WILL INFLUENZA VACCINATION PROTECT OBESE ADULTS?

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ABSTRACT

Scott D. Neidich: Will influenza vaccination protect obese adults?
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Obesity is a significant public health problem, affecting over one third of the United States, and more than one in ten worldwide. Obesity is associated with a number of comorbidities including cardiovascular disease, diabetes, and importantly, infection. Influenza has been recognized since the 2009 H1N1 influenza pandemic to cause increased severity in obesity, however use of influenza vaccine to reduce influenza-related risk has not been studied specifically in the context of obesity.

In this dissertation, we report that obese adults are at an increased risk for influenza-like-illness despite vaccination. We further report that this deficiency is not due to impairments in response to influenza vaccination, as subjects with confirmed influenza and influenza-like illness produce vaccine-specific antibodies in comparable levels, as assessed by hemaglutination inhibition and microneutralization.

Additionally, we report no apparent defects in influenza-vaccine specific immunoglobulin types, suggesting obesity causes no defects in antibody class switching during influenza vaccine response in a population of Caucasian female adults. Strain-specific response was also assessed, and obese Caucasian women were found to responds similarly to control populations. Finally, B-cell populations were not found to be altered in frequency by vaccination or obesity, however

expression of the activation marker CD38 was found to be lower on CD38 expressing B-cells from obese participants.

This dissertation calls into serious question the use of antibody measures as correlates of protection. If antibodies are indeed major drivers of immunity in influenza vaccination, then subjects who presented with clinical influenza or influenza-like-illness should have been protected against influenza. But as we report here, healthy weight and obese subjects with influenza-like-illness had similar levels of seroprotection assessed by hemaglutination inhibition. Taken in combination with elevated risk for influenza-like-illness in obesity, this suggests that hemaglutination inhibition antibody's status as a correlate of protection against influenza should be reexamined, especially in context of obesity.

To Mom, Dad, Matt, Allie, Lillian and Erin:
Thanks for believing in me.

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Adipo-R	Adiponectin Receptor
ANOVA	Analysis of Variance
ART	Anti-Retroviral Therapy
AID	Activation-Induced Cytidine Deaminase Protein'
BAFF	B-cell Activating Factor
BCR	B-Cell Receptor
BMI	Body Mass Index
CCR7	C-C Chemokine Receptor 7
CD-	Cluster of Differentiation (type indicated by number)
CI	Confidence Interval
CLS	Crown-Like Structure
COPD	Chronic Obstructive Pulmonary Disorder
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
Glut-1	Glucose Transporter 1
HA	Hemaglutinin
HAI	Hemaglutination Inhibition
HIV	Human Immunodeficiency Virus
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G (subtype indicated by number)

IgM	Immunoglobulin M
IL-1	Interleukin-1
IL1β	Interleukin-1 Beta
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-10	Interleukin-10
kD	kilo Dalton
M1	Macrophage Type 1
M2	Macrophage Type 2
MCP-1	Monocyte Chemoattractant 1
MDCK	Madin-Darby Canine Kidney cells
MEM	Minimal Essential Media
MFI	Mean Fluorescent Intensity
MHC	Major Histocompatibility Complex
NA	Neuraminidase
NIH	National Institutes of Health
NK	Natural Killer Cells
OB-R	Leptin Receptor
PBS	Phosphate Buffered Saline
LAIV	Live Attenuated Influenza Virus
QIV	Quadrivalent Influenza Virus (Inactivated)

RDE   Receptor Destroying Enzyme

TCR   T-Cell Receptor

TGFB1   Tissue Growth Factor Beta 1

TGFβ   Tissue Growth Factor Beta

TIV   Trivalent Influenza Virus (Inactivated)

Th1   T-Helper 1

Th2   T-Helper 2

Th17   T-Helper 17

TLR   Toll Like Receptor

TNFα   Tumor Necrosis Factor Alpha

Tregs   T-Regulatory Cells

US   United States

CHAPTER I: BACKGROUND AND SPECIFIC AIMS

A. Obesity and Public Health

A.1: The Burden of Obesity

Obesity is a growing public concern, affecting 13% of the population worldwide[1] and 35% of United States[2] in 2014. Between 2011 and 2012, 8.1% of infants and toddlers under 2 years of age were found to fit the definition for obesity, compared with 16.9% of children between 2 and 18 years of age and 34.9% of adults[3]. Hispanic infants and toddlers were found to have a greater incidence of obesity than non-Hispanic infants, though no other race effects were observed at this age. Between 2 and 18 years of age, Hispanic and Black children had a greater incidence of overweight and obesity than Caucasian children, and Asian children had the lowest incidence of overweight and obesity. No differences were observed for gender within this age group. Non-Hispanic Black adults have a greater incidence of obesity than non-Hispanic White and Hispanic White adults, while non-Hispanic Asian adults had the lowest incidence of obesity. Among all adults an estimated two-thirds of adults are overweight or obese, and the number of severely obese (BMI>40) is increasing. By most estimates, obesity related diseases are now a major burden on the US Healthcare systems, accounting for 4-7% of US Healthcare expenditure in 2003[4], and is estimated to reach 14-16% by 2030[5].

A.2: **Comorbidities of Obesity**

These cost predictions are not for obesity alone, but include a number of co-morbidities. Obesity has been linked to cardiovascular disease for over 60 years[6], diabetes for over 85 years[7], and dyslipidemia for over 50 years[8]. Obesity's contribution to elevated serum lipid levels, and subsequent deposition of lipids onto blood vessel walls causing partial blockages that can cause myocardial infarctions have been long described. More recent research[9] has demonstrated that these plaques are made worse by infiltration of immune cells, which trigger inflammation, enlarging and solidifying plaques. This contributes to increased blood pressure, which can induce aneurysm or hemorrhagic stroke elsewhere in the circulatory system. Obesity's relationship to diabetes is relatively newer, but nonetheless very well established. Through an incompletely understood mechanism, excess nutrient availability causes many cell types (including skeletal muscle cells and adipocytes) to become resistant to insulin, causing a feed-forward mechanism with greater circulating levels of glucose. This elevated glucose level then contributes to inflammation systemically[10]. Other diseases including musculoskeletal[11], gallbladder[12], and certain types of cancers[13] also have well established causal relationships with obesity, and are likely linked to obesity related inflammation. Those diseases are included in these cost estimates mentioned previously[14]. Notably absent from these projections, however, is infection: given what we know about influenza infection, these projections may be underestimates.

A.3: Influenza and Public Health

Influenza is already a significant public health burden on its own. Between 1976 and 2006, influenza killed between 0.4-5.1 per 100,000 annually[15]. Obesity has been shown to be an

independent risk factor for increase in the risk of severe infection and death from influenza[16]. And while influenza vaccination is a moderately effective treatment for preventing pneumonia, hospitalization, and death from influenza[17], whether or not obesity affects the ability to respond to influenza vaccination has not been described prior to our investigation in Specific Aim 1. The public health burden of obesity related influenza infections has not been quantified, however previous and current research from our lab suggests that a substantial portion of influenza's $16.3 billion annual cost may be attributable to obesity[18].

B. Influenza Vaccination and the Immune System

There are two main forms of influenza vaccination: Live Attenuated Influenza Vaccine (LAIV), and the Subunit/Conjugate Influenza Vaccine, referred to in this dissertation as either TIV (Trivalent) or QIV (Quadrivalent). In both vaccination strategies, global circulating cases of influenza are observed and modeled in order to anticipate which influenza strains are likely to be predominant during the coming influenza season. Similar strains are then selected as the targets for the annual vaccination, and enter vaccine production. When matched accurately, they will induce generation of antibodies to the circulating strain of influenza, protecting those inoculated from circulating strains. But even when matched poorly, antibodies and cellular immunity generated may also be "cross reactive," potentially granting some protection against circulating strains despite the poor match.

Influenza virus for vaccine production are typically grown by infecting embryonated chicken eggs, however cell culture approaches are also in use[19] and may become more prominent in the future.

B. 1: Vaccine Production

For LAIV vaccine production, the influenza strains of interest are reasserted with a cold-adapted strain and used to infect eggs. influenza infected eggs are incubated at a lower temperature of 33 degrees Celsius, and reassortment causes the strain to lose its ability to effectively replicate in cells at higher temperatures[20]. Patients are inoculated with the attenuated viral vaccine intranasally, thereby infecting the epithelia of the upper respiratory tract. While the virus will be able to infect cells in the cooler, upper airway, it is unable to replicate in the warmer cells of the lower airway, thus preventing a severe infection in healthy patients. As LAIV provides a "natural infection" route, it was previously thought to be particularly effective in children who may not have been exposed to any influenza virus before[21]. While some researchers found greater vaccine response from LAIV in children, longer term epidemiological analysis later suggested that LAIV is actually less effective at preventing certain strains of influenza infection, and equally effective for others[22]. LAIV is therefore no longer listed as the preferred vaccination form for children, however parents often select LAIV over TIV because it does not require administration via injection. The same features of LAIV previously thought to make it ideal for children also make it potentially harmful to patients with immunosuppression, including HIV positive patients. Additionally, neutralizing antibodies present in the nasal epithelia from previous exposure may prevent the cold adapted virus from initiating a mild infection, preventing "boosting" of the immune system in subjects with a history of influenza exposure. This means that elderly subjects, and even adults, may not generate additional protection to LAIV. For these, TIV is the preferred vaccination strategy for adults and the patients with immunosuppression, and LAIV's contraindications includes adults

over 50 years of age.

TIV consists of viruses grown at 37 degrees Celsius, however the virus undergoes a subjugation process which cleaves the virus particles into subunits, which are later included in the vaccine. TIV is administered through intramuscular injection, typically into the deltoid muscle. It is worth noting that the influenza virus is only able to replicate if it reaches the respiratory mucosa and is intact, making infection from TIV impossible.

B.2: Primary and Secondary Response to Vaccination

The immune system response to vaccination with TIV or LAIV, and influenza infection functions in much the same way. Resident dendritic cells engulf the pathogen or antigen and digest it within intracellular vesicles. Antigens induce signals on specialized receptors on the surface of these vesicles known as Toll-Like Receptors (TLRs), which induce signaling that alters the behavior of the cells. Macrophages typically respond to antigens by becoming more inflammatory, seeking out other nearby pathogens more aggressively, and secreting pro-inflammatory cytokines that induce swelling and inflammation at the site of antigen exposure. This inflammation, combined with additional intracellular TLR signaling, causes resident dendritic cells to reprogram from a "seek and destroy" phenotype to an "observe and report" phenotype. The dendritic cells begin to present the acquired antigens on surface embedded in Major Histocompatibility Complex (MHC) proteins, and cease uptake in preparation for migration. Following signaling through the CCR7 receptor, the dendritic cell leaves the tissue and migrates to the T-cell zone of the nearest draining lymph node. There, it presents MHC-antigen to T-lymphocytes. T-lymphocytes express the T-cell Receptor (TCR), and each naïve T-cell generated has a unique region of the TCR with variable specificity for MHC presented

antigens. When a T-lymphocyte with an antigen-specific TCR binds to the dendritic cell presented antigen, that T-lymphocyte becomes activated.

Meanwhile, dendritic cells also release antigens into the lymphoid space in the vicinity of B-lymphocytes, and some antigen may flow freely into the lymph node from surrounding tissue. B-lymphocytes are named for the B-cell Receptor (BCR), which has a variable region similar to the TCR. But unlike the TCR, the BCR can recognize free peptides, and does not require MHC presentation. When a B-lymphocyte's BCR binds to antigen, the B-lymphocyte will engulf the antigen, partially digest it, and present peptides on its surface MHC class II proteins. While B-lymphocytes residing in inflamed tissues can undergo activation without further stimulation, B-lymphocytes in the lymph nodes will migrate from the B-cell zone to the T-cell zone, and seek an activated cognate T-lymphocyte: One which shares influenza specificity.

When an activated cognate T-cell encounters a B-cell, which it recognizes by presentation of antigen, the T lymphocyte provides activation signals including CD40L and cytokines including IL-4[23]. This stimulation and costimulation triggers a series of intracellular signals that ultimately results in the B-cell activating the *AICDA* gene and producing the Activation-induced Cytidine Deaminase protein (AID). This B-cell, which has some BCR specificity for influenza, uses AID to induce mutations to the BCR. Known as somatic hyper mutation, this process causes changes to the BCR that may induce greater, lesser, or equivalent affinity to the target antigen. The B-cell migrates into the dark zone of a lymph node, along with its cognate T-cell. Both multiply, and daughter B-cells compete for limited antigen availability on follicular dendritic cells within the germinal center. B-cells with the greatest affinity for antigen outcompete B-cells with lesser affinity, a micro-evolutionary event which produces

greater specificity B-cells over the course of a few cell generations. B-cells with lower affinity fail this competition for antigen, and undergo apoptosis.

The T-cells also undergo a similar process in a separate lymph node compartment. By the time the B-cells have completed affinity maturation, a population of cognate T-helper cells are ready to help the high-affinity B-cells. These T-helper cells can provide signals to the B-cells to undergo class switching and additional proliferation, allowing the B-cell population to expand and change their BCR's to the ones most relevant for the target antigen, which are reviewed section B.3.

A secondary response to antigen occurs when an antigen previously seen by the immune system is introduced, and can happen much more quickly. Secondary exposures to previously experienced antigens may not even require a reaction to occur, as long-lived plasma cells in the bone marrow secrete antibodies for their entire lives. If the pathogen a vaccine is made to protect against manages to get into the body, it may be quickly neutralized by circulating levels of antibody and never begin infecting cells, nor illicit an immune reaction. Should there be enough pathogen to make it through this layer of protection, circulating memory B and T cells will become activated upon antigen exposure in much the same way as in a primary infection, and quickly give rise to the same populations. Because the memory cells do not need to undergo gene editing, and because there are more circulating pathogen-specific memory cells than there were pathogen-specific naïve cells originally, the memory cells are able to give rise to a larger population of effector cells more quickly than in the primary reaction. This quicker response typically overwhelms the infectious agent, and also generates new memory cells for future exposures.

