The effect of yearly influenza vaccinations on the influenza-specific antibody response in healthy weight, overweight and obese individuals.

By

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Abstract

Obesity has increased tremendously over the past few decades, as the World Health Organization estimates nearly 500 million adults and 43 million children under the age of 5 to be obese. In addition, more than two thirds of the US Adult population is overweight or obese, which is a major area of concern, as obesity increases an individual's risk of cardiovascular disease, hypertension and other chronic health complications. Obesity has been shown to increase an individual's susceptibility to the influenza virus. In order to combat this virus, individuals receive an annual influenza vaccination. In the present study, the antibody responses to annual vaccinations of 24 subjects, classified as healthy weight, overweight or obese from the UNC Flu Study were measured, by comparing the percent fold increase in immunoglobulin G subtype levels from pre vaccination to post vaccination. Enzyme linked immunosorbent assay was performed on the subjects serum using the 2013-2014 influenza vaccination strains to assess the levels of immunoglobin G antibodies present. There was a significant relationship between the patient sub group and time in terms of the antibodies present for IgG1 and IgGTotal antibodies. However, there was no significant relationship demonstrated for the IgG2, IgG3, IgG4, and IgGM antibodies. Future studies by performing microneutralization assays and T cell responsiveness should be conducted to further probe the effect of repeated vaccination in obese individuals.

Chapter 1

Introduction

1.1 Obesity

Obesity is used to describe a medical condition in which a person is in a weight range in which he/she has accumulated an excess amount of body fat which results in a high risk of health impairments.[1] Obesity is a pressing issue in public health, as the World Health Organization estimates nearly 500 million adults and 43 million children under the age of 5 to be obese.[2] This problem has continued to rise as the prevalence of obesity has risen from 12% in 1990 to 35.7% in 2010. This weight range has been shown to increase the risk of developing diseases and other health implications, including hypertension, diabetes, hypercholesterolemia, cancer, and heart disease.[3] Studies by the National Health and Nutrition Examination Surveys have shown that the prevalence of hypertension is 2.9 times higher for overweight indivuals in comparison to those of healthy weight, while the prevalence of hypercholesterolemia was 2.1 times more likely in the overweight group. Every year over 300,000 adults in the United States die from obesity induced causes, as in addition to cardiovascular problems associated with obesity, obese individuals are also more susceptible to cancer.

Obesity is typically measured by assessing an individual's Body Mass Index, which has the formula of BMI = (Weight in Kilograms)/(Height in Meters)^2. According to the National Institute of Health, a healthy weight BMI is classified as between 18.5-24.9 kg/m^2, underweight is below 18.5 kg/m^2, overweight is classified as 25.0-29.9 kg/m^2, and obese is 30.0 kg/m^2 and above.[4] Although BMI tentatively can tell if a person is in healthy weight, overweight, or obese and is useful in comparing populations, it should be used with caution, as it does not directly measure body fat, and often times can classify someone with more lean muscle mass as overweight. Typically however, those with a BMI which identifies them as obese tend to have an excess amount of fat which contributes to their high BMI level. Other manners which use scientific methods of measuring obesity, such as through desitometry, hydrometry, or bioelectric impedance are more difficult to use to compare obesity within a population, making BMI the simplest manner of measuring obesity.[5]

Obesity is typically caused by a combination of factors, which can stem off of different factors such as behavior, environment, and genetic factors. The excess fat is typically from a positive energy balance, in which a greater amount of calories is consumed rather than expended. Environmental factors such as the sedentary lifestyle maintained by many Americans and reduction of physical activity also contributes to the obesity epidemic. The lack of access to healthy foods or affordability can also have an impact, as those in food deserts do not have access to healthy foods and as a result have to rely on fast food or processed packaged foods. Genetic factors can also have a major impact in regards to obesity, as people can have certain genetic factors and may be predisposed to develop obesity, including those with one or both parents who are overweight/obese.

Other things which can possibly effect obesity include age, race, and sex. As a person ages, he or she loses muscle, especially as inactivity increases. Muscle loss can slow down the

rate at which a person's body burns calories, meaning the overall net calories consumed is higher, which can result in overweight or obesity. In addition, women have a higher propensity to gain weight at various stages of their lives, as many women gain nearly at least 5 pounds during menopause and have more fat around the waist in this time.

1.2 Influenza Virus

Influenza is one of the most dangerous infectious diseases, as it causes 3-5 million cases of severe illnesses and 250,000-500,000 deaths every year around the world, even if a major pandemic does not occur for that respective year.[6] A pandemic is described when there is a global influenza outbreak which is able to spread rapidly from one person to another person worldwide. There are three main types of Influenza virus, which are classified as Type A, B, and C, depending on what organism(s) each one effects.[7] Type A influenza virus is the most detrimental and constantly changing, as it is can cause flu epidemics/pandemics and is further classified by the two glycoproteins which are found on the surface of the virus, neuraminidase (NA) and hemaglutinin (HA). These HA and NA help name a particular viral strain, as 17 different versions of HA and 9 different versions of NA are known. Type B influenza virus is normally found in humans and can be broken down to two different two lineages: B/Yamagata and B/Victoria., while Type C has also been found in other animals such as dogs and pigs.

The hemaglutinin (HA) and neuraminidase (NA) glycoproteins both recognize sialic acid. The HA glycoproteins on the surface of the virus help the virus attach to the cell, so that it can enter a host cell and initiate the infection process. The HA do this by binding to sialic acids on the carbohydrate side chains of cell surface of glycoproteins and glycolipids. Once the HA proteins have stimulated this process, antibodies will be present as an immune response to the virus. To spread the infection to other cells, the other glycoprotein present in the cell, the neuraminidase (NA) glycoprotein removes the sialic acid from the cell surface of infected cells, which helps the virus exit the host cell so that newly formed viruses in the host can be released to other cells for infection. The virus is able to replicate due to the RNA within the proteins within the virus, as the influenza virus itself is a segmented, single-stranded RNA. [8]

1.3 Immune Response to Influenza Virus

The virus spreads through the respiratory tract to infect an individual's nose, throat, and lungs. The viral HA glycoproteins bind to the sialic acid sugars on the epithelial cell surface and then the NA glycoprotein along with the sialic acid sugars on the epithelial cell surface and then the NA glycoprotein along with the replication machinery allows it to spread the infection to other cells. Once an individual is infected, influenza virus is able to easily spread from person to person through talking, sneezing and cough, which could spread the virus.

