g of MMF/day though the population size was noted to be small [98]. Scharpe et al. readdressed the potential issues of MMF and serological response post vaccination using data collected from the 2003-2004 influenza season. Multivariate analysis revealed that the rate of seroconversion to all 3 strains in the vaccine was affected by baseline titers and the use of MMF. MMF use led to a 2.6 to 5 fold lower rate for seroconversion and MMF ≥ 2 g was significantly worse than MMF < 2g. Rates of post vaccine seroprotection, a less stringent marker of vaccine efficacy, were not affected by MMF usage [50]. Analyzing a cohort of Brazilian kidney transplants whom received the 2005 seasonal influenza vaccine, Salles et al. found that corticosteroids, tacrolimus and cyclosporine did not have an effect on the rate of seroprotection or seroconversion. On the contrary, MMF use did lead to significantly lower rates of seroprotection for H1N1 and H3N2 strains as well as a lower rate of seroconversion for H1N1 compared to azathioprine usage [103]. Analyzing a different cohort from the 2005-2006 influenza season, Keshtkar-Jahromi et al. also showed that MMF was an

independent factor that affected the rates of seroprotection in kidney transplants against H1N1 and not the other 2 strains in their vaccine with a p-value of 0.028 also compared versus responses in those on azathioprine [48].

Vaccination after transplantation has to be optimally timed for maximum benefit. As discussed in the guidelines for vaccination of SOT candidates and recipients, patients tend to have fluctuating immunosuppressive regimens until approximately 3 to 6 months post transplant making this the ideal time point to consider resuming influenza vaccination [104]. One of the initial papers describing an effect of time post transplant on rates of seroprotection was Salles et al. who utilized ROC curves to find that those transplanted prior to 87 months (7.25 years) had worse rates of serological response [103]. Birdwell et al. also looked at their cohort of tacrolimus-treated kidney transplants whom were recruited for vaccination as early as 30 days post transplant. The kidney recipients who were less than 6 months post transplant - one-third of the cohort - were significantly less likely than their healthy controls to seroconvert to all 3 influenza strains [99]. As mentioned before though a small cohort of pediatric heart transplant recipients were vaccinated as early as 5 weeks post transplant during the pandemic H1N1 influenza outbreak of 2009 and still 3 of 5 patients mounted antibody levels consistent with seroconversion [102]. Meyer et al.'s group also did not find that age or time post transplant affected the rates of response to influenza vaccine [100]. The 2 papers describing heart transplant cohorts who did not find time from transplant as an independent factor affecting rates of seroconversion were measuring vaccine immunogenicity post adjuvanted influenza vaccine. The papers that did find time from

transplant as an independent poor risk factor were from cohorts where the majority were status-post kidney transplants and whom had received seasonal influenza vaccine.

Though there are numerous papers discussing a variety of variables that may or may not affect humoral immunity, there are still fewer papers discussing the effect of vaccination on cellular immunity and how it translates clinically in a change of rates of infection or influenza-like illness. McMichael et al. in 1983 was the first to note that immunocompetent adults with baseline cytotoxic T-cell immunity against influenza clear virus more effectively than those without pre-existing cell-mediated immunity. In addition cytotoxic T-cells may demonstrate cross-reactivity when responding to new influenza A virus subtypes [105]. Cell-mediated immunity (CMI) also provides the helper component in the development of humoral immunity [103]. Overall it is harder to assess CMI secondary to additional steps that are required to acquire and adequately process patients' blood samples in order to store peripheral blood mononuclear cells (PBMCs). Mazzone et al. is one of the first groups to analyze CMI in adult lung transplants, a group that is especially important considering the correlation of respiratory viral infections and the increased risk for clinical rejection, bronchiolitis obliterans syndrome (BOS). Using ELISA techniques to measure Il-2, IL-10, and interferon-gamma, they found that lung transplant recipients did not have a significant increase in CMI from pre- to post-influenza vaccine (of note the controls also had varying degrees of response based on the strain in the vaccine). On the other hand both Ballet et al. and Candon et al. found that their renal cohorts had a significant expansion of influenza-specific interferon-gamma producing T-cells at similar frequencies as their healthy controls [51, 90]. Candon et al. also remarked that after

further evaluation, CMI responses were mainly against the hemagglutinin and neuraminidase components of the influenza virus and less frequently against the internal proteins of the virion (such as M1, PB1 and NP). This would be consistent with a CMI response to the components included in the influenza vaccine and not necessarily secondary to natural infection or exposure. Lindemann et al. performed ELISPOT testing on 65 kidney transplant recipients and found that the majority of them induced significant CMI but the magnitude was smaller compared to healthy controls. In subanalysis Lindemann et al. found that tacrolimus had a more detrimental effect on CMI compared to cyclosporine (or other immunosuppressants) [91]. Both Lindemann et al. and Madan et al. agree that a particular antibody response in SOT recipients does not predict CMI responses as measured with ELISPOT. Madan et al. also showed that though controls and transplant recipients had nearly equivalent pre-vaccine CMI, CMI measurements after both the first and second IM influenza vaccine their CMIs became statistically different with healthy controls being statistically higher than their transplanted siblings [92].