B.3: Antibody Classes

Depending on the context of the exposure, and the types of antigens present, T-helper cells and intracellular TLRs provide different signals to the newly activated B-cells. These signals trigger the B-cells to make one final modification to the BCR. In its secreted form, the BCR is more commonly referred to as an immunoglobulin or antibody, and there are multiple forms serving multiple purposes. All naïve B-cells express two types of immunoglobulins: Immunoglobulin-D (IgD) and Immunoglobulin-M (IgM). While naïve B-cells express these immunoglobulins on their surface, they can also be secreted by plasmablasts and plasma cells. While IgD's function has remained largely a mystery since its' 1965 discovery, recent evidence suggests that IgD may recruit basophils to induce inflammatory factors including cathelicidin, pentraxin-3, IL-1, IL-2, and BAFF[24]. These factors upregulate proteins with antimicrobial, opsonization, and immune-stimulation functions. Meanwhile, IgM's function is well established to be agglutination, opsonization, and complement fixation. Through some combination of T-cell derived cytokines, toll-like receptor signaling and possibly other unknown mechanics, B-cells recombine the heavy chain elements of their immunoglobulin genes to change from IgM/IgD coexpression to expression of solely IgM, solely IgD, or solely one of the other immunoglobulin subtypes.

Viral particles typically illicit an Immunoglobulin-G (IgG) subtypes 1 (IgG1) and 3 (IgG3), although it is interesting to note that they are derived from opposing cytokines. IL-4 secreted from Th2 cells drives IgG1 class switching and inhibits IgG3, while IFN-gamma drives IgG3 and inhibits IgG1. The anti-inflammatory, T-regulatory derived IL-10 promotes both IgG1 and IgG3 class switching. Both IgG1 and IgG3 effectively activate the complement receptor C1q, leading

to greater C3b recruitment and even disruption of any associated bilipid membranes by the complement pathway[25]. For a bacterium or viral (including influenza) infected cell, this can mean destruction. IgG1 and IgG2 also effectively bind many Fc receptors, while IgG3 has the greatest affinity.

IgG2 inducement is not fully explained by cytokines or any particular class of T-cells in humans, although it does have a complex interaction involving IL-6, wherein the cytokine appears to both[26] upregulate and downregulate IgG2. IgG2 is typically seen in context of bacterial antigen and in response to bacterial polysaccharides, whereas IgG4 is found with chronic exposure to the same antigen, alongside Immunoglobulin-E (IgE). In contrast to IgG1 and IgG3, IgG2 and IgG4 have much lower C1q binding affinity and Fc receptor affinity, making them less potent drivers of inflammation. Neither is typically observed in response to influenza vaccination[27].

In mucosal infections and exposures, IgA has a unique distribution mechanism. While other immunoglobulins are produced by B-cells and then diffuse through circulation and lymph, IgA interacts with the polymeric immunoglobulin receptor of mucosal epithelial cells, which bind IgA and transport it into mucosal spaces. There, IgA neutralizes pathogens, but does not trigger inflammation due to an inability to fix complement. While the specific regulation of IgA class switching is not fully known, T-cell derived TGFB1 and CD40L play a crucial role in class switching[28]. IgA secretion into mucosal spaces may prevent LAIV from successfully triggering an immune response, and is one of the reasons behind that vaccine's contraindication in adults over 50 years of age.

C. Obesity and the Immune System

C.1. Adipose tissue as an immunomodulatory endocrine organ.

Obesity is characterized by an excess amount of adipose tissue, which is composed of adipocytes, macrophages, lymphocytes, fibroblasts and endothelial cells. Obesity induces not only increased adipocyte number through enlargement of the tissue, but increased adipocyte size. Immune cell infiltration is also increased, further adding to the adipose-tissue population. In addition to excess adipose tissue, obesity is characterized by an increase in pro-inflammatory immune cells, which secrete cytokines that have been shown to promote insulin resistance[29]. Although the exact timing and series of events leading to adipose inflammation in obese humans is not known, work in animal models has demonstrated that, as adiposity increases, neutrophils enter the adipose tissue first, followed by macrophages, and then T and B cells[30]. How much of the inflammation is induced by feeding a high fat diet vs. the actual obesity is unresolved, although likely both contribute to the inflammation. Indeed, mice that become obese on a low-fat chow diet develop similar immune dysfunction compared with obese mice fed a high fat diet[31].

In addition to increased cellular infiltrate into the adipose tissue, the phenotype of the immune cells is altered. Macrophages of the M2 type (anti-inflammatory, tissue repairing) become more M1 like (pro-inflammatory). T cells infiltrating the adipose tissue display activation markers[32] and T regulatory populations are increased within adipose tissue.

In addition to their role as a storage site for lipids, adipocytes secrete a variety of adipokines, including leptin and adiponectin. Although leptin and adiponectin are primarily

known for their roles in regulating food intake, body weight and metabolism, it has recently become clear that they also play a role in T cell regulation.

Leptin is a 16kD adipokine which is secreted directly in proportion to adipocyte mass, thereby increasing adiposity causes an increase in leptin levels. Leptin is a well-known regulator of food intake and energy expenditure, and more recently has received attention for its pro-inflammatory characteristics[33]. Leptin concentration in the blood for healthy weight adults typically ranges between 10 and 20 ng/mL and can fluctuate within this range based on food intake. In obesity, however, a greater volume of adipose tissue alters serum leptin levels. Average leptin concentration is reported to be over 30 ng/mL in obese humans, and is less responsive to dietary fluctuations[34]. Obese humans can be considered to be in a constant state of hyperleptinemia. Hyperleptinemia promotes T-effector cell function while inhibiting T regulatory cell function[35], whereas absence of leptin prevents T-cell activation. B cells live longer in the presence of leptin, and are more resistant to apoptosis[36]. Dendritic cells activate T cells more strongly in presence of leptin[37], and natural killer (NK) cells experience greater metabolism and are more cytotoxic with increasing leptin[38].

Leptin induces effects within a cell by binding to a specific cellular receptor, the OB-R leptin receptor, and transducing a signal into the cell through protein kinase and phosphatase cascades[39]. Which cascades are activated determines the cell's response to leptin, however, it is important to note that multiple isoforms of the OB receptor exist, each with specific effects. Expression of different OB receptors on different cells allows cells to respond in different ways from one another[36]. There are six known isoforms including four short, cell embedded isoforms (OB-Ra, OB-Rc, OB-Rd, and OB-Rf) and one long, cell embedded isoform (OB-Rb). Additionally,

there is one soluble form (OB-Re). The soluble OB-Re regulates circulating concentrations of leptin and cannot transduce signals into the cell. The short form leptin receptors are mainly involved in leptin transport throughout the body, although some intracellular signaling has been observed from some of these isoforms. The long-chain OB-Rb is the primary receptor involved in signal transduction, thanks to an enlarged intracellular region of the protein which facilitates signal transduction through multiple kinase pathways reviewed in Allison and Myers[40] (2014).

OB-Rb is found not only on the cells of the hypothalamus involved in satiety signaling, but in multiple immune cell populations including T and B cells, dendritic cells, monocytes, neutrophils, macrophages, and natural killer cells. Given the wide variety of cell types which are responsive to leptin, it should come as no surprise that the actual effects of leptin signaling on different cell types are varied. For this reason, leptin's function is pleiotropic.

T regulatory cells (Tregs) express the OB-Rb receptor, and their activation is diminished by leptin signaling[41]. Dendritic cells, monocytes, B cells and NK cells are all dependent upon leptin signaling for their formation prior to activation, and inflammatory conditions including obesity have been shown to divert production of some of these cell types from the bone marrow to the periphery[42]. This shift in origin may lead to a shift in immune population favoring macrophages "primed" to become M1. While Tregs are increased in number in adipose tissue, Treg frequency is reduced with leptin concentration in humans with auto-immune diseases such as multiple sclerosis[43]. The anti-apoptotic effects observed in B cells functions occurs similarly in neutrophils, albeit through different signaling cascades[44] Leptin can also alter the metabolism of macrophages[45].

Adiponectin behaves in a manner opposite to leptin. It is also secreted by adipocytes, but is released during low insulin, high glucagon states such as fasting. Despite being secreted from adipose tissue like leptin, adiponectin is significantly decreased in obese humans. Healthy weight humans have been shown to have serum concentrations around 12 ng/mL, whereas obese humans in the same study were closer to 4 ng/mL adiponectin in the serum

Adiponectin triggers orexigenic pathways in the hypothalamus, inducing hunger and food seeking behavior. It plays a role in generation of adipocytes, and can alter metabolism by decreasing gluconeogenesis and increasing glucose uptake. This alteration of metabolism may be involved in adiponectin's ability to reverse insulin resistance in mice[46], an effect observed independently of obesity and weight loss.

Just as adiponectin's function is opposed to leptin's on satiety, they share an antagonistic relationship for several immune cell types. Interestingly, when at rest, only 1% of T cells express adiponectin receptor (Adipo-R) on their surface, retaining the receptor in intracellular vesicles, where it is inactive[47]. Upon activation, the Adipo-R migrates to the cell surface, allowing the T cells to respond to adiponectin. When adiponectin signaling occurs, T cell activity is dampened by enhancing apoptosis of T effector cells and inhibiting their proliferation[48].

In contrast, 47% of B cells, 93% of monocytes, and 21% of NK cells express adipo-R on their surfaces[48]. When stimulated through this receptor, B cells secrete a soluble factor which impairs T cell migration into inflamed tissues, further diminishing inflammatory responses[49]. Monocytes undergo a greater degree of apoptosis, and secrete fewer pro-inflammatory cytokines when stimulated with adiponectin[50].

Given the general effects of adiponectin on immune cell types, we can consider

adiponectin to induce "anti-inflammatory" effects on the immune system. It is important to

note that, while many of these effects are in opposition to leptin, the balance struck between

adiponectin and leptin in the behavior of the immune system is non-symmetrical. Whether this

brings the immune system to a "pro-inflammatory" or "anti-inflammatory" state is dependent

not only on the relative abundance of these adipokines, but innumerable other factors

including presence of infection, availability of metabolic substrate, and likely many others.

Adiponectin and leptin are far from the only adipokines secreted from adipose tissue,

although they are the only ones thoroughly established to be regulated by energy balance.

Other pro-inflammatory adipokines include Interleukin 6 (IL-6), monocyte chemoattractant 1

(MCP1), chemerin, and Tumor Necrosis Factor α (TNFα).

While each of these is positively associated with obesity, IL6 is very strongly correlated

with obesity, and has a very important immunomodulatory role involving Tregs. Tregs are a

subset of T cells which play an important anti-inflammatory role. Tregs are induced after a

prolonged inflammation period, and the predominant cytokine causing their induction is TGFβ.

Following induction, Tregs will dampen inflammatory signals in other CD4+ T cells (Th1, Th2, and

Th17), causing the T cell response to be suppressed. Many other immune cells, including B cells,

dendritic cells and macrophages, depend upon T cell signaling for their inflammatory response,

placing Tregs at a crucial point in inflammation progression. But in combination with the

adipokine IL6, naïve T-cells exposed to TGFβ will undergo differentiation into the pro-

inflammatory Th17 cell type, depriving the inflammation site of anti-inflammatory Tregs[51]. The

replacement of anti-inflammatory Tregs with pro-inflammatory Th17 cells cause acute inflammation, potentially an over-reaction.

The adipokines MCP1 and chemerin play important roles in monocyte recruitment to adipose tissue. MCP1[52] and chemerin[53] are both associated positively with human obesity. Recruitment of monocytes is the first step of adipose-tissue macrophage generation. Adipose tissue macrophages have a tremendous impact on the microenvironment of adipose tissue, which will be discussed in the next section. TNFα is positively associated with obesity[54], although it is secreted from pro-inflammatory M1 macrophages[55] resident in the adipose tissue. TNFα stimulates phagocytosis[56], as well as M1 polarization and angiogenesis.

From leptin's pro-inflammatory effects, to the lack of adiponectin to dampen inflammation, obesity triggers a pro-inflammatory cytokine secretion that results in a persistent low-grade inflammatory state. We speculate that this long term, chronic inflammatory state may have an impact on the response to pathogens, although the mechanisms remain undetermined. Explanations on the interaction between inflammation and infection outcome include damage from an excessive inflammatory response to a delayed immune response[57], which may allow greater pathogen replication in the lung[58]. Next, we will discuss how the innate immune system is affected by these primary characteristics of obesity.

C.2. Innate immune cells: local and systemic effects of obesity

While the precise origin of obesity's immunological consequences remains open to debate, it is widely held that cells of the innate immune system mediate physiological changes present in obesity. In the microenvironment of adipose tissue, macrophage infiltration and

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polarization to a pro-inflammatory subtype (M1 macrophage) is greatly increased. It remains unclear whether increased presence of M1 macrophages is the cause, or merely an important step in the creation of the low-grade inflammation characteristic of metabolic syndrome.

What is clear is the difference in macrophage phenotype. Lean adipose tissues include a relatively low number of anti-inflammatory M2 macrophages, which metabolize fatty acids and dampen immune activation through secretion of IL10 and TGFβ[59]. M2 macrophages tend to be dispersed throughout the adipose tissue, and may play a role in wound healing in the event of injury[60]. However, the obese adipose environment recruits and polarizes M1 macrophages to five times[61] the level of M2 in lean adipose tissue. M1 macrophages utilize glucose as their fuel source, and secrete pro-inflammatory molecules including TNFα, IL1β and MCP1. Using glucose as a fuel source allows M1 macrophages to generate greater amounts of energy without consuming as much oxygen, which may be necessary considering the way M1 macrophages distribute. Unlike M2 macrophages, distribution of M1 macrophages tend to cluster around a specific feature, called a crown like structure (CLS). Whether due to hypoxia, inflammation, or unmanageable hypertrophy, each dying adipocyte recruits dozens of adipose tissue macrophages which degrade the damaged cell and consume its fat content. The M1 macrophages that facilitate this process share many morphological characteristics with foam cells in atherosclerosis, and are believed to contribute to inflammation in a similar feed-forward mechanism. To clear lipids from the dead adipocyte, M1 macrophages secrete fatty acids into the tissue surrounding the CLS. Paradoxically considering their fuel sources, this may then facilitate conversion of M2 macrophages to M1, further shifting the adipocyte macrophage population toward the M1 phenotype[62].