The influenza virus's ability to resist host immunity is mainly due to the processes of antigenic drift and shift. There are three main flu strains within the influenza vaccine: an influenza A (H1NI) virus, an influenza A (H3N2) virus, and an influenza B virus. Once vaccinated, an individual produces infection-fighting antibodies, against the three flu strains in the vaccine. This way, if a person is exposed to any of the three flu strains within the vaccine during flu season, antibodies produced by the B cells will attach to the virus's HA antigens,

preventing the flu virus from attaching to healthy cells and infecting them. However, influenza virus genes are made of RNA rather than DNA, so they are much more prone to mutation. Therefore if the HA gene changes, the antigen that it encodes can also change, causing it to shape through a process called antigenic drift.

Once a cell is infected by the influenza virus, the body's non-specific immune system, the innate immune system is able to respond to the viral antigens. In the early phases of viral infection, the innate immune response serves a major role in initiating an immune response, through natural killer (NK) cells. These NK cells have a vital role in the first line host defense against acute viral infections by directly destroying infected cells, even without antigen stimulation.[9] The infected cells are able to sense the viral infection using pattern recognition receptors (PPRs) which are able to recognize the viral RNA and recognize the main pathogen associated marker pattern of Influenza A viruses. Pattern recognition receptors consist of toll like receptors (TLRs) and retinoic acid inducible gene 1 (RIG-1).[8] Double stranded viral RNA binds to TLR3 and RIG-1, while single stranded RNA binds to TLR7. This then produces pro inflammatory cytokines such as IFN-B and IFN-a, which is important as they limit viral replication by inhibiting protein synthesis in host cells. In addition apoptosis, mediated by both Fas-mediated and independent mechanisms help initiate a caspase cascade with activate TGF-B to up regulate pro apoptotic genes, which helps downregulate the virus' host cell protein synthesis.

As part of the immune response, the innate immune system produces antigen-presenting cells such as dendritic cells, macrophages, cytokines and chemokines.[10] These are able to activate cytotoxic T cells (CD8 T cells), a component of the adaptive immune response, which serve to destroy the virus. These cells also activate B cells, which are components of the adaptive immune response, which produces antibodies against the virus. In the lymph nodes, CD4+ helper T cells recognize antigen-presenting cells, class II MHC glycoproteins. Viral clearance is enhanced by cloning and differentiation of CD8+ T cells by the complex formed by virus derived peptides and the class I MHC glycoproteins. The class II MHC antigen complex stimulates helper T cells to induce B cell proliferation, which secrete antibodies as a result to the virus infection. This is done as once the B cell encounters an antigen, it produces plasma cells, which produce antibodies, including various Immunoglobulin G subclasses.

1.4 Influenza Vaccine

To prevent the risk of getting influenza, many individuals receive the annual influenza vaccination. The process of developing the correct and effective influenza vaccine is quite comprehensive, as several months before each flu season researchers predict which strains will be circulating during the upcoming season and begin development of a vaccine against those strains. These strains are predicted by scientists from the available evidence at the time the selection is being made. Therefore flu vaccine prediction may be incorrect about the actual circulating influenza strains during flu season, making the particular formulated flu vaccine for that season less protective. Most traditional flu vaccines, coined with the term trivalent vaccines, are formulated to protect against three major flu viruses: an influenza A (H1NI) virus, an influenza A (H3N2) virus, and an influenza B virus.[7]

These vaccines are typically recommended by public health leaders or physicians as it allows an individual to develop immunity without causing illness. The vaccine typically contains a dead virus so that once an individual is vaccinated, helper T cells and B cells are able to produce the antibodies needed to combat the antigens presented by the vaccine. Memory B cells signal the body to produce antibodies so the next time the body encounters the virus strain and antigens, the antibodies are present so that the virus is killed and is not able to spread.

1.5 Immunoglobulin G Subclassses

The B cells from the humoral immune response produce immunoglobulin glycoproteins, which are proteins produced from the plasma cells to recognize and bind to the virus antigen. The major immunoglobulin produced is the Immunoglobulin G (IgG), which consists of even smaller subclasses (IgG1, IgG2, IgG3, IgG4).[11] IgGtotal is a measure of all the subclasses together, without differentiation of the IgG subclasses. Other immunoglobulins (IgA, IgM, IgD) consist of 25% of the total immunoglobulin. The human IgG antibody is expressed on mature B cells and is the most prevalent antibody in serum. The major difference between the IgG subclasses is in the structure of the hinge region of the amino acid composition of these proteins, as this region can vary in length and flexibility. The relative serum concentrations of the IgG subclasses are: IgG1 > IgG2 > IgG3 = IgG4. Antibody responses to antigens which are soluble and membrane proteins induce IgG1 antibodies, but this can also be accompanied with low levels of IgG3 or IgG4. Immunoglobulin G responses to bacterial capsular polysaccharide antigens on the other hand, typically induces IgG2. IgG3 antibodies are pro-inflammatory antibodies while IgG4 are typically induced by allergens, and are formed after long term exposure to a particular antigen.[12]

Chapter 2

2.1 Purpose and Hypothesis

Purpose: To determine if the antibody response (total IgG, IgG1, IgG2, IgG3, IgG4, IgM) to repeated influenza vaccinations differs based on gender or BMI.

Hypothesis:

The antibody response to repeated influenza vaccinations should improve over time for all healthy, overweight and obese subject groups, with the obese response normalizing with time to match the response of the healthy group.