Influenza can cause significant morbidity in the organ transplant population and lead to allograft dysfunction [106-109]. Influenza vaccine responses, however, are quite variable in this immunosuppressed population and are dependent on several factors [13, 50, 76, 83, 99]. These factors include time from transplant, type of organ, and specific immunosuppressives such as MMF. The presence or absence of seroprotection at baseline has also been shown to be an important factor for vaccine responses in the transplant population. The use of high-dose ID vaccine is a novel strategy that has not previously been studied in solid organ transplant recipients. The

intradermal strategy takes advantage of higher density of dendritic cells present in the skin as compared to the deltoid muscle of the arm to enhance immunogenicity. During the influenza vaccine shortage in 2003, randomized clinical trials showed that as little as 3 μg of vaccine antigen injected into the dermis induced a comparable response to 15 μg antigen injected intramuscularly [85, 110]. However, intradermal injection technique is limited by the volume that can be safely injected. In 2010, an intradermal injection system (BD Biosciences) coupled with influenza vaccine antigen in 0.1mL volume became available (Intanza®). In healthy adults, low dose (9 μg) of seasonal ID vaccine showed comparable efficacy to standard dose (15 μg) of IM vaccine [86]. In an older population (≥ 60 years) with senescent immune system, they generally have poorer responses to influenza vaccine such that a 15 μg intradermal preparation was required to reach similar immunogenicity [87]. Therefore, we postulated that high-dose ID vaccine may increase immunogenicity in the transplant population. Although the ID vaccine is now available in a 15 μg preparation, we used two successive doses of 9 μg each. This was because at the time of the study, the 15 μg preparation was not available in Canada. Given the data in the elderly and our previous studies of low-dose intradermal injection in transplant, we felt that the use of a single 9 μg injection would not provide sufficient immunogenicity in SOT recipients.

As of 2012, there are only a few studies analyzing the use of intradermal (ID) influenza vaccine in the SOT population. Where the 6 μg ID vaccine and 15 μg IM vaccine are equivalent in immunocompetent persons [26], 85 lung transplant recipients failed to have an equivalent response as healthy controls of the same influenza season. In addition 2 of the 3 strains in the influenza vaccine had a statistically poorer result than

healthy controls [111]. There are multiple possible reasons for the failure of low-dose ID vaccine in lung patients compared to IM in controls such as lung transplant recipients requiring more overall immunosuppression compared to other types of SOTs. As lung transplants in general respond poorly to vaccination, it might be harder to achieve a benefit in this population with the utilization of a new style of influenza vaccine. Using the ID vaccine with 15 μg of HA/strain compared to standard IM vaccine, Morelon et al. were able to show that the ID vaccine created superior results in kidney transplant at 21 days post vaccination in 2 of the 3 strains in the vaccines. The rate of seroconversion to influenza A/H1N1 was 35% post ID vaccine but only 19% post IM: both were still below the recommended 40% rate for a vaccine to meet CHMP requirements for persons aged less than 60 [112].

We enrolled a wide variety of solid organ transplants but lung transplant recipients comprised almost one-third of our study population. Therefore, we decided a priori to analyze this subgroup separately. Indeed, seroconversion rates were lower in this group compared to the remaining component of the cohort. The low responses in this population also were highlighted by the fact that all three vaccinated patients who developed influenza infection in our study were lung transplant recipients. The lung transplant recipients were all vaccinated at least 4 weeks prior to infection and were at least 1 year post transplant. As one lung transplant recipient fell ill while in China for work, only 2 of the recipients were able to be typed. The samples were typed as H3 derivatives though at least one of the vaccine recipients had a post titer to H3N2 of 1:80. It is important to also note that during the 2010-2011 influenza season the CDC measured and published the amount of correlation between circulating strains and that

which was in the vaccine. Of the viruses tested by the CDC, 99.8% of influenza A/H1N1, 96.8% influenza A/H3N2 and 94% of influenza B were matched between the vaccine and circulating virus [113]. With such a good correlation those who sustained an appropriate immunologic response after vaccination should have been immune to the circulating strains of influenza during the 2010-2011 season.