This shift in macrophage population is not without consequence. M2 macrophage secretion of IL4 and IL10 promotes insulin sensitivity in adipocytes, whereas TNFα secretion by M1 macrophages acts locally, diminishing insulin sensitivity in adipocytes and other immune cells locally. And in addition to its feed-forward loop role in recruiting monocytes, MCP1 also acts to recruit other immune cells including neutrophils, basophils and mast cells. While these effects are highly pronounced within the adipose tissue, many of the adipokines generated have systemic effects when they leave the adipose tissue. Leptin and adiponectin's endocrine effects on satiety have already been discussed, but TNFα[63] and IL6[64] also escapes adipose tissue into the bloodstream, contributing to systemic insulin resistance. Macrophages may also be the primary drivers behind other immune cell infiltration into obese adipose tissue: Neutrophils exhibit elevated activity[65] and greater numbers[66] in obesity, as do mast cells[67]. Both neutrophils[68] and mast cells[69] are also established to drive insulin resistance in murine models.

C.3. Adaptive immunity: local and systemic effects of obesity

Cells from the adaptive immune system play critical roles in both lean and obese adipose tissues. In lean, Th2 and Treg cells provide anti-inflammatory cytokine signals to infiltrating monocytes, promoting anti-inflammatory M2 cells. It is the action of these anti-inflammatory T cells that maintains the inflammation-free environment of lean adipose tissue, which promotes insulin sensitivity and a healthy function of the endocrine organ. In obesity, however, Tregs and Th2 do not form as readily, and instead Th1, Th17, CD8+ T effector cells, and B cells are the predominant adaptive immune cells, which give rise to M1 macrophage

polarization, greater inflammation, and the pro-inflammatory state. While the mechanism of this transition from anti-inflammatory to pro-inflammatory adipose phenotype in obesity remains to be determined, the only question of the adaptive immunity's role is whether it is the cause, or merely a significant contributor.

Both in adipose tissue and systemically, Th1 cells generally act in concert with macrophage and CD8 T cells to combat intracellular bacteria and viruses, whereas Th2 cells promote B cell, eosinophil and mast cell activation, prompting a wide response to a wide array of extracellular pathogens including viral, bacterial and fungal. Th17 cells are pro-inflammatory and aid in clearing pathogens.

However, obesity affects the adaptive immune system beyond its changes to the adipose microenvironment as well, which may be attributable, in part, to nutrient availability. CD4 T cell subtypes Th1, Th2 and Th17 are glycolytic cell types, and increasing glucose availability causes greater T effector function[70]. While each of these T cell subtypes causes a distinct inflammatory response, together they all contribute to inflammation. Conversely, Treg cells depend on fatty acid oxidation for their metabolism, and do not increase their anti-inflammatory effects during increased glucose availability[71].

Given T cell function dependency upon glucose availability, it should come as no surprise that T cell activation includes upregulation of glucose transporters. Specifically, T cells will become insulin sensitive during activation, and use insulin signaling in a classical manner to activate Glut-1. Indeed, activated T cells from hyperglycemic subjects produce greater levels of pro-inflammatory cytokines *in vitro*[72] and *in vivo*. Insulin deficiency due to poor diet or genetic

defect results in poor T cell function, increasing susceptibility to infection[73]. The extent to which this can be attributed to insulin resistance remains unclear.

While relatively little has been characterized about B cell metabolism and obesity, numerous studies have pointed to B cell alterations related to obesity, including greater infiltration of mature, class-switched B-cells into visceral adipose tissue[74,75] and increasing B-regulatory cell activity in obese mice has been shown to reduce inflammation[74,76]. It remains to be determined whether these are secondary effects from T cell alterations, distinct effects brought on by obesity through B cells directly, or a more complicated mechanism yet to be proposed.

D. Viral Infection and Obesity.

Obesity is believed to have numerous synergistic effects with viral infections. For example, hepatitis C infection combined with obesity induces greater degree of hepatic steatosis.[77] While both obesity and hepatitis C can cause steatosis on their own, the combined effect is more pronounced than either disease individually. But in HIV infection, obesity has a complex interaction with the associated disease AIDS. Prior to the advent of anti-retroviral therapy (ART), which has effectively turned HIV from a death sentence into a serious, chronic, but survivable viral infection, obesity was associated with prolonged lifespan following HIV infection. Obesity can lead to greater numbers of CD4$^+$ T helper cells, which are also the cells HIV targets.. Therefore, unsurprisingly, obesity is positively associated with CD4+ T cells levels in HIV infected adults. Before ART, this also meant greater longevity[78]. However, ART has been shown to exacerbate dyslipidemia by destroying the mitochondria of adipocytes[79], leading to

greater occurrence of atherosclerosis in HIV positive adults, especially obese adults [80]. So while

obesity may give some protections against HIV progression, it comes at a greater cost.

Obese humans are at a greater risk of hospitalization and death from influenza than

healthy weight individuals[81], and obesity is linked with greater need for mechanical ventilation

and time spent in the Intensive Care Unit[16]. While there are changes to the muscular

involvement of breathing caused by obesity[82], the immunological rather than physical

conditions of obesity are likely to be substantial contributors to complications. Given previous

work in our lab, we can speculate on obesity's effects: Chronic inflammation associated with

obesity may delay the specific immune response, allowing the infection to become more

virulent. Alternatively, obesity may cause the immune system to overreact to the infection and

induce excessive tissue damage, paradoxically causing secondary infection and severe

pneumonia. Indeed, in obese models of influenza infection, there is greater inflammation in

lung tissues of obese mice[83], likely attributable to diminished Treg activity and increased

systemic levels of CD4+ T cells. Additionally, despite generating a normal response to influenza

vaccination initially, higher weight is correlated with a greater decrease in antibody levels 1

year post vaccination in humans[84] suggesting that although the antibody response is not

acutely affected, obese individuals are unable to maintain the antibody response over time.

Influenza virus is not a single, immutable infectious agent: multiple strains which can

infect humans exist, and immunity—especially humoral immunity—to one strain may not

equate to immunity to another due to differences in protein composition of the virus. Humoral

immunity primarily targets external viral proteins, while T-cell immunity primarily responds to

internal viral proteins, and proteins expressed during the virus's intracellular replication phase.

For this reason, T cells can produce cross-reactive immunity, as, in contrast to external viral proteins, internal influenza viral proteins are similar among strains. However, after priming obese mice with an influenza H3N2 strain and then re-infecting with what would otherwise be a lethal dose of influenza pandemic H1N1, 25% of obese mice die with survivors experiencing 10 to 100 times greater viral titers in lung tissue, as well as greater pro-inflammatory cytokines. In contrast, 100% of lean mice survive[85]. In humans, T cells isolated from overweight and obese humans show diminished markers of activation, while dendritic cells express reduced MHC-II, the protein responsible for antigen presentation to T-cells[86]. Despite these deficits in T-cell responses, following vaccination, obese adults produce an antibody response equivalent to healthy weight adults, however the antibody response declines more rapidly[84].

Taken in sum, obesity exacerbates dyslipidemia associated with some infections, inhibits memory response to infection, and contributes to a pro-inflammatory environment that licenses greater tissue damage from infection and inflammation.

E. Specific Aims

There remain specific questions unanswered by existing research. While there is ample epidemiological evidence that obese individuals are at greater risk for severe illness and death from influenza, it has not been determined if vaccination mitigates this disparity. We will address this question in **Specific Aim 1,** where we hypothesize that, compared with vaccinated healthy weight adults, obese adults will have a greater risk of influenza infection despite vaccination.

Secondly, although obesity's effects on innate immune cells have been closely studied, and the impact on T-cells in the context of influenza infection has been investigated by our laboratory, humoral Immunity has not been thoroughly investigated. Presently, published literature on influenza and infection is largely limited to T-cell immunity, with only one publication assessing vaccination and B-cell response in obese populations to date[87], with a narrow target focus, small experimental size, and findings that differ substantially from our laboratory's previously published work in antibody variation. Specific aim 2 will expand characterization of the B-cell response to influenza vaccination by specifically investigating B cell subpopulations and IgG serotypes. **Specific Aim 2** is to characterize alterations in humoral response to influenza vaccination driven by obesity in humans. We hypothesize that obese adults will exhibit impaired memory B-cell numbers despite normal plasma cell generation.

CHAPTER II: OBESITY DIMINISHES EFFICACY OF INFLUENZA VACCINE IN ADULTS

Overview

Influenza infection represents a significant public health problem for the general public, and is especially dangerous to individuals with obesity. Although obesity has been identified as an independent risk factor for influenza infection for over 5 years, protection offered by vaccination has not been specifically examined in this at-risk population. Here, we used a prospective observational study to assess whether influenza vaccination modifies the risk of influenza infection associated with obesity. Using multiple measures to assess influenza vaccine response, we found that obese adults generate antibodies at least as effectively as healthy weight adults. However, despite this equivalent humoral response, when compared with vaccinated healthy weight adults, vaccinated obese adults are approximately twice as likely to develop clinical influenza infection.

Introduction

Infection with influenza represents a worldwide public health problem, with 15% of the global population infected annually. In addition, pandemic outbreaks of influenza can greatly magnify the number of infections and death. Historically, the highest risk groups for increased morbidity and mortality from influenza infection include the elderly[1], the very young[88], individuals with chronic diseases such as COPD[89] or congestive heart failure[90], and pregnant women[91]. Recently, obesity has also been recognized as an independent risk factor for influenza[92]. This is significant, because at present 37% of the US population is obese[93], and

worldwide about 13% of the adult population is obese[94]. Obesity is defined as excessive adiposity as a result of prolonged positive energy imbalance, and is typically defined by Body Mass Index (BMI) in excess of 30 kg/m^2.

Vaccination is the best, and currently only, medically available prevention against influenza virus infection. Neutralizing antibodies against the two surface proteins of the influenza virion, hemagglutinin (HA) and neuraminidase (NA), are generated in response to influenza vaccination and are considered to be protective[95]. However, year-to-year changes in the HA and NA result in the influenza vaccine being less effective in subsequent years, often requiring a yearly reformulation of the influenza vaccine. This also means that in the event of a pandemic, in which viral reassortment causes major changes in the HA and NA, there may be little protection, contributing to a pandemic spread of the virus[96]. Generating protection against the most likely viruses to be encountered is facilitated by inclusion of three influenza types: typically one influenza A virus with Hemaglutinin type 1 and Neuraminidase Type 1 (H1N1), a second Influenza A virus with H3N2, and an Influenza B virus are included in the trivalent influenza vaccine. In the past several years, a second B virus has been added to make the quadrivalent vaccine.

The protective effect of influenza vaccine can vary year to year, because its effectiveness depends on the antigenic match between the viruses in the vaccine and the strain(s) circulating in the community. For example, in the 2014-2015 season a mismatch in the northern hemisphere between the H3N2 vaccine strain and the H3N2 strain circulating in the community reduced estimated vaccine effectiveness to 23%[97]. In contrast, the 2011-2012 year matched correctly and achieved protection rates of 39-65% varying by vaccine strain component, and 47% overall[98]. Even with proper matching as in 2011-2012, other factors affecting the general health of vaccine

recipients, including age,[106] immune dysfunction[99], and other uncharacterized factors limit full protection including unexplored effects of obesity.

Vaccine induced-antibody protection against influenza infection is generally assessed by quantifying homospecific antibody titer against the HA of viral strains included in the vaccine. In addition to homospecific antibodies, limited hetero- and poly-specific antibodies are also observed from prior exposures[100] and vaccinations[101]. Although there is an ongoing research effort to generate a universal cross-protective vaccine in which antibodies would be broadly cross-reactive[101], no such vaccine is currently available. For measuring a successful immunization with influenza vaccine, concentrations of antibodies against the HA is conveniently measured. HA inhibition assays (HAI) measure anti-HA antibodies; these levels correlate with protection[102]. Seroconversion is defined as a 4-fold increase in antibody titer from pre to post vaccination and is considered to be an immunogenic response[103]. Seroconversion is also considered a measure of potential vaccine efficacy. However, older adults have been shown to develop influenza despite undergoing seroconversion[104], demonstrating that antibody titers do not necessarily translate into clinical protection.

Seroprotection is defined as an HAI above a certain threshold, typically 32 or 40 HAU/50 μL serum. Seroprotection is more commonly referred to as a correlate of protection than an immunogenic marker because pre-vaccination HAI measures often meet this definition [105]. Despite being considered a correlate of protection, seroprotection suffers the same deficiencies as seroconversion in elderly participants. Additionally, seroconversion rates after vaccination have been reported as low as 58% in healthy adults, and seroprotection following vaccination

has been reported to be as low as 78%[106]. This suggest that the influenza vaccine fails to illicit humoral protection in approximately 1 in 5 healthy recipients[106].

We have previously shown that vaccinated obese individuals have an impaired response to influenza vaccination[86]. Although both healthy weight, overweight (BMI between 25 and 30) and obese adults developed equivalent levels of HAI antibodies to vaccine strains of influenza at 30 days' post vaccination, obese adults failed to maintain this response. At one-year post vaccination, greater BMI was associated with a steeper decline in antibody titer. Compared with healthy weight individuals, obese individuals were more likely to have a greater than 4-fold drop in antibody titer at one year. However, this analysis did not determine whether vaccinated obese adults were more likely than healthy weight adults to develop influenza. Here, we demonstrate that, compared with healthy weight adults, influenza-vaccinated obese adults had a higher rate of laboratory-confirmed influenza and influenza-like illness. Importantly, as has been reported for elderly adults, the increase in infection in obese adults occurred with antibody titers considered to be protective. These findings question the validity of HAI as a correlate of protection in obese adults, and makes clear that while HAI may still be an effective correlate of protection in some populations, it is certainly not the cause of protection.

Materials and Methods

Subject Population

Participants were recruited as a part of a prospective observational study carried out at the University of North Carolina at Chapel Hill Family Medicine Center, an academic outpatient primary care facility in Chapel Hill, North Carolina. Recruitment criteria for this study included

adults 18 years of age and older receiving the seasonal trivalent inactivated influenza vaccine (IIV) for the years 2013-2014 and 2014-2015, with exclusion for immunosuppression, immunomodulatory or immunosuppressive drugs, acute febrile illness, history of hypersensitivity to any influenza vaccine components, history of Guillain-Barre syndrome, use of theophylline preparations, or warfarin.