Chapter 3

Methods:

3.1 Study Design and Collection

The subjects for this study were chosen from those who received the influenza vaccine for the 2010-2011 year, 2012-2013 and the 2013-2014 year. These subjects were part of the UNC flu study in which they were administered the respective vaccine for that year. The eligibility requirements included greater than or equal to 18 years of age, and subjects did not have acute febrile illness, HIV, Hepatitis C, were pregnant/breastfeeding, or under immunosuppressive medicines within the past 4 weeks, or had a disease such as cancer. Upon enrollment, the nurse administering the shot took the informed consent, gender, race, age, weight, height and a baseline blood sample. Then each subject one at a time, was administered the dose of the inactive trivalent vaccine for that particular year. For example in 2010, the patients were administered the 2010-2011 trivalent inactive vaccine. The patients then returned between 25-28 days after this initial vaccination so that the clinic could retrieve a post vaccination blood sample from the patient. This study used patients who participated in the flu study and were administered the 2010-2011, 2012-2013, and 2013-2014 year.

Each subject's blood serum was collected prior to receiving the influenza vaccination and collected again 25-28 days after when the patient came back so that this could be collected from them. A vacutainer tube was used to collect from the patient, which was then centrifuged at 800 x g for 10 minutes at 4°C using the IEC Centra MP4R. This allowed serum to be aliquoted into 500 microliter amounts using serum tubes, which were then placed into an -80°C freezer.

3.2 Enzyme Linked Immunosorbent Assay

To identify the level of influenza-specific antibodies in the subjects' serum, an indirect enzyme linked immunosorbent assay (ELISA) was performed. Prior to beginning the ELISA, the materials and reagents had to prepared. These are listed below:

a. Materials:

96 well Falcon plates

Adhesive plate covers

Multi-channel precision pipettes with disposable plastic tips

ELISA Plate Reader

b. Reagents Used:

Coating Buffer	0.2 M sodium carbonate and bicarbonate solution in Phosphate Buffer Solution
Block Buffer	2.4 g of Nonfat Dry Milk in 80 mL of Coating Buffer
Dilution Buffer	3.45 g of Nonfat Dry Milk in 115 mL of PBS

Plate Washing Buffer (PBSt)	330 mL phosphate buffer solution, 3000 mL of distilled H_2O, and 1.6 mL of Tween-20
Vaccine Antigen	Diluted in coating buffer (1:160 dilution)
Primary Antibody	Antibodies from subject's serum
Detection/Secondary Antibody	Goat anti-human antibody for IgG (IgGtotal, IgG1, IgG2, IgG3, IgG4, and IgM antibodies
Horseradish Peroxidase	Attached to detection/secondary antibody acting as enzyme conjugate
Enzyme Substrate	TMB Substrate in horseradish peroxidase solution
Stop Solution	2M Sulfuric Acid

The entire process of preparing the plates and solutions for the ELISA up to measuring the color intensity level using the plate reader took nearly three days to complete. The amount of subjects we wished to perform the ELISAs for varied between 4-6 subjects at a time. Before beginning this process, an optimization assay was run for each component of the ELISA (vaccine antigen, serum, secondary antibody with horseradish peroxidase were determined). The optimal dilution for the vaccine antigen was 1:160. Each subclass of the IgG immunoglobulin (IgG1-4) in addition to IgGtotal and IgM was optimized individually, as shown below:

	IgG 1-3	**IgG 4**	**IgG Total**	**IgM**
Vaccine Antigen	1:160	1:160	1:160	1:160
Sample Serum	1:800 (in triplicate)	1:200 (in triplicate)	1:2000 (in triplicate)	1:40,000 (in triplicate)
Conjugate	1:1000	1:500	1:1000	1:1000

This process used antigen-antibody binding to immobilize antibody proteins, from which they could be analyzed on 96 welled plates. On the first day of doing the ELISA, The vaccine containing the antigen (2013-2014 influenza vaccine) was diluted in a 1:160 dilution with the prepared coating buffer. Each plate was then coated with diluted vaccine according to the following table:

Step	**Serum Sample (µL)**	**Dilution Buffer (µL)**	**Diluted Sample**	**Final Dilution**
1	**10**	990	**DS1**	1:100
2	**500 of DS1**	500	**DS2**	1:200
3	**250 of DS2**	750	**DS3**	1:800
4	**300 of DS3**	750	**DS4**	1:2000
5	**50 of DS4**	950	**DS5**	1:40,000

Once these diluted samples were formulated, specific amounts of the dilutions were added to the different plates (Plates A-F), with 50 µL of DS2 added per well to plate 1, 50 µL of DS3 per well to plates A, B and C, 50 µL of SD4 per well to Plate E, and 50 µL of SD5 per well to plate 5. The plates were then placed in a moist plastic Ziploc bag and stored overnight in a 4°C refrigerator.

In the second day of this process, the solution within the wells was discarded and each well was covered with 200 µL block buffer. The plates were then put back into the moist plastic bag and placed in the incubator at 37°C for two hours. The plates were then taken out and washed with PBSt solution. The subject's diluted serum was then added to the wells in triplicates of 50 µL, meaning three wells were filled with the same diluted serum, three wells were filled with a control serum, while three other wells were filled with no serum and just dilution buffer. This was so there could be a standard for comparison once the antibody levels were measured. Each plate then received a specific secondary antibody conjugated with horse radish peroxidase. The dilute goat anti-human IgG conjugated with horseradish peroxidase was diluted in dilution buffer according to the following table:

Antibody Name	**Antibody (µL)**	**PBS/BSA (mL)**	**Final Dilution**	**Plate**
IgG-1, 2, 3, Total, IgM	**5.5**	**5.5**	1:1000	A, B, C, E, F
IgG-4	**11.0**	**5.5**	1:500	D

Once each plate received of it's respective secondary antibody, the plates were put in the incubator for 1 hour at 37°C, taken out and put it the refrigerator overnight.