When lung transplants were excluded, we noted a benefit of ID vaccine only for the B strain of the influenza vaccine. The B strain is historically known to be less immunogenic than A vaccine strains, therefore, and may benefit from the use of novel strategies. This is an especially important finding since quadrivalent influenza vaccines (containing two A strains and two B strains) will likely be marketed in the future for public use. In the non-lung subgroup, protection to A strains was slightly greater with ID vaccine but did not reach statistical significance.

In the overall cohort, the use of MMF ≥ 2g and time from transplant < 6 months were independent factors for poor vaccine responses. These findings have previously been noted in the literature and support the current recommendation to administer influenza vaccine starting at least 3-6 months post-transplant when the patient has achieved stable immunosuppression [50, 99]. Age > 60 did not significantly influence vaccine responses likely because the immunologic decline associated with transplant outweighs immune senescence associated with age.

Our study has a number of limitations. First, we used two successive doses of the 9 μg preparation of ID vaccine in order to reach a total of 18 μg/HA. This may have increased the rate of local reactions observed in our study although it is still lower than

the rate observed in the general population status post a single dose. The dose of influenza vaccine also differs slightly between the two intervention groups (18 μg ID vs. 15 μg IM). The reasoning for the dose selection has been discussed above but the contribution of the higher dose to vaccine immunogenicity is not clear. Despite the higher dosing, the high-dose ID vaccine did not show superiority in the overall cohort. Higher doses or booster doses in the same season have also not been shown to increase influenza vaccine immunogenicity in transplantation [50, 76]. In addition, we enrolled a wide variety organ transplants which may have created an overly heterogeneous population. Although transplant recipients are on similar maintenance immunosuppression, certain groups such as lung transplant recipients are generally much more immunosuppressed compared to liver and kidney recipients and may have lead to inadvertent skewing of the data. However, despite this, we were able to show that ID vaccine is more immunogenic for B strain in the non-lung subgroup.

Lastly there are 2 other main limitations. First is the lack of immunocompetent controls with both HIA and HLA alloantibody measurements during the same influenza season. As there are often year-to-year differences between influenza vaccine compilations and percentages of circulating virus, it would have been ideal to have simultaneous controls who received either high-dose ID or IM vaccines. Though it is most likely due to endogenous issues that lead to the SOT recipients to have poor immunologic outcomes post influenza vaccine, theoretically it would have been an issue inherently due to the vaccine of the particular season in question. If there had been a poor match between the influenza vaccine and circulating virus, one would not be able to make assumptions about the number of influenza infections post vaccination. The

last limitation to be mentioned is the duration of follow-up with bloodwork. Though the percentage of full follow-up falls with increased numbers of bloodwork, it would have been ideal to have had a second bloodwork at 6 months. The trends of not only serology but also HLA alloantibody could have been followed to see if the trends seen at 1 month were still present at 6 months. For example, attenuation of the serological response post vaccination may occur leading to an increased risk for infection with influenza at a specific time point post-vaccine yet unknown. A similar scenario was discussed with data collected during the 2009-2010 influenza season but within a cohort of transplant patients who had microbiologically proven pandemic influenza A/H1N1 at University of Alberta, Canada. There was an inverse correlation between the rate of positive HIA and the time from infection to bloodwork. In those with positive responses, time to follow-up was 7.5 ± 2.2 months versus 9.1 ± 2.6 months in negative responders (p=0.047). Currently the rate of attenuation of serological response as measured by HIA is not clearly defined within the SOT population.

In summary, ours is the first study to show that high-dose ID vaccine was comparable to IM vaccine in solid organ transplant recipients and may even be more immunogenic in kidney and liver transplants. We also showed that neither high-dose ID nor IM influenza vaccine lead to a clinically relevant level of HLA antibodies or DSA. Based on this, we suggest that high-dose ID vaccine is a viable alternative to standard-dose IM vaccine and may be the preferred vaccine for non-lung subgroups.