Trivalent IIV during the 2013-2014 influenza season was formulated on recommendations for an A/California/7/2009(H1N1)-pandemic-09-like virus, an A/Victoria/361/2011(H3N2) or A/Texas/361/2011(H3N2)-like virus (which are antigenically similar), and a B/Massachusetts/02/2012-like virus.

Trivalent IIV during the 2014-2015 influenza season was formulated on recommendations for an A/California/7/2009(H1N1)-pandemic-09-like virus, an A/Texas/361/2011(H3N2)-like virus, and a B/Massachusetts/02/2012-like virus.

Participants were contacted weekly for 6 months when influenza activity was first reported in the community. Participants were contacted by phone or email and asked to report any symptoms of fever, cough, runny nose, sore throat, muscle aches, headaches and fatigue to assess for influenza symptoms. Medical records of all study participants, whether they reported influenza-like symptoms or not, were reviewed at the end of each season for medically reported influenza-like illness or laboratory confirmed influenza. In addition to weekly calls/emails, participants were also instructed to contact the study nurse if they developed influenza-like symptoms. Laboratory confirmed influenza infection was determined from the medical records which reported a positive influenza specimen using either PCR or a rapid-influenza detection system. All participants who tested positive for influenza were diagnosed

with influenza A. Influenza-like illness was defined as fever greater than 100 F with a cough and in the absence of any other medical diagnosis. For participants who became infected with clinically diagnosed influenza, we identified vaccinated controls matched for age, sex, race, BMI, that did not report any influenza-like illness during the reporting period.

Serum sample collection

Participants recruited to the study provided a blood sample prior to vaccination and 28-35 days post vaccination. Blood was collected via antecubital puncture. Sera were collected using non-heparinized 10 mL vacutainers, which were allowed to clot at room temperature for 2 hours before being separated by centrifugation at 800 x g for 10 minutes. Sera were then frozen at -80 C for subsequent analysis.

Antibody quantification

Hemaglutination Inhibition assay. A/California/04/2009 (CA/09, pdmH1N1), A/Texas/50/2012 (H3N2) and A/Switzerland/H3N2 viral stocks were propagated in the allantoic cavity of 10-day-old specific pathogen-free embryonated chicken eggs at 37°C. Allantoic fluid was harvested, cleared by centrifugation, and stored at -80°C as described previously[107]. The HAI titer was determined in accordance with World Health Organization guidelines[108]. Microneutralization assay: Madin-Darby Canine Kidney (MDCK) cells were cultured in Eagle's minimum essential medium (MEM, MediaTech, Manassas, VA) supplemented with 2 mM glutamine and 10% fetal bovine sera (FBS, Gemini BioProducts, West Sacramento, CA) and grown at 37°C under 5% CO_2. Sera were treated with receptor destroying enzyme (RDE; Denka

Seiken, Cambell California n) and standard microneutralization (MN) were performed according to WHO guidelines[108]. Luminescent microneutralization (MN) assays were performed as previously described using a reverse genetics A/California/04/2009 virus containing an NLuc on its polymerase segment[109].

Statistics

Results from surveillance and medical records were analyzed using log-binomial and logistical regression in Stata statistical software. Microneutralization and HAI results were analyzed via 2-way analysis of variance (ANOVA) in Graphpad Prism Software. A statistical cutoff of $p<0.05$ was used.

Results

Obesity was associated with increased risk of developing laboratory-confirmed and influenza-like illness.

Demographics for all participants in 2013-2014 and 2014-2015 influenza participants can be found in Table 1 and Table 2, and include a total of 1022 vaccinated adults. During 2013-2014, 3 participants had laboratory confirmed influenza, 2 of whom were obese and 1 was overweight. This increased to 7 during 2014-2015, with 3 obese, 3 overweight, and 1 healthy weight. Although this sample was not large enough to consider greater risk in obese participants compared to healthy weight, we did find greater risk when adults with laboratory confirmed influenza were considered alongside influenza-like illness. Combined, there were 30 cases of influenza-like illness during the 2013-2014 seasonal influenza monitoring period, and

43 cases during 2014-2015.   The number of combined laboratory-confirmed influenza and influenza-like illnesses for both years are reported in Table 3 where obese participants had approximately twice the risk for laboratory confirmed or influenza-like illness despite vaccination (p=0.0310, 95% CI 1.0156-4.0756).   Additionally, logistic regression of BMI using influenza season as a covariate found that although there were more cases during the 2014-2015 year than in 2013-2014 (p=0.00731), BMI remained a significant predictor of influenza-like illness (p=0.00438). Consistent with elevated risk for influenza infection in the elderly[106], age was correlated with a greater risk (logistic regression p=0.0498), however this effect was not observed in the larger category of influenza-like illness. Using age as a covariate did not eliminate the effect of obesity.

Antibody response to vaccine and circulating influenza viruses did not predict susceptibility to infection.

For participants with laboratory confirmed or influenza-like illness, there were no differences in HAI pre and post vaccination titers to vaccine strain H1N1 (Figure 1A and B) or H3N2 (Figure 1C and D) among healthy weight, overweight, or obese individuals, despite the increased risk for the obese individuals to develop influenza or influenza-like illness. However, in the 2014-2015 vaccine year, the H3N2 vaccine strain was a poor match for the circulating strains, resulting in a vaccine efficacy reported at only 23%[97]. Therefore, we measured HAI titers against the circulating strain of influenza, A/Switzerland/9715293/2013 for all participants reporting laboratory confirmed or influenza-like illness. As we noted for H3N2 vaccine strains, there was no difference in HAI titers to the circulating strains among healthy weight, overweight or obese adults (Figure 1E and F).   In addition, we examined percent of adults in each weight classification

for seroprotection (HAI $\geq$40) or seroconversion ($\geq$4-fold rise in HAI pre to post). As shown in Table 4, Obesity did not affect odds of seroconversion or seroprotection. For California/H1N1 and H3N2/Texas/50, there were no statistical differences that could explain the higher rate of infection in the obese group.

Comparison of HAI and microneutralization titers between laboratory confirmed influenza participants and matched, uninfected controls.

Differences in antibody responses between laboratory-confirmed influenza infected participants and controls matched for age, race, sex and BMI who did not develop influenza during the study period were also investigated. Table 5 provides the demographic information for the infected participants and their controls. As seen in Figure 2, HAI titers did not differ between infected adults and non-infected adults. Influenza-specific antibodies were assessed by microneutralization, and again no differences were detected in titers between infected and non-infected adults (Figure 3)

DISCUSSION

This prospective analysis of participants receiving influenza vaccine used a convenience sample to monitor influenza and flu-like illness over two influenza vaccination seasons. Obese adults were approximately two times more likely than healthy weight participants to present with influenza or influenza-like illness, demonstrating that S-IIV does not confer the same levels of protection to obese participants. Furthermore, our antibody findings suggest that this is not due to lack of immunogenic response to vaccination. Indeed, clinically confirmed influenza participants had similar HAI titers to vaccination as controls who did not report symptoms.

Of particular interest are findings concerning the 2014-2015 influenza season. That year, a mismatch between the H3N2 circulating strain and World Health Organization recommendations drove an increase in influenza cases[97], however our findings showed that participants who were infected with influenza during this season actually had similar levels of protective antibodies as defined by HAI. In fact, 68.3% had a protective level (40 HAI or greater) against the predominant circulating A/Switzerland/H3N2 strain, calling into question HAI antibody's definition as protective. Although HAI antibodies have been well established as correlates of protection, protective HAI levels generated from vaccination against the predominant strain clearly demonstrate that HAI antibodies are not solely protective in the infected population. The clinically confirmed population in this study was 86% obese or overweight, however more data are necessary to determine if HAI antibodies are poorly correlated with protection solely in non-healthy weight populations.

Obesity has been recognized as an independent risk factor for influenza infection for 5 years, however this is the first study to our knowledge to monitor influenza vaccination and subsequent outcomes in obesity prospectively. Without prospective monitoring, studies looking at predictors of influenza infection and outcome are unable to determine response to influenza vaccine. Although this study would be better informed with unvaccinated controls, for ethical reasons, an unvaccinated group was not included.

In conclusion, this study demonstrates the need for greater influenza vaccination monitoring techniques to determine vaccine's extent of protection in at-risk populations, especially the obese. This clinical study provides a framework for future vaccination and risk group investigations, and provides justification for the necessity of testing alternative influenza

vaccination formulations for use in obese populations. The high-dose vaccine, for example, has been shown to induce greater HAI antibody to influenza among adults 65 years age or older[110] and may be a viable candidate for future investigations in obese participants, despite HAI's inability to protect individuals in this study. The newly approved adjuvanted influenza vaccine, which consists of inactivated influenza virus and a squalene-oil mixture (MF59)known to induce CD4+ T-cell activation[111] may be of particular interest in obesity, where CD4+ T-cells have previously been shown to be impaired in context of influenza vaccination[84, 86, 87]. This study underscores the importance of immune measures going beyond HAI and microneutralization to inform such changes in vaccination recommendations and approvals for influenza. As demonstrated here, microneutralizing titers and even HAI against the circulating strain did not prevent participants in this study from becoming infected, as has been reported previously for elderly populations[106]. While greater correlation of protection has been observed from antibodies inhibiting the other major influenza surface protein, neuraminidase[112], our previous work has also suggested that T-cell impairment occurs in obese populations[86,87] which suggests that regardless of humoral antibody generation, other immune considerations should also be evaluated as better correlates or even drivers of protection. Given the lack of B-cell derived differences between cases and controls seen here, and T-cell's role as critical immune drivers of the entire immune system, T-cell related correlates of protection should be prioritized in exploring alternative correlates of protection.

Acknowledgements

Table 1: 2013-2014 Influenza Season Enrollment

		Underweight	Healthy weight	Overweight	Obese	Total
	Enrolled	6 (1.0)	143 (24.9)	170 (29.6)	256 (44.5)	575(100)
	Age*	53.0 ± (19.9)	55.8 ± 18.3	53.5 ± 16.2	54.1 ± 12.2	
Gender						
	Male	3 (0.5)	53 (9.2)	73 (12.7)	75 (13.0)	204 (35.5)
	Female	3 (0.5)	90 (15.7)	97 (16.9)	181 (31.5)	371 (64.5)
Race						
	Caucasian	6 (1.0)	103 (17.9)	115 (20.0)	140 (24.3)	364 (63.3)
	African American		24 (4.2)	43 (7.5)	108 (18.78)	175 (30.4)
	Other		16 (2.8)	12 (2.1)	8 (1.4)	36 (6.3)
Diabetes						
	Yes- Type 1		3 (0.5)	1 (0.2)	1 (0.2)	5 (0.9)
	Yes- Type 2		11 (1.9)	30 (5.2)	86 (15.0)	127 (22.1)
	Pre-Diabetes	1 (0.2)	7 (1.2)	10 (1.7)	29 (5.0)	47 (8.2)
	No	5 (0.9)	122 (21.2)	129 (22.4)	140 (26.3)	396 (68.9)

Table 1: Demographics for the 2013-2014 seasonal enrollment. *Age is reported as average as average at day of years (standard deviation). All other values are number of participants (percent makeup of total).

Table 2: 2014-2015 Influenza Season Enrollment	Underweight	Healthy weight	Overweight	Obese	Total
Enrolled	6 (1.3)	134 (30.0)	112 (25.1)	195 (43.6)	447 (100)
Age*	54.4 ± 20.9	54.1 ± 18.6	59.4 ± 16.7	54.4 ± 13.6	
Gender					
Male	2 (0.4)	45 (10.1)	54 (12.1)	56 (12.5)	157 (35.1)
Female	4 (0.9)	89 (19.9)	58 (13.0)	139 (31.1)	290 (64.9)
Race					
Caucasian	3 (0.7)	98 (21.9)	74 (16.6)	112 (25.1)	287 (64.2)
African American		20 (4.5)	33 (7.4)	74 (16.6)	127 (28.4)
Other	3 (0.7)	16 (3.6)	5 (0.9)	9 (2.0)	33 (7.4)
Diabetes					
Yes- Type 1			1 (0.2)	2 (0.4)	3 (0.7)
Yes- Type 2		9 (2.0)	19 (4.3)	57 (12.8)	85 (19.0)
Pre-Diabetes	1 (0.2)	2 (0.4)	7 (1.6)	18 (4.0)	28 (6.3)
No	5 (1.1)	123 (27.5)	85 (19.0)	118 (26.4)	331 (74.0)

Table 2: *Demographics for the 2014-2015 seasonal enrollment. *Age is reported as average as average at day of years (standard deviation). All other values are number of participants (percent makeup of total).*

Table 3: Influenza-Like Illness during 2013-2015 Flu Seasons

		Underweight	Healthy Weight	Overweight	Obese	Total
2013-2014	Laboratory confirmed influenza	-	1	1	1	3
	Influenza-like Illness (excludes confirmed)	-	4	6	17	27
	No Flu-like Illness	6	138	163	238	545
2014-2015	Laboratory confirmed influenza	-	1	3	3	7
	Flu-like Illness (excludes confirmed)	-	8	6	22	36
	No Flu-like Illness	6	125	104	169	404
Total		12	277	282	451	1022
Odds Ratio vs Healthy Weight		1.40	1	1.13	1.98	
95% CI		(0.04-12.90)	(0.47-2.14)	(0.54-2.35)	(1.06-3.40)	
Significance		n.s.	n.s.	n.s.	*	

*Table 3: Summary of which participants acquired influenza during the subsequent season. Data incorporates participants from both 2013-2014 and 2014-2015. *Obese vaccinated participants were at a significantly greater risk of Flu-like illness (Odds ratio=2.03, 95% CI 1.02-4.08 p=0.0451). Note: Underweight participants are not included on this table.*

Table 4: Seroconversion among influenza-like Illness during 2013-2014 and 2014-2015

	A/California/09/H1N1					A/Texas/50/H3N2					A/Switzerland/H3N2				
	Seroconversion[a]	Odds Ratio	Seroprotection[b]	Odds Ratio	N	Seroconversion[a]	Odds Ratio	Seroprotection[b]	Odds Ratio	N	Seroconversion[a]	Odds Ratio	Seroprotection[b]	Odds Ratio	N
Healthy Weight	41.67%	ns	66.67%	ns	12	33.33%	ns	66.67%	ns	12	75.00%	ns	62.50%	ns	8
Overweight	42.86%	ns	64.29%	ns	14	35.71%	ns	78.57%	ns	14	55.56%	ns	100.00%	ns	9
Obese	40.54%	ns	70.27%	ns	37	51.35%	ns	89.19%	ns	37	33.33%	ns[c]	58.33%	ns	24
Combined	41.27%		68.25%		63	44.44%		82.54%		63	46.34%		68.29%		41

Table 4: Seroconversion and Seroprotection rates in participants who presented with Influenza-like illness. Data are shown as percentage of data points. Only participants from 2014-2015 were assessed for A/Switzerland/H3N2. Data from 10 subjects were not analyzed for pH1N1 and a/Texas/50/H3N2, including one eligible for A/Switzerland/H3N2 analysis. a: Seroconversion is defined as 4-fold or greater increase in HAI. b: Seroprotection is defined as HAI$\geq$40 at 30 days post vaccination. c: Seroconversion was insignificantly lower in obese subjects compared with healthy weight, p=0.0525.