On the final day of the ELISA process, the plates were taken out of the refrigerator and washed with PBSt. After washing, 100 µL of the TMB substrate solution was added to each well. This substrate catalyzed the reaction of the horseradish peroxidase enzyme to induce a color change, with color intensity correlating to a higher antibody level. This reaction was done to every plate as they were put under aluminum foil for 30 minutes. The reaction was then stopped by adding 100 µL of 2M sulfuric acid to every well. The plates were then taken to Dr. Styblo's lab to use the ELISA plate reader to measure the absorbance of these samples at a wavelength of 450 nm, as Microsoft excel was used to record this data. Once each plate was read, the blank measurements in the wells was compared to the positive control wells. If the measurements form the control wells were significantly higher, the ELISA protocol for that particular subject was rerun.

Data Analysis:

The data from the plate reader was saved onto a flash drive on Microsoft Excel, and then using the Graphpad Prism 6 program, the data for all the antibodies was able to be imported. This

program allowed us to display the data in a chart form, in addition to performing T tests and 2 way ANOVA tests for all the various immunoglobulins in the study. Two subjects in the study, who were missing serum from the data were removed to improve the accuracy of the results.

The data was further separated by gender and weight status using Microsoft Excel. The average percent increase between pre and post vaccination for all the weight classes (healthy, overweight, obese) for each respective antibody and subclass (IgGtotal, IgG1-4, IgM) was calculated with the following formula:

$$Percent\ Increase = \frac{Post\ Vaccination\ Value - Pre\ Vaccination\ Value}{Pre\ Vaccination\ Value} x\ 100$$

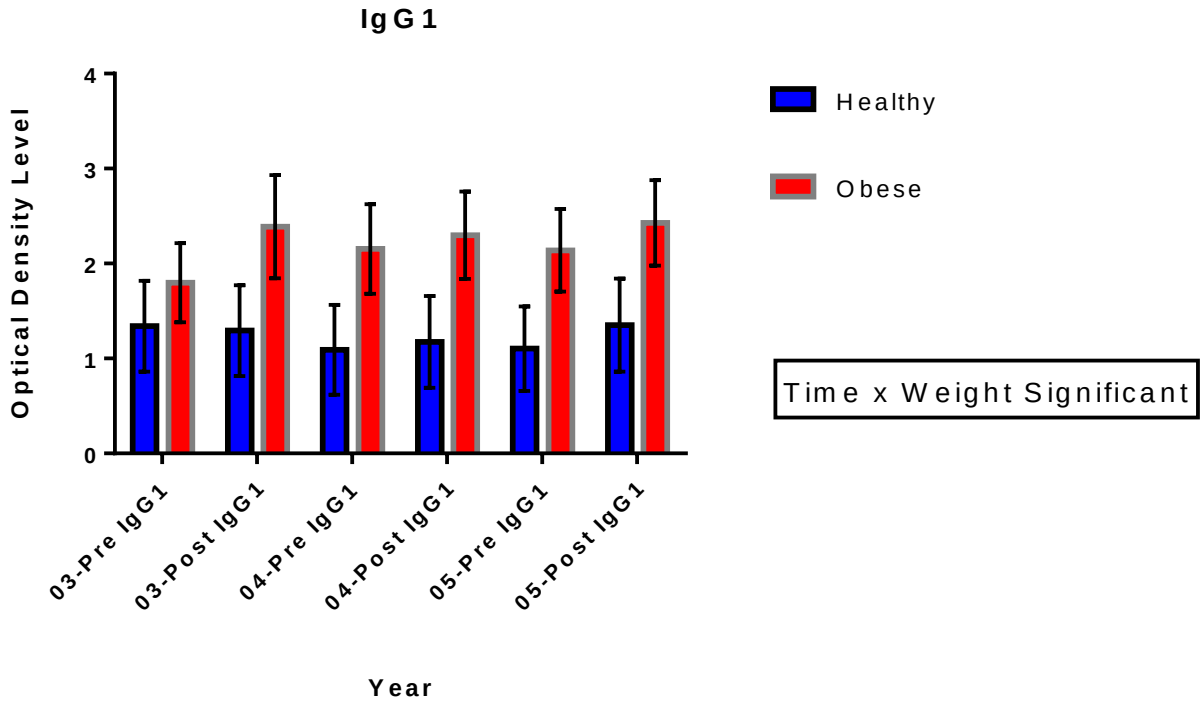
This allowed us to calculate the mean percent increase using excel. These values were then used to construct graphs in Microsoft Excel and two sample t tests assuming unequal variances were performed to determine significance within the data, with data with a P value of < 0.05 classified as significant.

Chapter 4: Results

The null hypothesis was that both, the optical density level of the antibodies would increase every year due to reoccurring vaccinations including the Immunoglobulin G subclasses in addition to the IgM and IgGtotal antibodies. We also surmised that the optimal density level of the particular antibody would be higher for the healthy in comparison to the obese group . Our results found a significant interaction for the time and weight status of the subject for the optimal density levels of the IgG1 and IgGTotal antibodies. This means that there was a significant interaction between the weight status over time in the optimal density level expressed by these subjects.