Bibliography

1. Kwong JC, Stukel TA, Lim J, et al. The effect of universal influenza immunization on mortality and health care use. PLoS Med **2008** Oct 28;5(10):e211.
2. Schanzer DL, Tam TW, Langley JM, Winchester BT. Influenza-attributable deaths, Canada 1990-1999. Epidemiol Infect **2007** Oct;135(7):1109-16.
3. Gordon A, Saborio S, Videa E, et al. Clinical attack rate and presentation of pandemic H1N1 influenza versus seasonal influenza A and B in a pediatric cohort in Nicaragua. Clin Infect Dis **2010** Jun 1;50(11):1462-7.
4. Cordero E, Perez-Romero P, Moreno A, et al. Pandemic influenza A(H1N1) virus infection in solid organ transplant recipients: impact of viral and non-viral co-infection. Clin Microbiol Infect **2012** Jan;18(1):67-73.
5. Kunisaki KM, Janoff EN. Influenza in immunosuppressed populations: a review of infection frequency, morbidity, mortality, and vaccine responses. Lancet Infect Dis **2009** Aug;9(8):493-504.
6. Vilchez RA, McCurry K, Dauber J, et al. Influenza virus infection in adult solid organ transplant recipients. Am J Transplant **2002** Mar;2(3):287-91.
7. Mauch TJ, Bratton S, Myers T, Krane E, Gentry SR, Kashtan CE. Influenza B virus infection in pediatric solid organ transplant recipients. Pediatrics **1994** Aug;94(2 Pt 1):225-9.
8. Rossman JS, Lamb RA. Influenza virus assembly and budding. Virology **2011** Mar 15;411(2):229-36.
9. Nichol KL. The efficacy, effectiveness and cost-effectiveness of inactivated influenza virus vaccines. Vaccine **2003** May 1;21(16):1769-75.
10. Robertson JS. 5' and 3' terminal nucleotide sequences of the RNA genome segments of influenza virus. Nucleic Acids Res **1979** Aug 24;6(12):3745-57.
11. Fouchier RA, Munster V, Wallensten A, et al. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol **2005** Mar;79(5):2814-22.
12. Noda T, Kawaoka Y. Structure of influenza virus ribonucleoprotein complexes and their packaging into virions. Rev Med Virol **2010** Nov;20(6):380-91.
13. Dormitzer PR, Galli G, Castellino F, et al. Influenza vaccine immunology. Immunol Rev **2011** Jan;239(1):167-77.
14. Tong S, Li Y, Rivailler P, et al. A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci U S A **2012** Mar 13;109(11):4269-74.
15. Chen BJ, Leser GP, Morita E, Lamb RA. Influenza virus hemagglutinin and neuraminidase, but not the matrix protein, are required for assembly and budding of plasmid-derived virus-like particles. J Virol **2007** Jul;81(13):7111-23.
16. Hayden FG. Antiviral resistance in influenza viruses--implications for management and pandemic response. N Engl J Med **2006** Feb 23;354(8):785-8.
17. Moscona A. Neuraminidase inhibitors for influenza. N Engl J Med **2005** Sep 29;353(13):1363-73.
18. McGeer AJ, Lee W, Loeb M, et al. Adverse effects of amantadine and oseltamivir used during respiratory outbreaks in a center for developmentally disabled adults. Infect Control Hosp Epidemiol **2004** Nov;25(11):955-61.
19. Dharan NJ, Gubareva LV, Meyer JJ, et al. Infections with oseltamivir-resistant influenza A(H1N1) virus in the United States. JAMA **2009** Mar 11;301(10):1034-41.
20. Ison MG, Sharma A, Shepard JA, Wain JC, Ginns LC. Outcome of influenza infection managed with oseltamivir in lung transplant recipients. J Heart Lung Transplant **2008** Mar;27(3):282-8.