Table 5: Demographics of Case-confirmed Influenza Participants	Age	BMI	Gender		Race		Study Year		Total Participants
			Female	Male	Caucasian	African American	2013-2014	2014-2015	
Confirmed Flu	64.31 ± 16.71	32.65 ± 6.71	7	3	5	5	3	7	10
Matched Controls	62.42 ± 16.02	33.16 ± 9.07	7	3	5	5	3	7	10

Table 4: Demographics of participants who acquired clinically verified influenza infection and matched controls. Age reported in years ± standard deviation, BMI in kg/m^2 ± standard deviation.
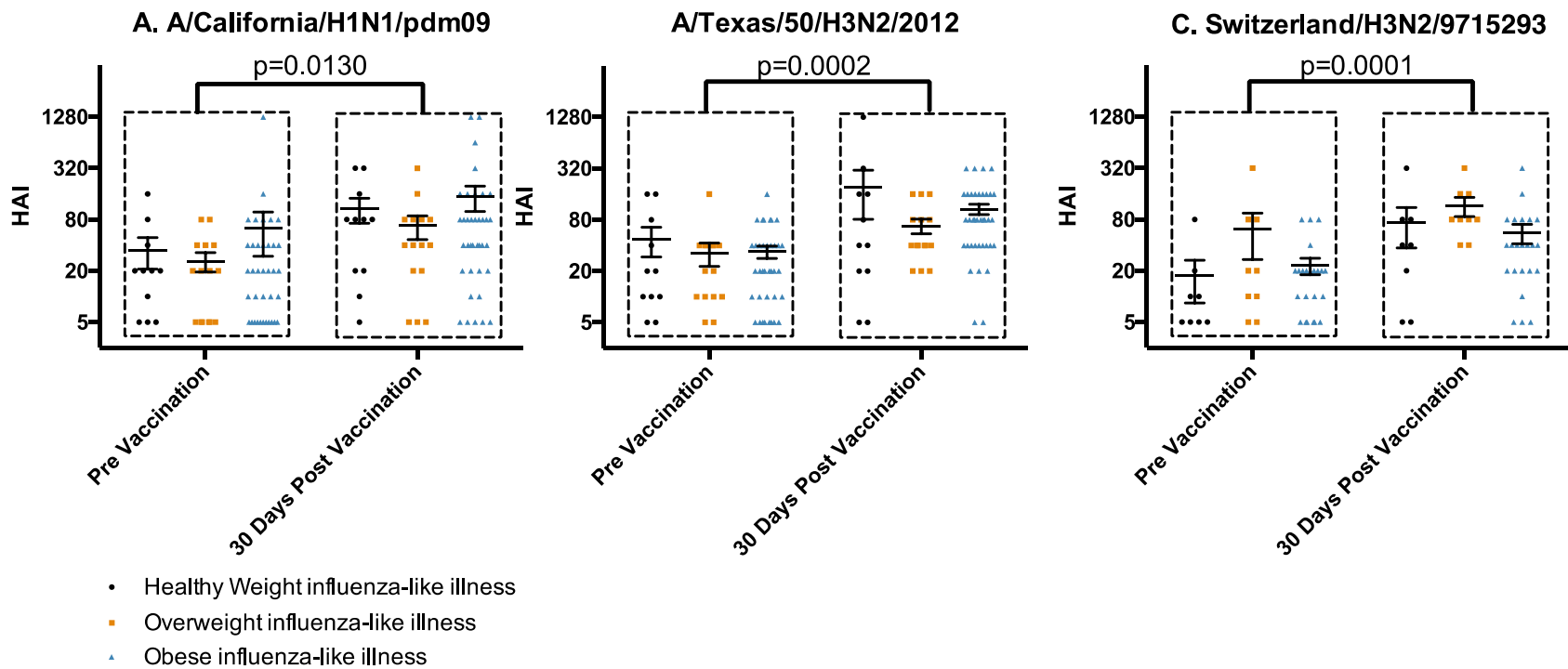
Figure 1: Weight does not impact Influenza HAI antibody generation. HAI for all subjects with flu/flu like illness displayed by weight status. HAI shown against vaccine-included strains A/California/H1N1pdm09 (A) and A/Texas/50/H3N2 (B), and major circulating strain A/Switzerland/9715293 (C). Comparison between weight and time post vaccination was assessed by ANOVA, p<0.05 is indicated.
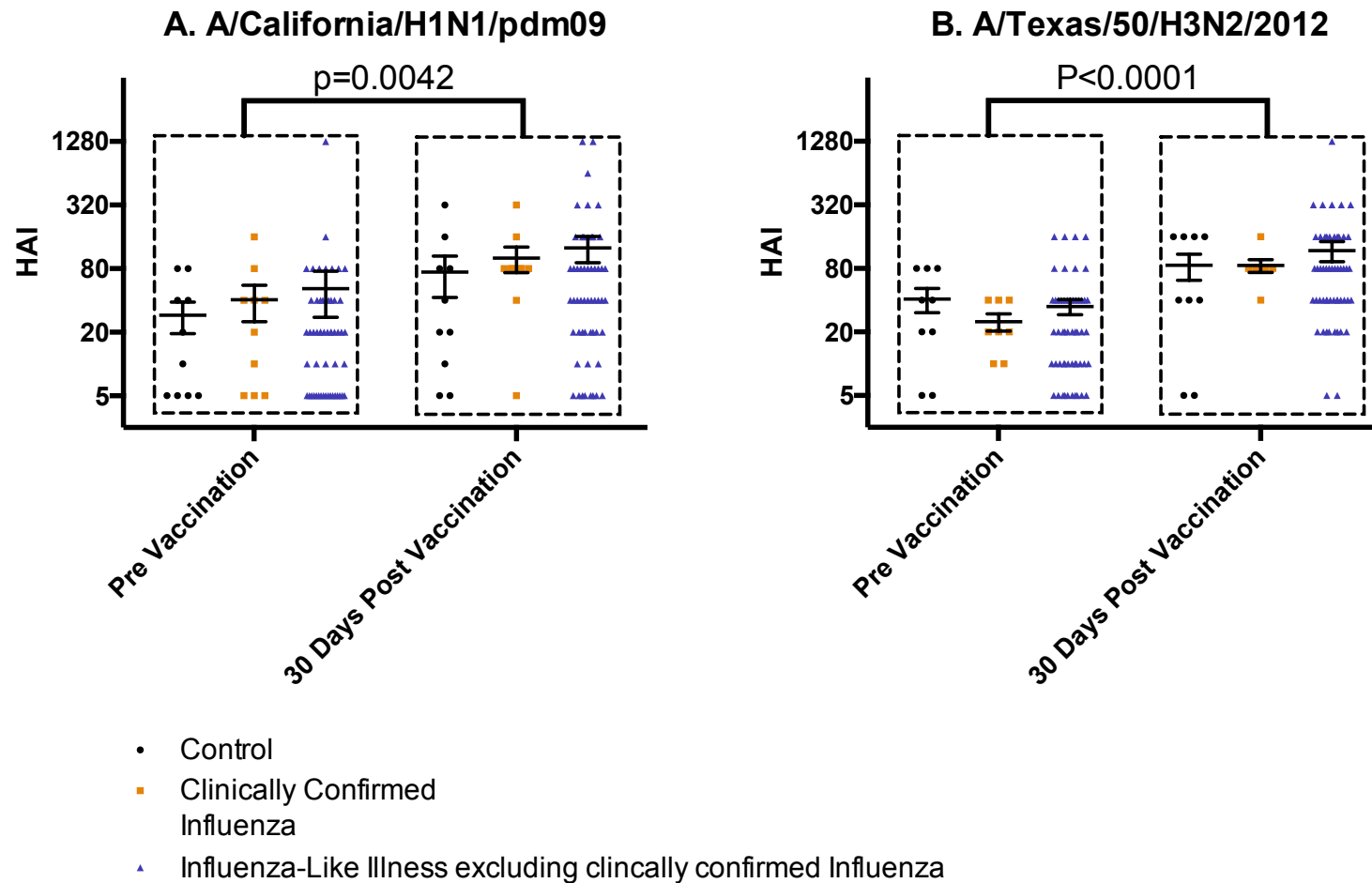
A. A/California/H1N1/pdm09

p=0.0042

B. A/Texas/50/H3N2/2012

P<0.0001

- Control
- Clinically Confirmed Influenza
- Influenza-Like Illness excluding clincally confirmed Influenza

Figure 2: Confirmed Influenza was not predicted by HAI. Hemaglutination Inhibition against A/California/H1N1/pdm09 (A) and A/Texas/50/(H3N2) (B) broken down by influenza outcome. Data shown reflects sera from participants with laboratory-confirmed influenza infection (Yellow, n=10) with matched controls (black, n=10), and influenza-like-illness excluding confirmed influenza (purple, n=54). Statistical values reflect 2-way ANOVA pwith a statistical cutoff of p=0.05.
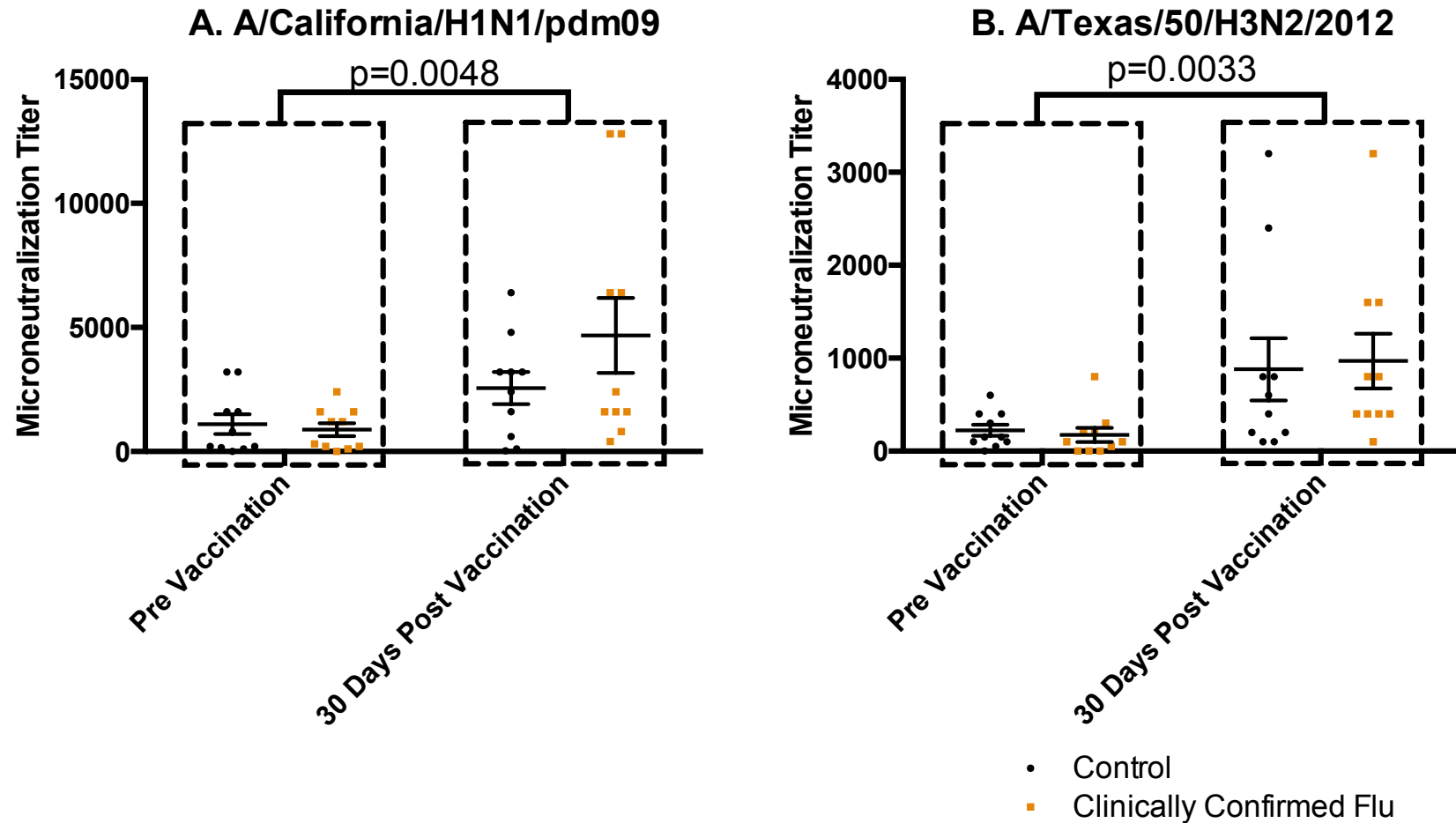
Figure 3: Confirmed Influenza was not predicted by Microneutralizing Antibodies. Microneutralization against A/California/H1N1/pdm09 (A) and A/Texas/50/H3N2 (B). Data shown reflects sera from participants with laboratory clinically influenza infection (red, n=10) with matched controls (black, n=10). Both subject groups responded to vaccination (indicated in A, C), but no differences or interaction between infected and uninfected were observed. Statistical values reflect 2-way ANOVA with a statistical cutoff of p=0.05.
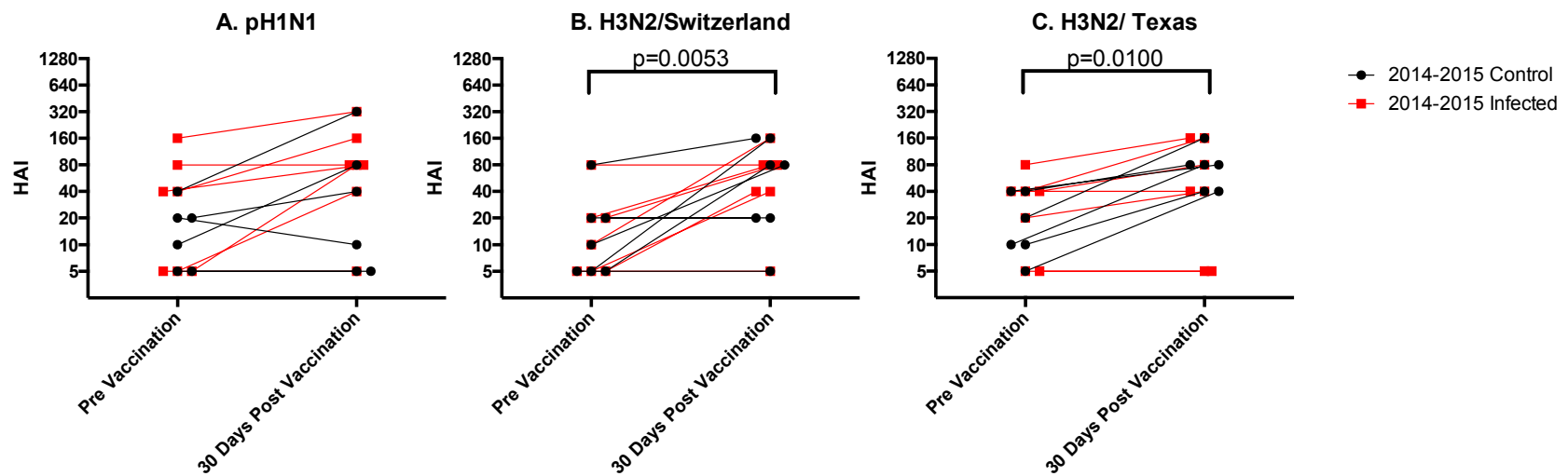
Figure 4: Subjects who later became infected had unimpaired HAI against Influenza Vaccine and Circulating Strains. Subsequent Influenza outcome and Hemaglutination inhibition against vaccine-included viruses A/California/H1N1pdm09 (A), vaccine-included virus A/Texas/50/H3N2(C), and circulating strain A/H3N2/Switzerland. Statistical values reflect 2-way ANOVA with a cutoff of p=0.05.