4.1 Overall Optical Density Levels

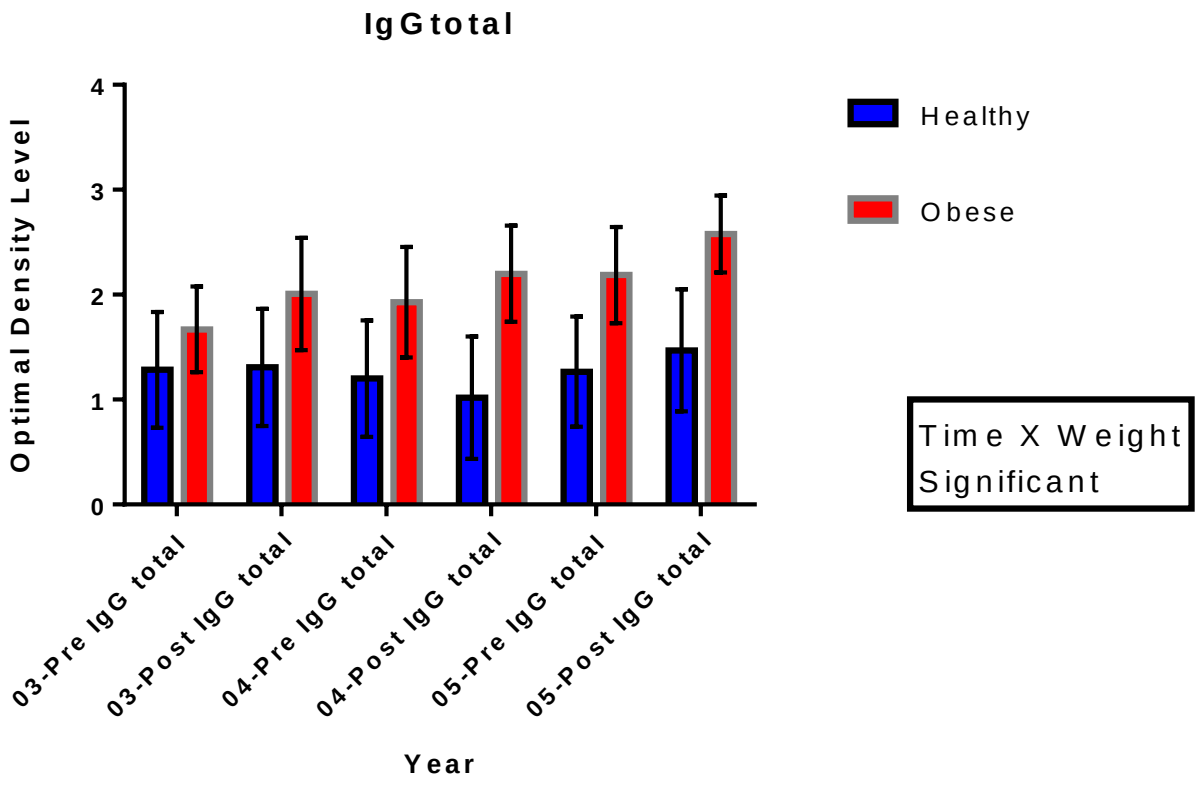
Figure 1:



Significance between Healthy and Obese weight classes for the IgG1 antibody, with a P value interaction of P = 0.0263, using 2 way ANOVA test

We found that obese individuals actually had a higher optical density level in comparison to the healthy individuals, which we did not expect. In addition, we can see that the optical density level for the obese group increased consistently from one year to another, as expected but the healthy group fluctuated and was not as consistent. This suggests that the obese group actually had a greater IgG1 antibody response than the healthy individuals.

Figure 2



Significance between the interaction of time vs weight status in terms of the overall Optical Density levels for the IgGTotal antibody levels between healthy and obese individuals.

P value 0.0168, Using ANOVA two way test

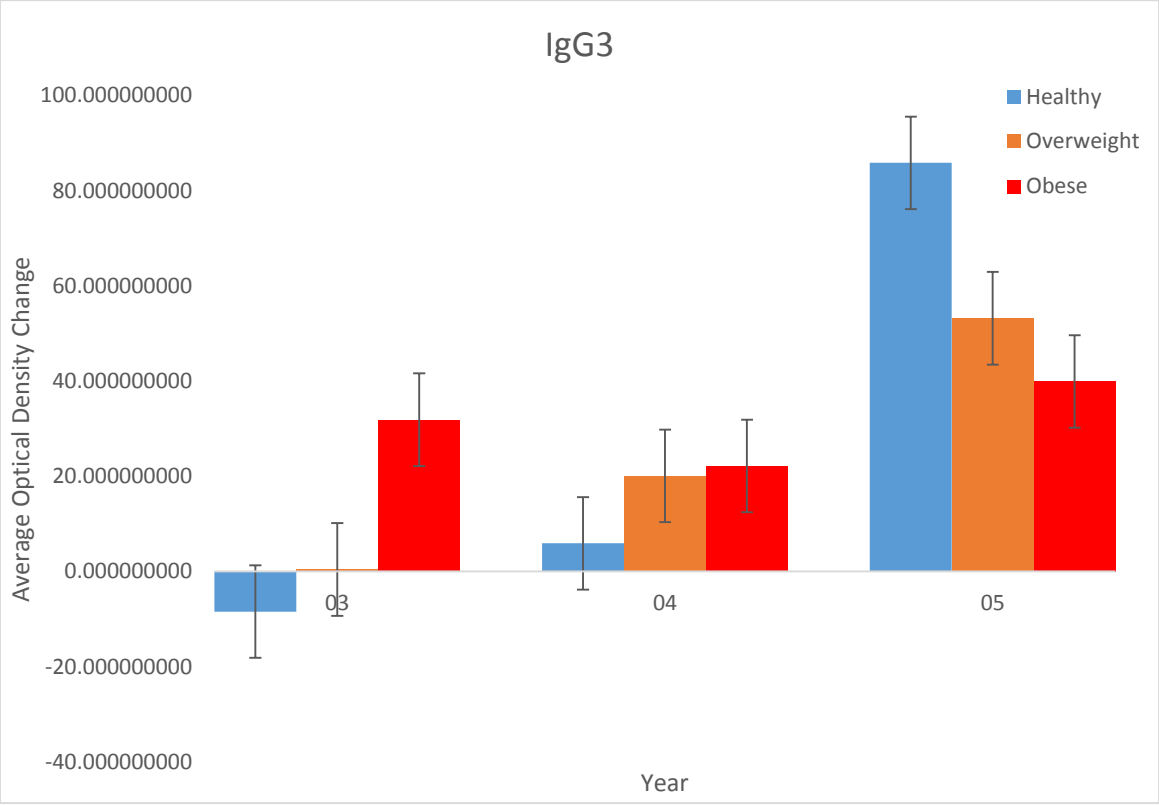
The results from the ANOVA two way test also showed a significant interaction between time and weight status for the IgGtotal group. This provides us with a good measure, as this measures all the subclasses together instead of differentiating between the IgG subclasses. As shown, the obese group had a consistent increase from one year to another, while again the healthy group varied, suggesting a greater total IgG antibody response by the obese group. In addition, it shows that although repeated vaccination helped the response in the obese group, it did not result in increased antibody response in the healthy weight group. We did not find any statistical differences in levels of IgG2, IgG3, IgG4 and IgM between healthy weight and obese individuals.