21. Gangappa S, Kokko KE, Carlson LM, et al. Immune responsiveness and protective immunity after transplantation. Transpl Int **2008** Apr;21(4):293-303.
22. Gunther M, Stark K, Neuhaus R, Reinke P, Schroder K, Bienzle U. Rapid decline of antibodies after hepatitis A immunization in liver and renal transplant recipients. Transplantation **2001** Feb 15;71(3):477-9.
23. Vogel FR. Immunologic adjuvants for modern vaccine formulations. Ann N Y Acad Sci **1995** May 31;754:153-60.
24. Stark K, Gunther M, Schonfeld C, Tullius SG, Bienzle U. Immunisations in solid-organ transplant recipients. Lancet **2002** Mar 16;359(9310):957-65.
25. Committee for Proprietary Medicinal Products, **1997** 12 March 1997.
26. INTANZA, Influenza Vaccine (Split Virion, Inactivated) [package insert]. Lyon, France: Sanofi Pasteur SA, **2010**.
27. Guidelines for vaccination of solid organ transplant candidates and recipients. Am J Transplant **2004**;4(Suppl 10):160-3.
28. Ballout A, Goffin E, Yombi JC, Vandercam B. Vaccinations for adult solid organ transplant recipient: current recommendations. Transplant Proc **2005** Jul-Aug;37(6):2826-7.
29. Ueno H, Schmitt N, Palucka AK, Banchereau J. Dendritic cells and humoral immunity in humans. Immunol Cell Biol **2010** May-Jun;88(4):376-80.
30. Liard C, Munier S, Joulin-Giet A, et al. Intradermal immunization triggers epidermal Langerhans cell mobilization required for CD8 T-cell immune responses. J Invest Dermatol **2012** Mar;132(3 Pt 1):615-25.
31. Kupper TS, Fuhlbrigge RC. Immune surveillance in the skin: mechanisms and clinical consequences. Nat Rev Immunol **2004** Mar;4(3):211-22.
32. Alarcon JB, Hartley AW, Harvey NG, Mikszta JA. Preclinical evaluation of microneedle technology for intradermal delivery of influenza vaccines. Clin Vaccine Immunol **2007** Apr;14(4):375-81.
33. Valladeau J, Saeland S. Cutaneous dendritic cells. Semin Immunol **2005** Aug;17(4):273-83.
34. Brown H, Kasel JA, Freeman DM, Moise LD, Grose NP, Couch RB. The immunizing effect of influenza A/New Jersey/76 (Hsw1N1) virus vaccine administered intradermally and intramuscularly to adults. J Infect Dis **1977** Dec;136 Suppl:S466-71.
35. Josefsberg JO, Buckland B. Vaccine process technology. Biotechnol Bioeng **2012** Mar 7.
36. Principi N, Esposito S. Adjuvanted influenza vaccines. Hum Vaccin Immunother **2012** Jan 1;8(1).
37. National Advisory Committee on Immunization (NACI). Vaccine Safety and Adverse Events Following Immunization Recommended Immunization Active Immunizing Agents - Influenza Vaccine Canadian Immunization Guide. 7th Ed ed: Her Majesty the Queen in Right of Canada represented by the Minister of Public Workds and Government Services Canada, **2006**:80-4, 110, 31-3, 209-20.
38. Potter CW, Jennings R, Clark A. The antibody response and immunity to challenge infection induced by whole, inactivated and tween-ether split influenza vaccines. Dev Biol Stand **1977** Jun 1-3;39:323-8.
39. Rhorer J, Ambrose CS, Dickinson S, et al. Efficacy of live attenuated influenza vaccine in children: A meta-analysis of nine randomized clinical trials. Vaccine **2009** Feb 11;27(7):1101-10.
40. Ambrose CS, Wu X, Belshe RB. The efficacy of live attenuated and inactivated influenza vaccines in children as a function of time postvaccination. Pediatr Infect Dis J **2010** Sep;29(9):806-11.
41. Beyer WE, Palache AM, de Jong JC, Osterhaus AD. Cold-adapted live influenza vaccine versus inactivated vaccine: systemic vaccine reactions, local and systemic antibody response, and vaccine efficacy. A meta-analysis. Vaccine **2002** Jan 31;20(9-10):1340-53.
42. Rao SS, Kong WP, Wei CJ, et al. Comparative efficacy of hemagglutinin, nucleoprotein, and matrix 2 protein gene-based vaccination against H5N1 influenza in mouse and ferret. PLoS One **2010**;5(3):e9812.