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CHAPTER III: INFLUENZA VACCINATION OF OBESE ADULTS RESULTS IN LESS ACTIVATED CD38+ B CELLS BUT UNIMPAIRED ANTIBODY RESPONSE TO INFLUENZA VACCINATION.

Overview

Background.

Obesity is a known risk factor for increased morbidity and mortality from influenza infection and, following vaccination, the antibody response is not maintained. What is not known is whether or not obesity impacts the B cell response to vaccination.

Objective.

We tested the hypothesis that obesity would alter the B cell response to vaccination.

Methods.

Obese and healthy weight Caucasian women were recruited and received the 2015-2016 seasonal influenza inactivated vaccine. Participants provided blood samples pre- and 7 and 30 days' post-vaccination. Plasma antibody responses to each vaccine strain were measured by hemaglutination inhibition assay. Vaccine-specific antibody subtypes, IgM, IgG, IgG1, IgG2 and IgG3 were assessed by ELISA. Lastly, B cell phenotypes were identified using flow cytometry.

Results.

HAI antibody titers and vaccine-specific antibody subclass response did not differ between obese and healthy weight subjects. Although B cell phenotypes numbers and percentages were not altered by obesity, the expression level of CD38, a marker of activation,

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was decreased among CD38+ activated B-cells, suggesting incomplete activation of this phenotype.

Conclusions.

Obesity was not shown to impair the influenza-specific antibody response at 30 days post vaccination, nor the generation of antibody secreting cells at 7 days post vaccination. However, decreased expression of the activation marker CD38, may suggest an impairment in the ability of B cells to achieve full activation in obese adults.

Introduction

Obesity is a growing public concern, affecting 13% of the population worldwide[113] and 34.9% of United States[3] in 2014. Between 2011 and 2012, 8.1% of infants and toddlers under 2 years of age were found to fit age specific definitions for obesity, compared with 16.9% of children between 2 and 18 years of age and 34.9% of adults[2]. By most estimates, obesity and its co-morbidities such as cardiovascular disease, diabetes and dyslipidemia are now a major burden on the US Healthcare systems, accounting for 4-7% of US Healthcare expenditure in 2003[4], and is estimated to quadruple by 2030[5]. In addition to these well-known co-morbidities of obesity, increased susceptibility to infection among obese is also a growing concern. Compared with healthy weight individuals, obese humans are at a greater risk for hospitalization and death from influenza infection[81]. Our lab has previously demonstrated that diet-induced obese mice have greater lung inflammation and lung damage and impaired T and B cell functions during influenza infection[31]. In humans, both CD4 and CD8 T cells isolated from overweight and obese subjects show diminished markers of activation and function[86]. In

addition, following influenza vaccination, obese adults generate levels of influenza-specific antibodies equivalent to healthy weight individuals at 30 days post vaccination. However, increasing BMI is correlated with a greater decrease in antibody levels 1-year post vaccination, suggesting that although the B-cells from obese individuals respond appropriately initially, they fail to generate or maintain adequate humoral memory[84]. The drivers of this B-cell dysfunction are not currently known, but one possibility is impairments in initial B-cell generation, which is the focus of the current investigation. Humoral response to antigen exposure consists of three main cell types derived from naïve B-cells: Plasmacells, which can be short or long lived, memory B-cells which can respond to future antigen exposures, and plasmablasts which are responsible for generating plasma and memory cells. Plasmablasts and plasma cells are generally grouped together as antibody secreting cells (ASCs), as memory B cells do not secrete antibody. The peak of the ASC population occurs 7 days post influenza vaccination[114,115]. Initial functional generation of antibodies followed by impaired antibody maintenance long term suggests that obesity may not impact short lived plasma cell generation and function, but may impede long lived plasma cell survival or function. Additionally, it is possible that memory B-cell function is impaired. Given the previously observed changes in antibody dynamics associated with obesity, we sought to characterize the initial B-cell response to influenza vaccination in a human population during the 2015-2016 year. Another possible indicator of altered B-cell response is type of immunoglobulin generated: Initial plasma antibodies generated during a primary exposure against influenza are Immunoglobulin M (IgM), followed by Immunoglobulin G (IgG) and Immunoglobulin G subtypes 1 (IgG1) and 3 (IgG3)[116]. We were also interested in Immunoglobulin G subtype 2 (IgG2) which is not typically elicited against viral antigens, but has

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been shown to be elevated in HIV infected adults following influenza vaccination[117], allowing us to consider it a possible marker of immune dysfunction in obesity.

Materials and Methods

Patient Recruitment

Participants were recruited as a part of a prospective observational study carried out at the University of North Carolina at Chapel Hill Family Medicine Center, an academic outpatient primary care facility in Chapel Hill, North Carolina. Original recruitment criteria for the general study included adults 18 years of age and older receiving seasonal Inactivated Influenza Vaccine (S-IIV), with exclusion for immunosuppression, immunomodulatory or immunosuppressive drugs, acute febrile illness, history of hypersensitivity to any influenza vaccine components, history of Guillen-Barre syndrome, use of theophylline preparations, or warfarin. Patients recruited to the study provided a blood sample pre vaccination and 28-35 days post vaccination. The vaccines administered consisted of a A/California/7/2009(H1N1)pdm09-like virus, an A/Switzerland/9715293/2013(H3N2)-like virus, and a B/Phuket/3073/2013-like virus. During the 2015-2016 recruitment, 45 previous participants who were Caucasian, female, between the ages of 35 and 66, and had a previous weight history of obese or healthy weight were asked to provide an additional sample 7 days post vaccination. Forty-four subjects completed the study, with one withdrawing due to a scheduling conflict.

Blood Samples

Peripheral blood mononuclear cells (PBMCs) and plasma were collected from the same blood samples, gathered in heparinized vacuum tubes. PBMCs were isolated using a Ficoll-

Histopaque gradient and frozen in 10%DMSO/90% Fetal Bovine Serum at -80° C and transferred to liquid nitrogen within 24 hours. Plasma was gathered during following initial centrifugation of Histopaque separation and immediately stored at -80° C until subsequent analysis.

Hemaglutination Inhibition Assay (HAI). In order to assess antibody response to specific influenza strains included in the vaccine, HAI was performed in accordance with World Health Organization guidelines[108]. Briefly: Human plasma samples were thawed and incubated with Receptor Destroying Enzyme (Denka Seiken, Cambell California) for 18-20 hours at 37C, followed by heat inactivation at 56C for 1 hour. Plasma were diluted to 1:10 using sterile physiological saline, and used as the start of a serial dilution on a 96-well V-bottom plate. Following a 1:2 serial dilution, 4 HAU of virus (either Cal/09/H1N1, Switzerland/H3N2, or B/Phuket grown in embryonated chicken eggs) in 25 μL of Phosphate Buffered Solution was added to each well. After 15 minutes at room temperature, 50 μL of a 0.5% turkey red blood cell solution was added to each well, and hemaglutination inhibition was blindly assessed via visual inspection. Reciprocal of HAI is reported.

ELISA

In order to assess antibody class response, an ELISA for influenza vaccine-specific immunoglobulins for IgG1, IgG2, IgG3, total IgG, and IgM was performed. Briefly: IgG and IgM antibodies were quantified by enzyme-linked immunosorbent assay using the 2015–2016 seasonal TIV as antigen. Vaccine was diluted and adsorbed to micro titration plates in a carbonate coating buffer. After washing, triplicate serum dilutions in PBS were allowed to react with antigen, and bound antibodies were detected by a peroxidase-conjugated goat anti-human IgM, IgG, IgG1, IgG2, and IgG3 (Abcam, Cambridge, MA, USA), followed by a

chromogenic substrate. Color intensity was measured by absorbance at 450 nm. Internal control plasma were included in each run. Pre- and post-vaccination plasma from each participant were tested in the same run. The intra-assay coefficient of variation using this assay is 4%.

Flow Cytometry

Frozen PBMCs from a representative subgroup were thawed in a 37 C water bath and reconstituted in RPMI complete with 10% Fetal Bovine Serum and Penicillin/Streptomycin (Thermo Fisher, Watham Massachusetts). Cells were centrifuged at 350 x g and washed three times and 1 x 0^6 cells were incubated in a 96 well plate at 37 C, 5% CO2 for 2 hours. Cells were spun at 350 x g, and treated with Human Fc Block (eBioscience, San Diego California) before being stained for viability (Life Technologies, Carlsbad California). Cells were subsequently stained for extracellular proteins.

Protein targets and fluorophores used included CD19-PE-Alexafluor 610 (Life Technologies), CD10-PE (BD Biosciences, San Jose California), IgG-APC (BD Biosciences), IgD-FITC (BD Biosciences), CD138-Brilliant Violet 605 (Biolegend, San Diego California), CD38-Pacific Blue (Exbio, Vestec Czech Republic), and CD27-Brilliant Violet 711 (BD Biosciences). After staining, the cells were fixed for no more than 15 minutes using a formaldehyde-based fixation buffer (eBioscience) before being centrifuged and reconstituted in PBS + 1% FBS and stored at 4 C. Samples were analyzed within 36 hours on a LSR Fortessa at the UNC Flow Cytometry Core Facility. Gating and analysis were performed using FlowJo flow cytometry software. Samples were run in duplicate.

Statistics

Data were analyzed using Graphpad Prism. Effects of obesity and vaccination were assessed by two-way ANOVA using a statistical cutoff of $p<0.05$. If an effect was observed, a Student's T-test was employed to determine which timepoints the effect of obesity was present.

Results

Demographics.

Subject demographics are included in Table 6. Because subjects were selected based on BMI status from 2014 medical records, several subjects who were initially classified as healthy weight or obese had weight change that would reclassify them as overweight (4 formerly healthy weight, BMI at vaccination=25.3, 25.4, 26.4, 26.4 and two formerly obese, BMI at vaccination=28.0, 29.0). No subject from the healthy weight group achieved a higher weight than the obese group however, and so the original classifications were used in this analysis. Participants classified as Obese had a greater BMI than subjects classified as Healthy Weight (Table 6, BMI $p<0.0001$).

Obesity did not affect the immunoglobulin subtypes induced by vaccination.

ELISA analysis shows an increase in both healthy weight and obese population's influenza-specific total immunoglobulin G (IgG-total), subtypes 1 (IgG1) and 3(IgG3), and immunoglobulin M (IgM) (Figure 5). Influenza specific immunoglobulin 2 (IgG2) did not increase in response to influenza vaccination (not shown), and no differences were observed between

healthy weight and obese groups. Additionally, there was no correlation found between BMI and IgG subtypes.

HAI titers unaffected by obesity.

Strain specific HAI titers did not differ between obese and healthy weight subjects before vaccination, seven days post vaccination, or twenty-eight days post vaccination. As shown in Figure 6, vaccination increased the titers for all 3 vaccine strains in both healthy weight and obese subjects. In addition, seroconversion (4-fold increase from pre-vaccination titer) and seroprotection (HAI $\geq$ 40) levels did not differ between vaccinated obese and healthy weight subjects (data not shown).

Obesity resulted in decreased CD38 expression on B cells.