4.2 Average Percent Increase of Antibody for Time/Weight Class

We then looked at the percent difference between the pre and post vaccinations for the various antibodies and subclasses to determine if there were any differences between BMI classes. We again expected that the samples from the 05 year would have a greater immune response, and show a greater percent change, as this would suggest that there is an enhanced response for the presence of the antibodies following repeated vaccination. The IgG3 antibody showed significance for the percent change between the 03 and 05 years, so a 3 year difference in which the patients were vaccinated yearly. As expected, every group had a very high increase in the percent increase from the pre and post vaccinations for the 05 year. In addition, as expected, the healthy weight group had the highest percent increase from the 03 to the 05 year, as the antigen used in the ELISA was from the 05 vaccine. while the obese had the least change.

Although the t tests all had values above 0.05 for the remaining IgG subclasses, IgG1, IgG2, and IgG3 all showed trends that the percent increase was smallest in the obese group compared with the healthy weight group.

Figure 3



P Value .04777

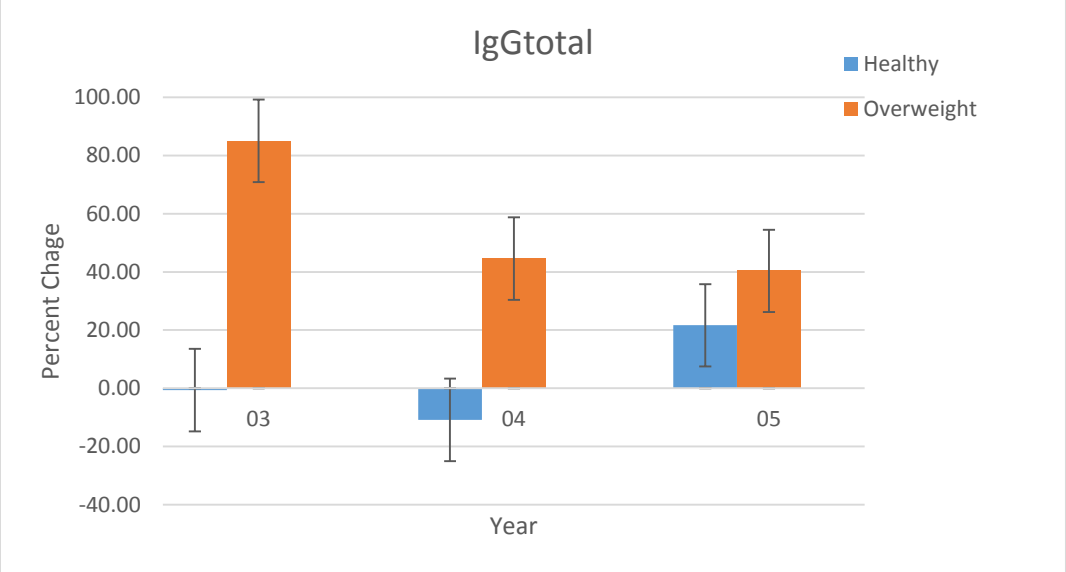
There was a significant change in the Percent change between the 03 and 05 samples for the IgG3 antibodies as seen by the graph above. For the 03 samples, the percent change for the

healthy patients was actually negative at -8.44%, while the overweight subjects had a percent change of 0.42% and 31.89%, respectively. The 05 samples for healthy, overweight, and obese all showed significant changes as the percent increase for the IgG3 present significantly increased, including a 85.87% percent increase for the healthy weight individuals. The overweight and obese subjects also had an increase in the amount of IgG3 present from pre to post vaccination, as there was a 53.19% change for the overweight and 39.94% increase for the obese subjects.

IgGTotal Based on Weight Status

We also looked at the IgGtotal percent change from pre to post vaccinations based on weight classes. We expected that the percent change would be higher for the healthy group in comparison to the overweight group. However our results for the IgGtotal chart showed that the overweight group had a higher percent change between the pre and post in comparison to the healthy group consistently for the 03, 04, and 05 years. This is the opposite of what we expected. However our data also showed an increase in the percent change for the healthy group from the 03 year to the 05, while the percent change for the overweight group decreased, as expected.

