DO OBESE INDIVIDUALS HAVE LESS CROSS-REACTIVE, NON-NEUTRALIZING ANTIBODIES TO THE 2009 PANDEMIC H1N1 INFLUENZA VIRUS?

By:

-Mahreed Rahman Khan-

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Department of Nutrition

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Approved:

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Advisor-Melinda A. Beck

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Reader- Justin Milner

ABSTRACT

Mahreed Rahman Khan

DO OBESE INDIVIDUALS HAVE LESS CROSS-REACTIVE, NON-NEUTRALIZING ANTIBODIES TO THE 2009 PANDEMIC H1N1 INFLUENZA VIRUS?

(Under the direction of Melinda A. Beck)

Background: Obesity has recently become a major global health concern as the worldwide population of those who are obese has increased to over 1 billion overweight adults. Recent studies suggest that obesity is a risk factor for severity of influenza virus, especially during the 2009 pandemic H1N1 outbreak. During the 2009 influenza pandemic, it was found that while overall severity to pH1N1 was mild, obese individuals had higher rates of hospitalization and severity than healthy weight individuals. The mechanism for impaired immunity in obese individuals will be explored.

Objective: To determine if naïve obese individuals have less pre-existing cross-reactive, non-neutralizing antibodies to pH1N1 proteins NP, M1 and HA than healthy weight individuals.

Results: The average M1 antibody OD value in healthy weight patients was found to be 1.12 while average OD value in obese patients was found to be 1.01. The average NP antibody OD value in healthy weight patients was found to be 0.91 while the average antibody OD value in obese patients was found to be 0.97. The average HA antibody OD for healthy weight patients was 1.32 while the average antibody OD value for obese patients was 1.28. None of these differences were significant.

Conclusion: Obese individuals had similar levels of pre-existing, non-neutralizing, cross-reactive antibodies to pH1N1 compared with healthy weight individuals. Mechanism for impaired immunity in obese individuals likely lies elsewhere.

ACKNOLWEDGMENTS

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“If you learn from defeat, you have never truly lost”

–Zig Ziglar

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LIST OF ABBREVIATIONS

pH1N1	Pandemic H1N1
CDC	Center of Disease Control
WHO	World Health Organization
BMI	Body Mass Index
NP	Nucleoprotein
M1	Matrix Protein 1
HA	Hemagglutinin
HRP	Horse Radish Peroxidase
HW	Healthy Weight
OB	Obese
OD	Absorbance
ELISA	Enzyme-linked immunoabsorbent assay
HAI	Hemagglutinin inhibition test
TIV	Trivalent influenza vaccine
LAIV	Live attenuated influenza vaccine

CHAPTER 1

STUDY AIMS AND HYPOTHESIS

Obesity has recently become a major global health concern as the worldwide population of those who are obese has increased to over 1 billion overweight adults, and nearly two-thirds of the US population has been reported as overweight or obese.[1] Obesity is a topic of concern because of the associated risk factors, which include diabetes mellitus, dyslipidemia, hypertension, osteoarthritis, metabolic syndrome disease, and cardiovascular disease. In addition, obesity is considered an immune-compromising condition.[1,7] The classification of obesity is determined by body mass index (BMI).[2] The Center of Disease Control (CDC) calculates BMI via height (m) and weight (kg) measurements, the formula: weight (kg)/ [height (m)]^2.[2]

The influenza virus is a contagious respiratory illness that is routinely spread among humans.[3] The virus ranges in severity, from mild to severe illness which can result in hospitalization and even death. According to the World Health Organization (WHO), it is estimated that up to 5 million cases of severe illness occur along with 250,000 to 500,000 deaths worldwide.[4] While most people recover within two weeks without serious medical treatment, those who are immunocompromised (such as obese individuals) are more prone to severe complications.[4] In the US, the CDC estimates that each year there are between 3,000 to 49,000 influenza related deaths. The most effective prevention method for influenza infection is via annual vaccination.[5] Vaccines contain weakened or attenuated (dead) antigens which are not strong enough to actually cause the disease, but do initiate immune response. The immune system produces antibodies against the antigen, producing memory cells that prevent re-infection when the disease is encountered in the future.[9]