The gating strategy used for B cell phenotyping is shown in figure 7. We analyzed two plasma cell populations (CD19+ CD38+ CD138+ and CD19+ CD38+ CD138+ CD27+), unswitched memory cells (CD19+ CD27+ IgD+ IgG-), switched memory cells (CD19+ CD27+ IgG+ IgD-), inactivated memory cells (CD19+ CD27+ CD38-), naïve cells (CD19+ CD27- IgG- IgD+), innate-like memory cells (CD19+ CD27- IgG+ IgD-) and activated cells (CD19+ CD38). No differences in numbers or percentages of these cells were detected between the obese and healthy weight populations (Figure 8). However, mean fluorescent intensity (MFI) for the activation marker, CD38, was significantly lower compared with healthy weight in the obese CD19+CD38+ cells (Figure 5).

Discussion

This study found lower CD38 expression among CD19+ CD38+ activated B cells despite no differences in B-cell type populations, immunoglobulin types, or vaccine strain specificity driven by obesity. It was observed that anti-HA antibodies against all three strains included in the vaccine treatment, as well as flu-specific immunoglobulin types G (total, 1, 3) and M increased following vaccination, demonstrating that the vaccine elicited a response regardless of weight group. Although obese and lean subjects had similar levels of circulating populations of B-lymphocytes regardless of weight status or time point post vaccination, obese subjects were observed to have lower CD38 intensity among CD38+ cells, suggesting that while obese subjects have similar numbers of CD38+ activated B-cells, the extent of activation is reduced. These findings were surprising because we would have expected to see shifts in overall B-cell population in response to antigen exposure with increases in plasma cells seven days post vaccination and an enlarged memory pool thirty days post vaccination, but the dynamics of B-cell phenotype shifts may have been missed because our study did not assess influenza specificity. The influenza specific measures of antibody did respond at each of these timepoints, however.

CD38's utility as a marker of activation was initially discovered[118] nearly a decade after the protein itself was first described in 1980[119]. CD38 has long been established to be upregulated on B-cells, as well as on most other peripheral blood mono- and poly-nuclear cells following stimulation with a variety of antigens and mitogens. CD38 has been shown to lower the threshold signals necessary to drive B-cell responses in a murine *in vitro* model[120], and CD38 also been shown to be expressed simultaneously with memory cell formation in mice[121]. A

limitation of this study was that we did not conduct flow cytometry for influenza specificity, so we were unable to conclude whether the observed reduction in CD38 from obese subjects' CD38+ B-cells cells was related specifically to other findings concerning influenza vaccine response.

Another groupl[87] and our laboratory have observed defects in B-cell and antibody dynamics associated with obesity, but our findings point to different time course and phenotypic targets for this observation. *Frasca et al* found obesity to be associated with a decreased numbers of switched memory and transitional B cells and an increased percentage of pro-inflammatory late/exhausted memory and naïve B cells in both young and elderly adults. We did not share these observations, however the CD38 findings presented herein may support this prior work. We attribute this to demographic differences in our respective patient populations: Our study was entirely Caucasian females in the state of North Carolina selected based on BMI from a previous convenience sample, whereas their recruitment methods were unspecified but achieved a racially diverse cohort. Additionally, differences may be explained by markers used in flow cytometric definition of memory B-cells. We relied on CD27 as a primary marker of B-cell memory, while *Frasca et al* used CD24 to exclude transitional cells from the B-cell memory definition. Differing sample storage methods were also relevant, as well as *Frasca et al*'s tightly clustered serological measures: while they found a reduction in antibody response at day 7 and 28 post vaccination among their 9 subjects, our findings in the larger but demographically dissimilar population (n=22) continue to support our previous findings (n=40)[84] that obesity does not impair initial generation of influenza specific antibodies.

The decreased MFI of CD38 observed in obese populations suggests that CD19+ CD38+ "activated B cells" are overall less active in obese populations and this may therefore contribute to the long-term decline of memory cells, resulting in the previously reported[84] drop in antibody titer. Further work examining additional time points and additional markers of B cell activation is needed to better understand the long-term loss of antibody protection in obese populations.

In summary, this study provides clear evidence that there are no differences in vaccine-induced immunoglobulin subtypes or strain-specific flue protection between average weight and obese populations. There is however a drop in CD38+ MFI in obese patients, which suggested impaired B-cell activation. Future work in murine models of obesity will be critical in determining the mechanisms behind the CD38 MFI and how it translates long-term to a drop in memory B cells, and the overall loss of protection from influenza in obesity.

Acknowledgements

Table 6: Participant Demographics	n	BMI Range	Average BMI	Average Age	Non-Diabetic	Pre-Diabetic	Type 2 Diabetic
Healthy Weight	22	18.85-26.37	22.4 ± 2.3	53.05 ± 10.3	22 (100%)	0	0
Obese	22	28.03-45.28	34.8 ± 4.6	54.77 ± 8.1	18 (82%)	2 (9%)	2 (9%)

Table 6: Demographic data of subjects. All subjects were female, non-Hispanic Caucasian. Average BMI and Age are reported ± standard deviation.

Table 7: Flow Cytometry Subset Demographics	n	BMI Range	Average BMI	Average Age	Non-Diabetic	Pre-Diabetic	Type 2 Diabetic
Healthy Weight	8	18.85-24.37	22.11 ± 1.96	55.11 ± 10.76	8 (100%)	0	0
Obese	8	29.00-37.92	33.01 ± 3.21	51.38 ± 8.42	7 (87.5%)	1 (12.5%)	0

Table 7: Demographic data of subjects included in flow cytometric analysis. All subjects were female, non-Hispanic Caucasian. Average BMI and Age are reported ± standard deviation.
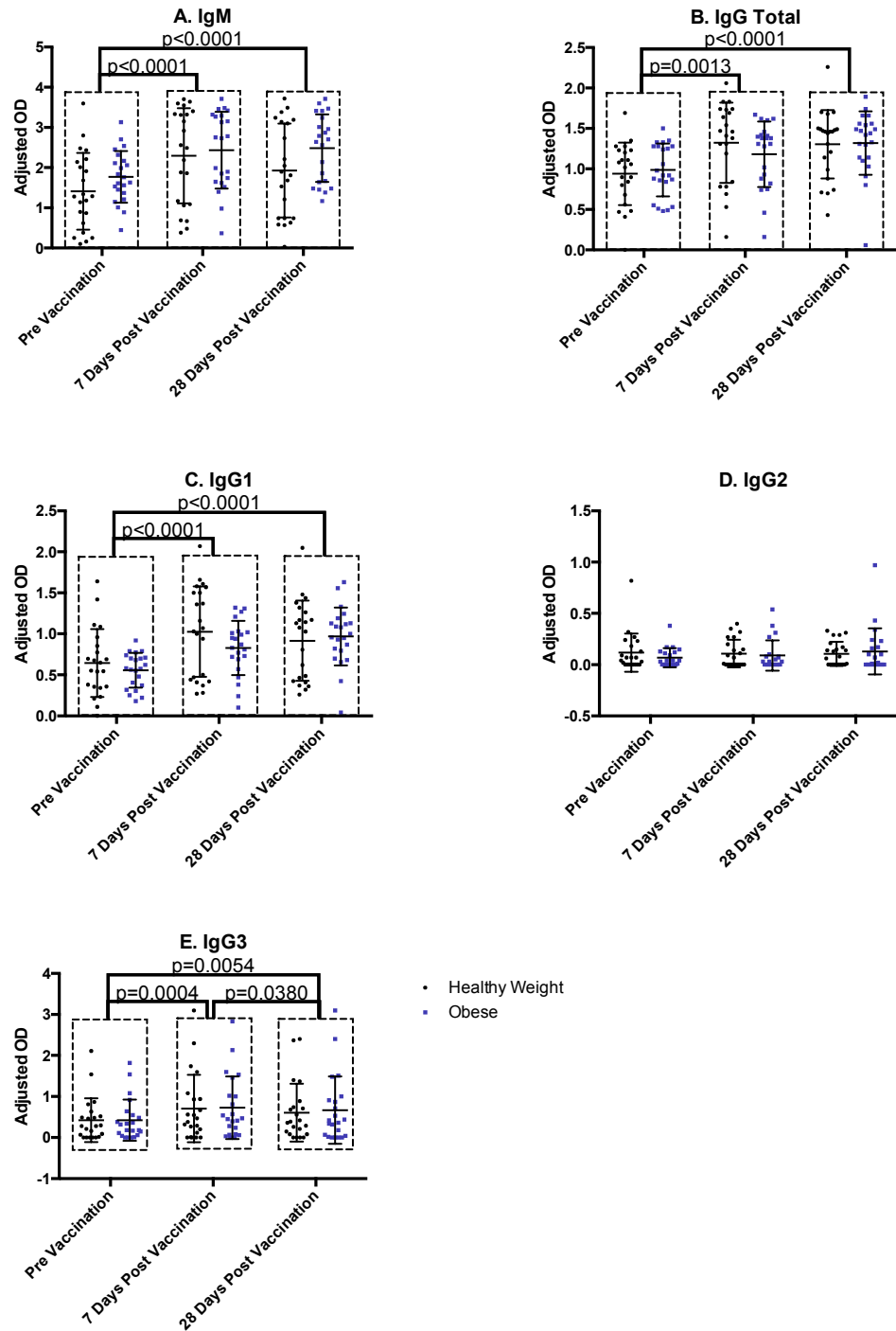
Figure 5: Influenza Vaccination Induced Antibodies by Class: IgM (A), IgG-total (B), IgG1 (C), and IgG3 (E) but not in IgG2 (D). Statistics reported by ANOVA with a statistical cutoff of p=0.05.

A. A/California/H1N1/pdm09

B. A/Switzerland/H3N2/9715293/2013

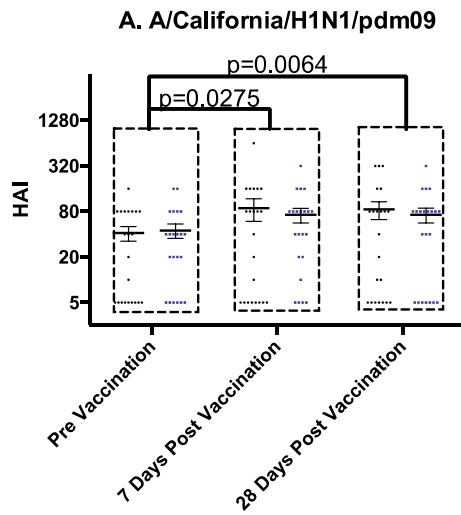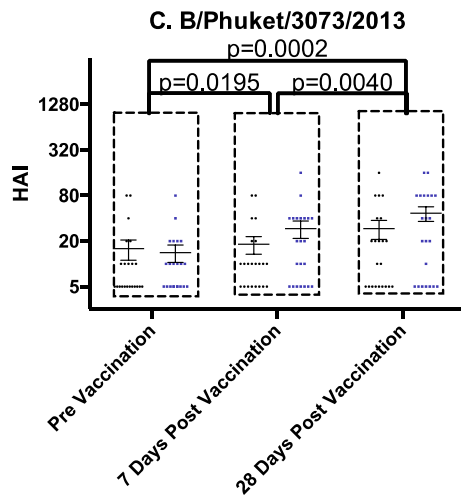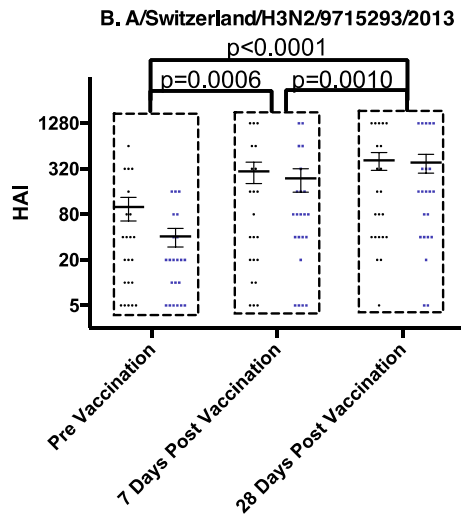## C. B/Phuket/3073/2013