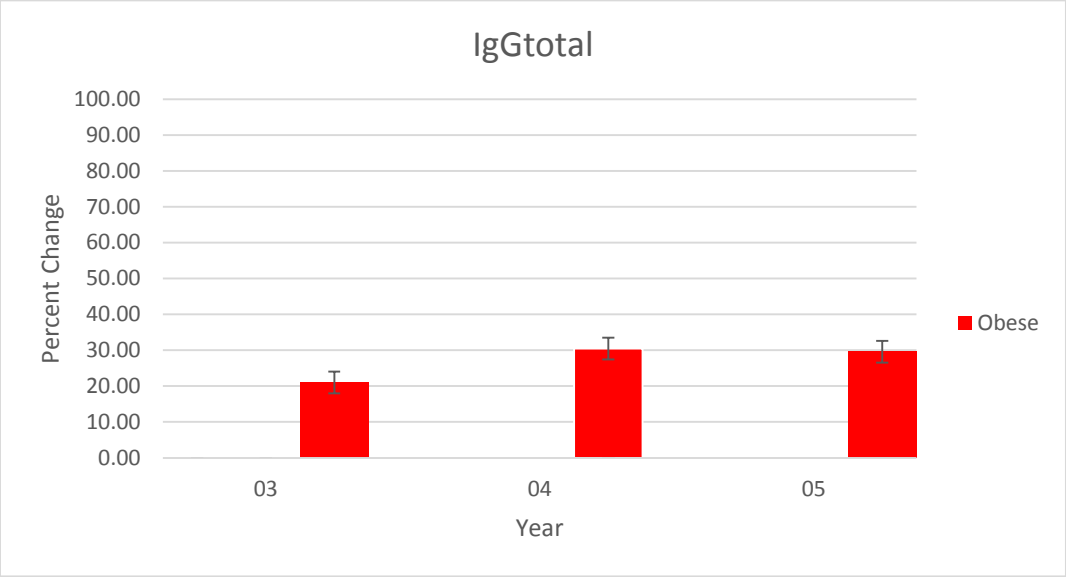
Figure 4



The percent change between the healthy and overweight subjects showed significance with a P value of 0.036241. The healthy subjects had a -0.62% change in the 03 year, which then actually decreased to a value of -10.86% in the following year and then went up to 21.64% in the 05 year. The overweight individuals on the other hand, started off at a very high percent increase between their 03 and 04 vaccinations, at 85.08%, which then decreased to 44.56% for the 04 samples, and decreased further to 40.34% in the 05 samples.

When the percent change is analyzed for the obese group, we found that the obese group did not increase to the same extent as the healthy weight and overweight subjects significantly for all three years (Figure 5), which shows that these individuals had an initial high response but did not get significantly better in their antibody response over the next three years.

Figure 5



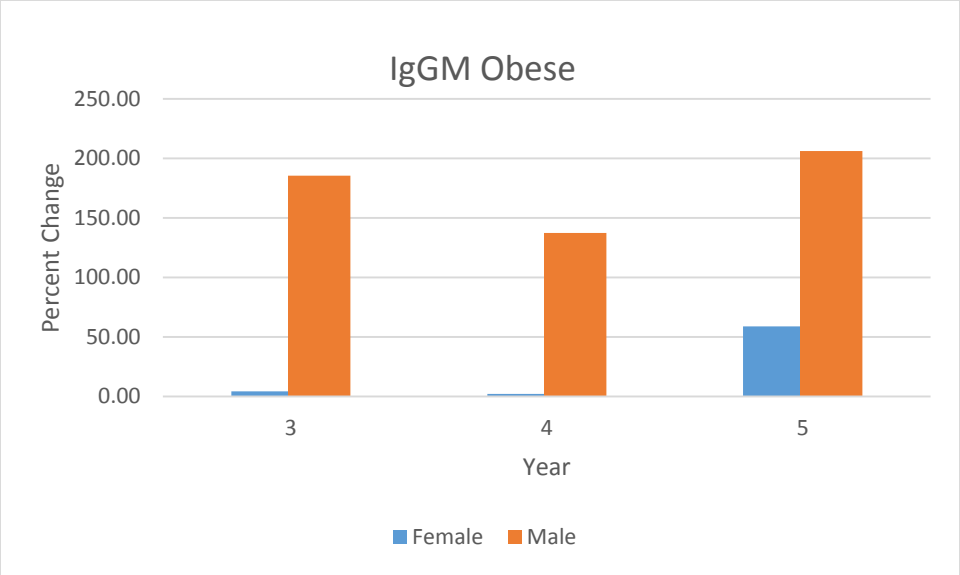
The IgGtotal percent change levels for the obese individuals on the other hand, remained fairly constant, as they changed from 20.98% in the 03 samples to 30.46% in the 04 to 29.60% in the 05 year. These values were all lower than the percent change from pre to post vaccination for the healthy and overweight subjects.

Gender Significance

The final factor I focused on was the differences in the percent change of the pre to post vaccinations for the antibody levels based on gender. I surmised that gender should not be a major factor in the antibody response since all these individuals were between the ages of 30-90. However for the IgM obese group, there was a significant difference between the males and females as the males had a significantly higher percent change in comparison to the females for that particular group. Both the males and female groups increased in the percent change from the 03 to the 05 year, but the males still had a very high percent change compared to the female.

However, although the data for the other classifications of weight class and antibody were not found significant, the data showed that there was no observable trend, as these results varied depending on whichever specific antibody I chose to look at.

Figure 6



The difference in the percent changes for the female and male obese patients in the IgGM antibody showed a major significance with a P value of 0.004948, as the females only had a 4.41% change in the IgGM levels for the 03 year, which stayed low the following year at a 2.30% change, and then increased suddenly to 58.99% in the 05 year. However these figures were miniscule compared to the percent increase in IgGM level between the pre and post vaccinations for the male obese subjects, as there was a 185.59% increase in the 03 samples. This value decreased to 137.55% in the 04 year, but then reached its highest point in the 05 sample with a very high antibody response, at 206.17% change in the 05 year.

Ch. 5 Discussion and Limitations

5.1 Discussion

This study looked at the immune antibody response for healthy, overweight, and obese subjects who were repeat patients in UNC's Flu Study for the 2010-2011, 2012-2013, and the 2013-2014 years respectively. We hypothesized that the antibody response would improve over time for all groups of people with the obese subjects eventually normalizing over time to match the response of the healthy weight group. We also hypothesized that gender should not have a major impact, as earlier studies have not shown sufficient proof to be able to generalize that gender does have a positive or negative effect.

After vaccination an individual's body produces infection-fighting antibodies against the flu strains within that particular vaccine.[13] This way if an individual is exposed to any of the flu strains during the influenza season, these antibodies would be able to attach to the virus' hemaglutinin antigens and prevent the infection initiation process. Even if the HA gene changes, the antibody it encodes may still have other binding sites which haven't altered in shape and is still able to bind to a certain antigen.[14] This phenomenon is known as cross reactivity. In addition, memory B cells are produced as part of the adaptive immune response, in which an individual's body will have B cells specific for a particular virus. This way if it encounters this virus again, it is able to have antibodies ready to combat this. Finally, the strains circulating for a particular year could be repeated from one year to another, in which an individual would already have antibodies ready to combat this as well.