Recent studies suggest that obesity is a risk factor for severity of influenza virus, especially during the 2009 pandemic H1N1 outbreak.[6] While obesity does induce a pro-inflammatory state in the human body, the exactly immunological consequences of obesity on the immune response to influenza infection are not specifically identified.[7] Using an animal model, studies in our lab have demonstrated that obese mice have less cross-reactive antibodies to pH1N1 compared with lean mice. Cross-reactivity is measured by the way in which the body's immune system can combat antigens which appear similar.[21] It is likely the increased severity seen in obese humans to pH1N1 was a result of decreased cross-reactive antibodies to the pandemic strain.[36]

Specific Aim: To determine if naïve obese individuals have less pre-existing cross-reactive, non-neutralizing antibodies to pH1N1 than healthy weight individuals.

Hypothesis: Naïve Obese individuals will have less pre-existing cross-reactive antibodies to pH1N1 as compared to healthy weight individuals. Specifically, the antibody response to intracellular conserved protein of the flu virus matrix protein 1 (M1) and nucleoprotein (NP) will be lower in the obese compared with the naïve healthy weight individuals. The titer for obese individuals will be on average lower than the titer for healthy weight individuals for both conserved intracellular proteins of the influenza virus.

CHAPTER 2

INTRODUCTION

2.1 Obesity

The worldwide epidemic of obesity has more than doubled since 1980. It was reported in 2008 that there were 1.4 billion overweight adults over the age of 20 around the world. More than 35% of adults aged 20 and over are overweight, and 40 million children under the age of 5 were reported as overweight in 2011.[1] Obesity is defined by a body mass index (BMI) of over 30. BMI is calculated by correlating height and weight to determine relative fat accumulation that may impair health. The Center of Disease Control (CDC) calculates BMI via height (m) and weight (kg) measurements (formula: weight (kg)/ [height (m)]^2).[2] BMI is one of the best methods for assessment of obesity and overweightness. The CDC defines four distinct categories of BMI: a BMI of < 18.5 indicates Underweight health status, BMI of 18.5-24.9 indicates Healthy weight or Normal, BMI of 25.0-29.9 indicates Overweight while a BMI of 30.0 and over indicates Obese.[2] Table 1 summarizes these classifications.

WEIGHT CLASSIFICATION	**BMI**
Underweight	Below 18.5
Normal/Healthy Weight	18.5-24.9
Overweight	25.0-29.9
Obese	30.0 and greater

Table 1. Weight classifications and respective BMI ranges

Being overweight and obese have been cited as the fifth leading cause of global deaths.[1] In addition to mortality rates, obesity has been linked to risk factors for stroke, cardiovascular disease, type 2 diabetes, hypertension, cancer, osteoarthritis and dyslipidemia. Obesity is one of the most preventable diseases as exercise and proper dieting can help lower BMI to a healthy range.[26] In addition medical care costs for those who are obese in the US alone exceeded 147 billion dollars.[27]

Fundamentally, BMI exceeding 25 is generally caused by an energy imbalance, where more calories are consumed than expended.[2] An increasing global trend is the consumption of fast foods or energy dense foods that are high in fat. In addition the rise in sedentary day jobs with limited physical activity and increasing sedentary modes of transportation has resulted in poor health among most populations. The combination of poor physical activity and increasing caloric consumption has resulted in the global obesity epidemic.[1,2] Current trends suggest that by the year 2030 more than 51% of the world population will be obese, a 33% increase in prevalence. Additionally 549.5 billion more dollars will be used on medical costs resulting in more problems for healthcare cost containment.[1]