43. Pandemrix, pandemic influenza vaccine (H1N1) (split virion, inactivated, adjuvanted) [package insert]. Belgium: GlaxoSmithKline Biologicals s.a., **2010**:1-9.
44. Smith H, Sweet C. Lessons for human influenza from pathogenicity studies with ferrets. Rev Infect Dis **1988** Jan-Feb;10(1):56-75.
45. Yetter RA, Barber WH, Small PA, Jr. Heterotypic immunity to influenza in ferrets. Infect Immun **1980** Aug;29(2):650-3.
46. Steinhoff MC, Fries LF, Karron RA, Clements ML, Murphy BR. Effect of heterosubtypic immunity on infection with attenuated influenza A virus vaccines in young children. J Clin Microbiol **1993** Apr;31(4):836-8.
47. Crockett F, Mal H, Amazzough K, et al. [H1N1 (2009) influenza A infection in transplant recipient patients: a comparative study versus non-transplanted patients]. Rev Pneumol Clin **2011** Sep;67(4):199-208.
48. Keshtkar-Jahromi M, Argani H, Rahnavardi M, et al. Antibody response to influenza immunization in kidney transplant recipients receiving either azathioprine or mycophenolate: a controlled trial. Am J Nephrol **2008**;28(4):654-60.
49. Kumar D, Blumberg EA, Danziger-Isakov L, et al. Influenza vaccination in the organ transplant recipient: review and summary recommendations. Am J Transplant **2011** Oct;11(10):2020-30.
50. Scharpe J, Evenepoel P, Maes B, et al. Influenza vaccination is efficacious and safe in renal transplant recipients. Am J Transplant **2008** Feb;8(2):332-7.
51. Candon S, Thervet E, Lebon P, et al. Humoral and cellular immune responses after influenza vaccination in kidney transplant recipients. Am J Transplant **2009** Oct;9(10):2346-54.
52. Belshe RB, Van Voris LP, Bartram J, Crookshanks FK. Live attenuated influenza A virus vaccines in children: results of a field trial. J Infect Dis **1984** Dec;150(6):834-40.
53. Powers DC, Murphy BR, Fries LF, Adler WH, Clements ML. Reduced infectivity of cold-adapted influenza A H1N1 viruses in the elderly: correlation with serum and local antibodies. J Am Geriatr Soc **1992** Feb;40(2):163-7.
54. Hirota Y, Kaji M, Ide S, et al. Antibody efficacy as a keen index to evaluate influenza vaccine effectiveness. Vaccine **1997** Jun;15(9):962-7.
55. de Jong JC, Palache AM, Beyer WE, Rimmelzwaan GF, Boon AC, Osterhaus AD. Haemagglutination-inhibiting antibody to influenza virus. Dev Biol (Basel) **2003**;115:63-73.
56. Powers RD, Hayden FG, Samuelson J, Gwaltney JM, Jr. Immune response of adults to sequential influenza vaccination. J Med Virol **1984**;14(2):169-75.
57. Hoskins TW, Davies JR, Smith AJ, Miller CL, Allchin A. Assessment of inactivated influenza-A vaccine after three outbreaks of influenza A at Christ's Hospital. Lancet **1979** Jan 6;1(8106):33-5.
58. Bray RA, Gebel HM. Strategies for human leukocyte antigen antibody detection. Curr Opin Organ Transplant **2009** Aug;14(4):392-7.
59. Kao KJ, Scornik JC, Small SJ. Enzyme-linked immunoassay for anti-HLA antibodies--an alternative to panel studies by lymphocytotoxicity. Transplantation **1993** Jan;55(1):192-6.
60. Pei R, Wang G, Tarsitani C, et al. Simultaneous HLA Class I and Class II antibodies screening with flow cytometry. Hum Immunol **1998** May;59(5):313-22.
61. Lin A, Xu H, Yan W. Modulation of HLA expression in human cytomegalovirus immune evasion. Cell Mol Immunol **2007** Apr;4(2):91-8.
62. Kuipers HF, Biesta PJ, Groothuis TA, Neefjes JJ, Mommaas AM, van den Elsen PJ. Statins affect cell-surface expression of major histocompatibility complex class II molecules by disrupting cholesterol-containing microdomains. Hum Immunol **2005** Jun;66(6):653-65.
63. West-Thielke P, Herren H, Thielke J, et al. Results of positive cross-match transplantation in African American renal transplant recipients. Am J Transplant **2008** Feb;8(2):348-54.