Cross reactivity and memory B cell production led us to the hypothesis of improved antibody response to repeated influenza vaccinations over time, including the obese group normalizing over time to match the response of the healthy group. Our results showed that there was a significance between time and weight for the IgG1 and IgGtotal. There was no significance found for any of the other immunoglobulin G subtypes or IgM antibodies. This could be due to the fact that IgG1 is the major isotype in influenza antibodies and constitutes most of the relative serum concentrations of the human IgG subclasses. IgG1 is also mainly responsible for the thymus mediated immune response against proteins and polypeptide antigens, as IgG1 binds to the Fc-receptor of phagocytic cells and activates a complement cascade by binding to C1.[12] IgGtotal is a measurement of all the various IgG subcategories considered together, which may reflect why total IgG also showed significance, compared to the subtype analysis which are not as prevalent in serum.

Our results demonstrated at a baseline level, the obese group had a higher level of antibodies present, and this response improved over time, as the following year the optical density levels for the obese groups was greater than that of the previous year. However this was not observed for the healthy subjects, as the optical density levels for IgG1 and IgGtotal for this subgroup of people varied from one year to another. Previous studies have shown that the baseline levels of a particular antibody may be independent of receiving a vaccination in a previous year. Sasaki et al., reported a study that showed that there was no significant difference in the baseline percentage of the memory IgG B-cells between an 03-T group, which received TIV in the

previous year, and the 03-L group, who did not receive the TIV.[15] These results showed that the percentage of circulating influenza-specific memory B cells was not affected by the status of having a previous vaccination. An explanation for the fact that the healthy subjects had a lower baseline antibody level in comparison to the obese subjects is that the obese individuals may have been exposed to the circulating influenza in the 2010-2011, 2012-2013 and 2013-2014 years, resulting in those individuals being infected and producing a greater amount of antibodies.

Our results for the percent change in regards to over time was consistent with what we hypothesized, in that over time the antibody response improved for all three groups: healthy, overweight and obese. By performing T tests, we found significance for the IgG3 data over time. It is interesting to consider that there was no significance found for the time for any of the other subcategories or IgM data, considering that IgG3 concentration in serum is lower than the other subclasses of immunoglobulin G proteins. In addition, our data showed that there was a significance between the healthy and overweight groups' antibody response over time for the IgGtotal antibody levels. Our results were consistent with the findings of many other studies which showed that there was a significant inverse correlation between the percent change of a particular antibody versus the baseline antibody levels. The study by Sasaki et al. also found an inverse correlation between baseline HAI titer and the fold change in HAI after 30 days. His study was conducted with subjects who received either the '05-LAIV or '05-TIV, however this inverse relationship was consistent with both groups. In addition, the study showed that higher levels of preexisting serum antibodies was associated with a reduced peripheral effector B cell response to TIV. These studies showed that serum antibody response to influenza vaccination could be affected by the levels of circulating antibodies when an individual is vaccinated, so these obese individuals had a higher baseline antibody level and greater circulating antibodies initially, which is why they did not have as high of a percent change and did not improve over time like the healthy group.

The disparity in antibody response by observing the percent increase between the overweight and obese groups could be explained by the innate immune system of obese individuals. A study done previously in the Beck lab demonstrated that BMI did not influence the initial fold increase in IgG antibodies following vaccination, however after a one year (12 month) period high BMI resulted in with a greater decline in the influenza antibody titer.[16] This was due to decreased cytotoxic (CD8+) T cell activation in these obese individuals, resulting in decreased expression of functional proteins. In our study, we noted that the percent change for the overweight actually declined over time, a trend that could be observed throughout our data for the obese and overweight subjects although the results were not significant for the IgG1, IgG2, IgG3, IgG4 and IgM data. This decline in antibody titers following repeated vaccinations can also be seen in this study, as subjects with declining antibody titers after repeated vaccinations had the highest postvaccination titers following the initial vaccination.

We also looked to see the role gender may play in this response. Studies reporting male and female immune responses has varied. In one study, done by World Health Organization, it was found that in both humans and rodents the inflammatory immune responses were higher in females compared with males, as clinical studies in India, Italy, Sweden and the United States

showed that men have lower , CD4+:CD8+ T cell ratios ,CD3+ and CD4+ T cell counts, and helper T cell type 1 (Th1) responses.[17] In addition, small animal models have shown that there has been elevated immunity post-vaccination in females as a result of cross-protection against influenza viruses. The main reason for these differences detected in gender is a result of the sex steroid hormones such as testosterone and estradiol which generally suppress the activity of immune cells. As a result there has also been data showing that males have a much more vigorous immune and behavioral response to influenza infection in comparison to females.[18] This led us to hypothesize that gender should not make a huge difference in the antibody response for the subjects. Our data further exemplified this as we did not see a significant trend between the percent changes for the males and females groups. The only significant data found was for the IgM obese group, which showed that the males had a much higher percent change consistently for all three years of the study. However when looking at the healthy IgM group, the results were nearly the opposite, with the female group exhibiting a much higher percent change. It is interesting the values for the IgM group were so high as IgM is the first antibody to appear in response when a person is exposed to an antigen.

5.2 Limitations and Recommendations

The major limitation of this study was the small sample size. This study consisted of only 24 subjects. When further looking at the data, it can be seen that there were only a total of 6 healthy weight subjects, 12 overweight, and 6 obese subjects. This small and uneven sample size amongst the groups limited the amount of significant data we were able to have from this study. Further studies could make sure to divide the subjects for each weight class evenly or perhaps pull in from other institutions or clinics performing a similar study to increase the sample size.

Another limitation was that confounding factors such as smoking or the subject's lifestyle was not accounted for and could make a difference in their immune response to the influenza vaccination, as smoking is associated with decreased antibody and immune response. This could have skewed our data as these individuals may have had impaired response.

In further studies, the process of microneutralization assays could be done on the subjects serum. This procedure is able to look at which antibodies neutralize certain viruses to give us a better idea of the subjects response to a particular virus strain. [18]

Ch. 6 References

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