2.2 Obesity and Immune Response

Immune dysfunction has been a recently cited associative health condition with obesity. How the immune system is affected by obesity is still relatively unknown as research has been limited. Current research suggests that obesity can be characterized as a low-grade, chronic inflammatory state which alters levels of metabolic hormones and circulating nutrients.[30] The influence the pro-inflammatory state has on the immune system in obese individuals is still largely unexplored. Additional studies suggest that obesity increases the risk for nosocomial infections;

furthermore obesity has recently been isolated as an independent risk factor for increased morbidity for pandemic H1N1 virus.[32]

It has been reported that obese individuals are more prone to infection than lean subjects. Obese individuals have been shown to be more susceptible to infections, bacteremia and poor wound healing.[30] Obesity, which can be considered a state of malnutrition, has also been known to affect both cell-mediated immune response and leucocyte count. Associated production of leptin and reduction of adiponectin affects the stimulation and activation of innate and adaptive immune response. Specific cytokine-like signaling pathways appear to be hindered with altered leptin levels. Other pathways which are affected include altered number of T-lymphocyte and B-cell synthesis and circulation, decreased dendritic cell function and decreased response to antigen stimulation.[29] Moreover, obesity results in a pro-inflammatory state associated with increased levels of circulating pro-inflammatory proteins, in both obese children and adults.[29,31]

2.3 Influenza Virus and Infection

Human influenza is a virus which has three separate strains, A, B and C. Influenza A and B are the only strains which are known to cause seasonal epidemics in humans. In addition Influenza A can be hosted in avian birds, humans and other mammals.[8] Influenza is a respiratory infection which is transmitted through unprotected coughing and sneezing. Crowded and enclosed spaces harbor the virus, as well as hand contamination and direct inoculation.[10] The influenza virus attaches to sialic acid receptors on the apical surface of human tracheal epithelial cells.[33] Upon infection the body generally takes 1-4 days to begin responding to the virus and then works to combat it; during this time humans are most contagious.[10] Severity of the infection ranges from asymptomatic to severe and even fatal outcomes. General symptoms include coughing, chills,

fever and headache. More complicated cases include development of pneumonitis, bacterial pneumonia and others. Generally, recovery from the infection occurs within 1 or 2 weeks, but sometimes the virus takes a more fatal tone. It is estimated that worldwide 5-10% of adults are infected annually with influenza, while 20-30% of children are infected. In addition anywhere from 3,000 – 49,000 deaths worldwide are attributed to the influenza virus.[10]

Influenza generally results in an immune response in populations that doesn't result in hospitalization unless the person is in some way immunocompromised. Generally speaking those who are most susceptible to influenza infection include children, the elderly, those with chronic disorders (CVD, asthma, hypertension, diabetes), pregnant women, and those who are obese.[11] In addition influenza is more commonly seen in large, crowded population centers such as large cities as the virus can move quickly while airborne and person to person contact is increased.

The influenza virus is a single-stranded RNA virus with a segmented genome that codes for specific proteins.[12] The subtypes of influenza are determined by the glycoproteins which are expressed on the virus's surface. Two specific glycoproteins, hemagglutinin (HA) and neuraminidase (NA) are the receptors and binding sites for the virus. Mutations in the genes that code for these glycoproteins results in altered HA and NA proteins that results in the need for new vaccinations each year. In addition to the surface proteins, the virus contains many internal proteins as well. Matrix protein 1 (M1), a highly conserved protein among strains of flu gives strength and rigidity to the lipid envelope of the flu.[12] RNA segments found within the virion of the virus are known as PB1 and PB2 and code for specific proteins. Nucleoprotein (NP), another highly conserved protein of the flu, is a structural protein. Many of the proteins of the flu virus and how conserved they are among different influenza strains are reported in Table 2.[13]

Influenza Protein	Percent Conserved between strains (%)
Hemagglutinin (HA)	12
Neuraminidase (NA)	17
Matrix Protein 1 (M1)	57
Nucleoprotein (NP)	56
Ion channel (M2)	13
PB1	74
PB2	40

Table 2. Influenza extracellular and intracellular proteins with percentage conserved between different influenza strains.