64. Katerinis I, Hadaya K, Duquesnoy R, et al. De novo anti-HLA antibody after pandemic H1N1 and seasonal influenza immunization in kidney transplant recipients. Am J Transplant **2011** Aug;11(8):1727-33.
65. Adams AB, Pearson TC, Larsen CP. Heterologous immunity: an overlooked barrier to tolerance. Immunol Rev **2003** Dec;196:147-60.
66. Roddy M, Clemente M, Poggio ED, et al. Heterogeneous alterations in human alloimmunity associated with immunization. Transplantation **2005** Aug 15;80(3):297-302.
67. Lachmann N, Terasaki PI, Budde K, et al. Anti-human leukocyte antigen and donor-specific antibodies detected by luminex posttransplant serve as biomarkers for chronic rejection of renal allografts. Transplantation **2009** May 27;87(10):1505-13.
68. Blumberg EA, Fitzpatrick J, Stutman PC, Hayden FG, Brozena SC. Safety of influenza vaccine in heart transplant recipients. J Heart Lung Transplant **1998** Nov;17(11):1075-80.
69. Brakemeier S, Schweiger B, Lachmann N, et al. Immune response to an adjuvanted influenza A H1N1 vaccine (Pandemrix(R)) in renal transplant recipients. Nephrol Dial Transplant **2011** May 25.
70. Broeders NE, Hombrouck A, Lemy A, et al. Influenza A/H1N1 vaccine in patients treated by kidney transplant or dialysis: a cohort study. Clin J Am Soc Nephrol **2011** Nov;6(11):2573-8.
71. Carroll RN, Marsh SD, O'Donoghue EP, Breeze DC, Shackman R. Response to influenza vaccine by renal transplant patients. Br Med J **1974** Jun 29;2(5921):701-3.
72. Danziger-Isakov L, Cherkassky L, Siegel H, et al. Effects of influenza immunization on humoral and cellular alloreactivity in humans. Transplantation **2010** Apr 15;89(7):838-44.
73. Villard J, Siegrist CA. Anti-HLA Antibody, Adjuvanted Vaccines and Efficacy of Influenza Immunization in Solid Organ Transplanted Recipients. Am J Transplant **2011** Nov;11(11):2539-40.
74. Kimball P, Verbeke S, Flattery M, Rhodes C, Tolman D. Influenza vaccination does not promote cellular or humoral activation among heart transplant recipients. Transplantation **2000** Jun 15;69(11):2449-51.
75. Kumar D, Campbell P, Humar A. Donor-Specific Alloantibody Upregulation After Influenza Vaccination in Transplant Recipients. Am J Transplant **2011** Sep 11.
76. Manuel O, Humar A, Chen MH, et al. Immunogenicity and safety of an intradermal boosting strategy for vaccination against influenza in lung transplant recipients. Am J Transplant **2007** Nov;7(11):2567-72.
77. Magnani G, Falchetti E, Pollini G, et al. Safety and efficacy of two types of influenza vaccination in heart transplant recipients: a prospective randomised controlled study. J Heart Lung Transplant **2005** May;24(5):588-92.
78. Sanchez-Fructuoso AI, Prats D, Naranjo P, et al. Influenza virus immunization effectivity in kidney transplant patients subjected to two different triple-drug therapy immunosuppression protocols: mycophenolate versus azathioprine. Transplantation **2000** Feb 15;69(3):436-9.
79. Schaffer SA, Husain S, Delgado DH, Kavanaugh L, Ross HJ. Impact of adjuvanted H1N1 vaccine on cell-mediated rejection in heart transplant recipients. Am J Transplant **2011** Dec;11(12):2751-4.
80. Schuurmans MM, Tini GM, Dalar L, Fretz G, Benden C, Boehler A. Pandemic 2009 H1N1 influenza virus vaccination in lung transplant recipients: coverage, safety and clinical effectiveness in the Zurich cohort. J Heart Lung Transplant **2011** Jun;30(6):685-90.
81. World Health Organization (WHO). Regulatory preparedness for human pandemic influenza vaccines 2007 [cited July 8, 2011]; Available from: http://www.who.int/vaccine_research/diseases/influenza/Guidelines_regulatory_preparedness_pandemic_influenza_vaccines.pdf
82. Baluch A, Humar A, Egli A, et al. Long Term Immune Responses to Pandemic Influenza A/H1N1 Infection in Solid Organ Transplant Recipients. PLoS One **2011**;6(12):e28627.

83. Blumberg EA, Albano C, Pruett T, et al. The immunogenicity of influenza virus vaccine in solid organ transplant recipients. Clin Infect Dis **1996** Feb;22(2):295-302.
84. Hayney MS, Welter DL, Francois M, Reynolds AM, Love RB. Influenza vaccine antibody responses in lung transplant recipients. Prog Transplant **2004** Dec;14(4):346-51.
85. Belshe RB, Newman FK, Cannon J, et al. Serum antibody responses after intradermal vaccination against influenza. N Engl J Med **2004** Nov 25;351(22):2286-94.
86. Arnou R, Eavis P, Pardo JR, Ambrozaitis A, Kazek MP, Weber F. Immunogenicity, large scale safety and lot consistency of an intradermal influenza vaccine in adults aged 18-60 years: Randomized, controlled, phase III trial. Hum Vaccin **2010** Apr;6(4):346-54.
87. Arnou R, Icardi G, De Decker M, et al. Intradermal influenza vaccine for older adults: a randomized controlled multicenter phase III study. Vaccine **2009** Dec 9;27(52):7304-12.
88. Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. J Hyg (Lond) **1972** Dec;70(4):767-77.
89. Bridges C. Inactivated influenza vaccines. In: Plotkin OaO, ed. Vaccines. 5th ed. Philadelphia, PA: WB Saunders, **2008**:p. 259-90.
90. Ballet C, Roussey-Kesler G, Aubin JT, et al. Humoral and cellular responses to influenza vaccination in human recipients naturally tolerant to a kidney allograft. Am J Transplant **2006** Nov;6(11):2796-801.
91. Lindemann M, Witzke O, Lutkes P, et al. ELISpot assay as a sensitive tool to detect cellular immunity following influenza vaccination in kidney transplant recipients. Clin Immunol **2006** Sep;120(3):342-8.
92. Madan RP, Tan M, Fernandez-Sesma A, et al. A prospective, comparative study of the immune response to inactivated influenza vaccine in pediatric liver transplant recipients and their healthy siblings. Clin Infect Dis **2008** Mar 1;46(5):712-8.
93. Grekas D, Alivanis P, Kiriazopoulou V, et al. Influenza vaccination on renal transplant patients is safe and serologically effective. Int J Clin Pharmacol Ther Toxicol **1993** Nov;31(11):553-6.
94. Briggs WA, Rozek RJ, Migdal SD, et al. Influenza vaccination in kidney transplant recipients: cellular and humoral immune responses. Ann Intern Med **1980** Apr;92(4):471-7.
95. Edvardsson VO, Flynn JT, Deforest A, et al. Effective immunization against influenza in pediatric renal transplant recipients. Clin Transplant **1996** Dec;10(6 Pt 1):556-60.
96. Versluis DJ, Beyer WE, Masurel N, Wenting GJ, Weimar W. Impairment of the immune response to influenza vaccination in renal transplant recipients by cyclosporine, but not azathioprine. Transplantation **1986** Oct;42(4):376-9.
97. Cavdar C, Sayan M, Sifil A, et al. The comparison of antibody response to influenza vaccination in continuous ambulatory peritoneal dialysis, hemodialysis and renal transplantation patients. Scand J Urol Nephrol **2003**;37(1):71-6.
98. Smith KG, Isbel NM, Catton MG, Leydon JA, Becker GJ, Walker RG. Suppression of the humoral immune response by mycophenolate mofetil. Nephrol Dial Transplant **1998** Jan;13(1):160-4.
99. Birdwell KA, Ikizler MR, Sannella EC, et al. Decreased antibody response to influenza vaccination in kidney transplant recipients: a prospective cohort study. Am J Kidney Dis **2009** Jul;54(1):112-21.
100. Meyer S, Adam M, Schweiger B, et al. Antibody response after a single dose of an AS03-adjuvanted split-virion influenza A (H1N1) vaccine in heart transplant recipients. Transplantation **2011** May 15;91(9):1031-5.
101. Manuel O, Pascual M, Hoschler K, et al. Humoral Response to the Influenza A H1N1/09 Monovalent AS03-Adjuvanted Vaccine in Immunocompromised Patients. Clin Infect Dis **2011** Jan;52(2):248-56.