· Healthy Weight
· Obese

Figure 6: Strain specific HAI response. Bars represent average +/- SEM. Vaccination drove an increase in all three strains. Reported p-values reflect ANOVA with a statistical cutoff of p=0.05.
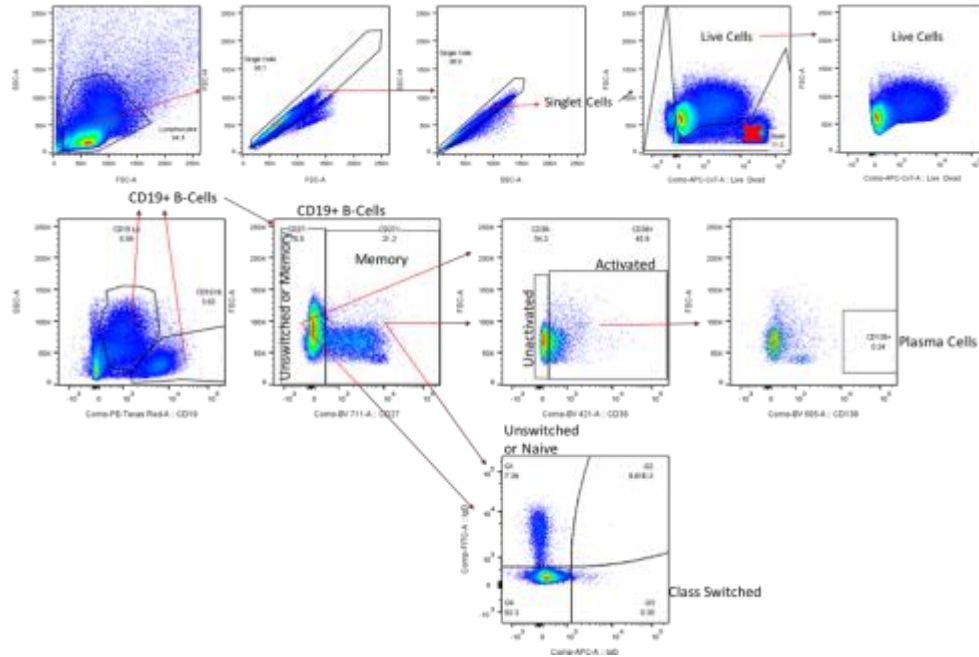
Figure 7: Representative Gating for Flow Cytometry Data. Arrows demonstrate which events were subsequently analyzed in the next gate. Only live cells as defined by the top row were considered. Multiple arrows from the same panel, as in the CD27 panel, means that the subsequent gate was applied equivalently to both the positive and negative populations and are considered separately. Positioning of gates was based on Fluorescent-Minus-One for that target.
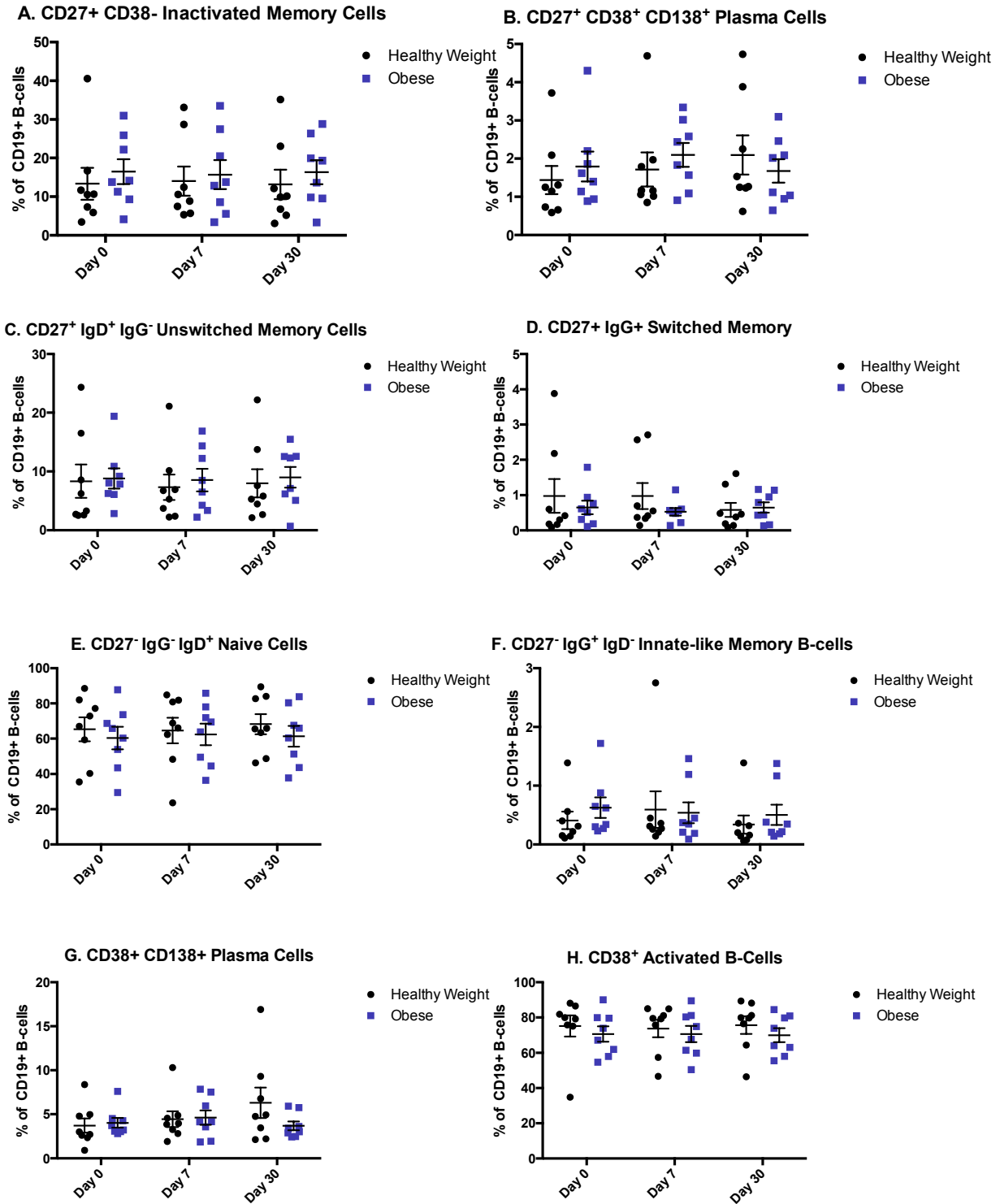
Figure 8: B-cell Subtype population sizes as a percentage of CD19+ B-cells. Neither obesity nor vaccination significantly altered any of these populations. (ANOVA, p<.05)

Expression of CD38 among CD38⁺ Cells
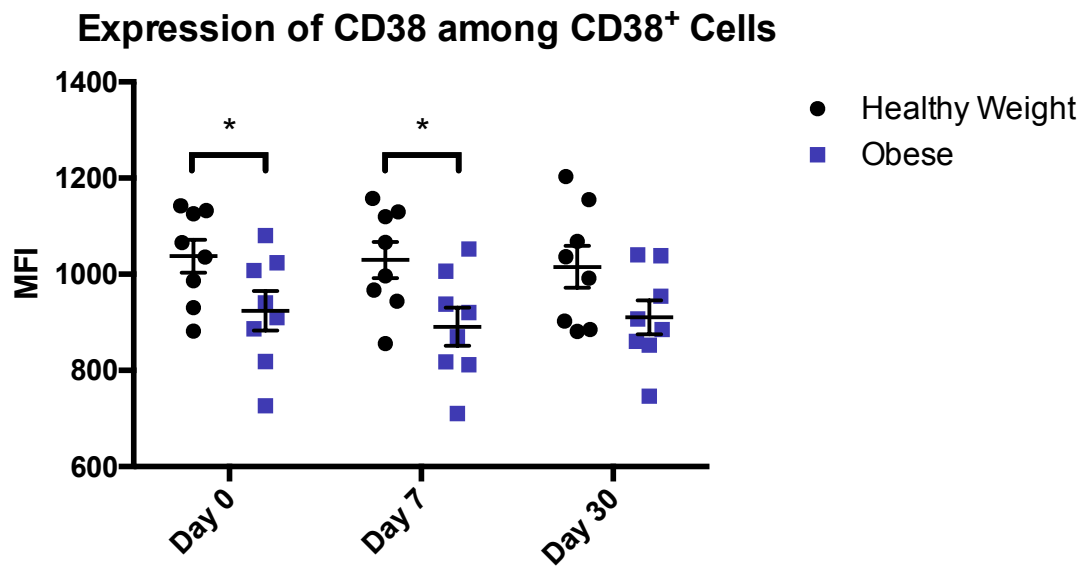
Figure 9: Mean Fluorescence Intensity of CD38 among CD38⁺B-cells. Reported p values reflect Student's T-test (Day 0: p=0.0368, Day 7 p=0.0438, thirty days p=0.0614) conducted post-hoc to ANOVA (Obesity p=0.0368). A statistical cutoff for significance of $p \leq 0.05$ was used.

CHAPTER IV: SYNTHESIS

Overview of research findings

This doctoral dissertation investigated two main questions about influenza vaccination in obese humans. Firstly, does vaccination mitigate the disparity in influenza outcomes among obese humans? And secondly, are there phenotypic differences in humoral response to vaccination among obese humans that may explain influenza vaccine response differences? In these investigations, we observed that obese humans were more susceptible to influenza-like-illness regardless of humoral response to vaccination through two separate measures. We did not observe phenotypic differences in B-cell response, both cellular and serological.

Do differences in antibody response to vaccine explain why some vaccinated individuals still get influenza?

The hemaglutination inhibition assay (HAI) is a quantitative method which assesses the ability of anti-influenza antibody's inhibition of the virus' inherent hemagluttinating ability, that is: ability to link red blood cells' sialic acid residues and form a lattice structure. It is commonly used as a correlate of protection and assessment of response to vaccination. However, our study and others[122] have remarked upon its' shortcomings as a correlate of protection against influenza. There have been suggestions in the literature that microneutralization may serve as a better correlate of protection. Rather than relying on antibody's ability to block solely hemaglutination, microneutralization measures antibody's ability to block viral replication in

live cells. While blocking a virus's ability to attach to sialic acid residues should prohibit viral replication, blocking other viral epitopes can also inhibit viral replication[123]. Microneutralization is therefore a more sensitive and biologically relevant assay and may be more useful for measuring susceptibility to influenza infection. For example, binding the stalk-region of hemagglutinin instead of the head region will inhibit merging of the viral and vesicular membranes, trapping endocytosed viruses inside vesicles fated for degradation. Neuraminidase-inhibiting antibodies can impair neuraminidase' role in both viral exit[124] and possibly viral entry[125], but our findings concluded that microneutralization titer was unimpaired in vaccinated subjects who were infected with influenza, demonstrating that a successful generation of microneutralizing antibodies 30 days post vaccination does not offer protection to obese subjects. In summary, neither HAI nor microneutralization at 30 days post vaccination offered protections against subsequent influenza infection.

Does obesity drive different B-cell phenotypic outcomes?

To answer this question, we used flow cytometry to measure B-cell population phenotypes, and ELISA and HAI to assess antibody phenotypes. Given the role of B-cells in generation of humoral immunity and obese subjects' demonstrated impairments in long-term humoral maintenance. We expected that a defect during initial B-cell response to vaccination might explain long-term effects, however this investigation did not identify any differences in B-cell population numbers. Importantly, the marker of activation CD38 was more highly expressed among CD19+ CD38+ B-cells from healthy weight subjects, suggesting that there may be impairments in B-cell activation among obese Caucasian women. CD38 is a multifunction

protein which is associated with activation on lymphocytes, and has also been found to dictate age-related decline in Nicotinamide Adenine Dinucleotide (NAD) and contribute to mitochondrial dysfunction related to aging in mice[126], which could suggest that B-cell impairments in metabolism may be worth investigation. CD38 is expressed on a variety of cells, and global expression of CD38 has also been implicated as necessary for induction of diet-induced obesity in mice[127]. However, our findings suggest paradoxical reduction in CD38 expression on obese B-cells, or perhaps that CD38 expression on B-cells is not involved in diet induced obesity.

In addition to an absence of B-cell population shifts, obesity did not drive differences in immunoglobulin class or subclass generation for influenza specific IgM, IgG, IgG1, IgG2, or IgG3 in the population, nor differences in strain-specific HAI. This differed from our preliminary findings presented at the Federation of American Societies for Experimental Biology[128] where young (18-35) obese participants had impaired IgG1 response compared to healthy weight, however the reduced sample size, differing age, and race selection in this follow-up suggests our original findings may still be valid in younger populations.

A recently published article did demonstrate defects in memory cell generation[87] which were also not observed in the studies reported here, likely due to differences in population, methods, and cell population definitions.

Recommendations for future research

There are two key outcomes from this dissertation which should be addressed in future studies:

1) Greater incidence of laboratory confirmed and influenza-like illness among obese vaccinated adults.

2) Lack of antibody or B-cell differences in obese subjects 30 days post vaccination.

In addressing greater incidence of influenza-like illness, a larger observational study will be necessary to refine this finding from influenza-like illness to clinically confirmed influenza cases. Continuing the subject recruitment our laboratory has been conducting over the last few years will provide more data on this phenomenon, however year-to-year variances in influenza may make conclusions elusive. Viable solutions include expanding the observational pool of participants from the previous five-hundred to one-thousand, and performing nasal-swabs to confirm cases of influenza for every subject who reports flu-like symptoms. In doing so, many subjects described as flu-like illness, or even mild-flu symptoms, may be redefined as clinically confirmed in future years.

In addressing lack of antibody or B-cell alterations driven by obesity, I suggest two future directions that may assess obese individuals' increased risk for influenza infection. Long-term maintenance of circulating antibody is provided by long-lived plasma cells within the bone marrow, wherein microenvironment is altered by obesity[129]. Given the challenges associated with human studies in general, a murine study of B-cell trafficking and bone-marrow microenvironment may be optimal for addressing this question. Our lab has demonstrated that diet-induced obese mice, similar to humans, can mount an adequate antibody response at 30 days post influenza infection, but that these antibodies are lost 3 months later[57]. Using this model, the bone marrow can be examined to determine if plasma cells fail to be maintained in the obese bone marrow environment. And finally, further characterizing dynamics of humoral

response to vaccination in obesity may be warranted: although obese subjects had similar levels of influenza-specific antibodies 7 and 30 days post vaccination, and subsequently infected subjects had similar humoral response to uninfected controls 30 days post vaccination, we do not have a clear picture of circulating antibody level at time of exposure to influenza. Examining this would require regular follow up with patients to gather serum at time points throughout the influenza season, but may also be able to identify subjects with sub-clinical illness and exposure events to serve as controls.

Future investigations could focus on verifying that infected individuals do not lose protective level of antibodies. Determining mechanisms of long-term deficiency and dynamics of influenza susceptibility would present an enormous public health opportunity: the possibility to prevent influenza infection with multiple doses of the seasonal influenza vaccination within at-risk populations. If antibodies are present at time of exposure, such a study may also suggest that this vaccination strategy would be ineffective at preventing illness, and further suggest that B-cell characteristics are less important to assessing influenza protection than other immune cells. It may therefore be necessary to prioritize T-cell phenotypic analysis as a better correlate or protection following vaccination.

T-cells are central to the acquired immune response, and have many interactions with innate immunity as well. T-cells form germinal centers with B-cells, provide signals to Macrophage[130] and Natural Killer Cells[131], and receive signals from dendritic cells[132]. CD8 T-cells also destroy viral infected cells. While assessing T-cells may be more technically challenging and expensive than assessing antibodies, measures based on their activity should nonetheless be considered candidates as correlates of protection following influenza vaccination.

In closing: influenza is a virus with a significant public health burden causing approximately 100,000 deaths annually[133]. Previous work has demonstrated that obesity is a risk factor for complications from influenza, but why these complications arise is not well understood[134]. This dissertation has begun to answer this question by delving into the subtleties and efficacy of humoral response in healthy weight vs obese individuals. Our current findings support the conclusion that initial humoral response shows slight differences between healthy weight and obese individuals, though overall humoral response is initially unchanged. Further work on this question should delve deeply into other time points to examine B cell memory response, as well as other aspects of the immune system through study of T cell response. These future studies will develop a more complete understanding of the impact of obesity on the immune system's response to infections, and critically, influenza.

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