102. Altamirano-Diaz L, West L, Humar A, et al. Early post-transplant vaccination with pandemic influenza A/H1N1 vaccine in pediatric heart transplant recipients. Pediatr Transplant **2011** Mar;15(2):172-5.
103. Salles MJ, Sens YA, Boas LS, Machado CM. Influenza virus vaccination in kidney transplant recipients: serum antibody response to different immunosuppressive drugs. Clin Transplant **2009** Jan-Feb;24(1):E17-23.
104. Danziger-Isakov L, Kumar D. Guidelines for vaccination of solid organ transplant candidates and recipients. Am J Transplant **2009** Dec;9 Suppl 4:S258-62.
105. McMichael AJ, Gotch FM, Noble GR, Beare PA. Cytotoxic T-cell immunity to influenza. N Engl J Med **1983** Jul 7;309(1):13-7.
106. Garantziotis S, Howell DN, McAdams HP, Davis RD, Henshaw NG, Palmer SM. Influenza pneumonia in lung transplant recipients: clinical features and association with bronchiolitis obliterans syndrome. Chest **2001** Apr;119(4):1277-80.
107. Kumar D, Michaels MG, Morris MI, et al. Outcomes from pandemic influenza A H1N1 infection in recipients of solid-organ transplants: a multicentre cohort study. Lancet Infect Dis **2010** Aug;10(8):521-6.
108. Smud A, Nagel CB, Madsen E, et al. Pandemic influenza A/H1N1 virus infection in solid organ transplant recipients: a multicenter study. Transplantation **2010** Dec 27;90(12):1458-62.
109. Billings JL, Hertz MI, Savik K, Wendt CH. Respiratory viruses and chronic rejection in lung transplant recipients. J Heart Lung Transplant **2002** May;21(5):559-66.
110. Kenney RT, Frech SA, Muenz LR, Villar CP, Glenn GM. Dose sparing with intradermal injection of influenza vaccine. N Engl J Med **2004** Nov 25;351(22):2295-301.
111. Manuel O, Humar A, Berutto C, et al. Low-dose intradermal versus intramuscular trivalent inactivated seasonal influenza vaccine in lung transplant recipients. J Heart Lung Transplant **2011** Mar 4.
112. Morelon E, Pouteil Noble C, Daoud S, et al. Immunogenicity and safety of intradermal influenza vaccination in renal transplant patients who were non-responders to conventional influenza vaccination. Vaccine **2010** Oct 4;28(42):6885-90.
113. CDC. 2010-2011 Influenza (Flu) Season. 2011 [cited 2012 May 24, 2012]; Available from: http://www.cdc.gov/flu/pastseasons/1011season.htm