Upon exposure to the influenza virus, the virus binds to surface epithelial cells of the respiratory system. Glycoprotein HA has multiple sialic binding sites, making it a prime receptor for host cells (erythrocytes and surface epithelium). The virus is then endocytosed into the host cell and transcription of viral mRNA occurs.[14] The body, upon detection of viral infection by RIG-1 (recognizes viral infections) will initiate innate and adaptive immune responses through production of pro-inflammatory cytokines IFN alpha and beta. Next, respiratory dendritic cells (RDC's) capture the viral antigen, present it on MHC-II and migrate towards lymph nodes. In the lymph nodes, RDC's interact with naïve T and B cells, stimulating white blood cells producing plasma and memory cells, helping the body fight infection.[15] Antibodies are created for

glycoprotein HA on the surface of the flu virus, preventing binding of the virus to host cells in the body. Additional antibodies are produced to other proteins of the virus.

2.4 Influenza Vaccination

The severity of the flu virus has resulted in recommended protection from the infection through vaccination. The most effective way to prevent infection is to get vaccinated every year. The CDC recommends that everyone 6 months and older should receive a flu shot yearly.[17] This is especially true for high risk patients such as the elderly, children, pregnant women and the immunocompromised.[17] Due to the constantly changing nature of the influenza virus, antibodies from previous viruses are not as effective and may not result in proper protection against the current virus. The seasonal flu vaccine consists of two influenza type A strains and one influenza type B strain based on research and predictions of the circulating strain that season. In 2013 the very first quadravalent vaccine was distributed, consisting of two A viruses and two B viruses.

Two types of administration are provided for the flu vaccine, trivalent inactivated influenza vaccine (TIV) and live attenuated influenza vaccine (LAIV). LAIV is a nasal spray which has weakened, but still live influenza virus which will more closely resemble normal infection.[16] TIV is more common as it is the inactive virus administered subcutaneously or intramuscularly via needle. Vaccination has been reported to prevent anywhere from 70% to 90% of influenza-related illness in those who are healthy, and nearly 60% in older adults (over the age of 65).[17] Vaccines contain weakened or attenuated (dead) antigens which are not strong enough to actually cause the disease, but do initiate immune response. The immune system produces antibodies against the antigen, producing memory cells that prevent re-infection when the disease is encountered in the future.[10]

2.5 2009 Pandemic H1N1 Outbreak

During the 2009-2010 flu season, the influenza strain H1N1 was identified in the US.[16] This virus is often referred to as the "swine flu" because many of the genes seen in the virus were similar to influenza found regularly occurring in US swine (pigs). Further research suggested that two more genes of the H1N1 were from avian and swine genes of European and Asian descent, making the virus a "quadruple reassortant". The nature of this quadruple re-assortment resulted in a new influenza virus which had not been seen in over 40 years.[16] Scientists and physicians were concerned because there would be no pre-existing immunity to a new strain such as H1N1, thus resulting in increased severity across all demographics.[17] The novel strain of influenza A (pH1N1) is genetically distinct from any previous seasonal influenza virus, thereby rendering antibodies from previous seasons non-protective.[19]

During the subsequent flu season of the pandemic, the CDC reported the virus peaked from October to March and spread relatively quickly as compared to other flu seasons.[18] However as the flu season continued, Dr. Jhung, of the CDC's flu division told CNN that the spread and severity of the disease was typical of an influenza season.[18] The CDC estimated that between 188,000 to 381,000 US citizens were hospitalized due to the pandemic, not stemming from the average of 250,000-500,000 annually. In addition the estimated death toll of the pandemic ranged from 8,520 to 17,620.[20] Both the number of infected and the death toll did not spike as highly as was expected from a new strain of flu. The relatively mild effects overall could be attributed to increased rate of vaccination and possibility of cross-reactive immunity from previous influenza strains.

It was found, however, that the pandemic did affect those at higher risk more so than previous flu seasons. The obese, immunocompromised, young and pregnant faced much worse

outcomes than healthy adults. It was found that elderly had greater protectively to pH1N1, which is uncommon for any flu strain. It is likely that older adults had been exposed to a flu strain similar to pH1N1 during the first half of the 20th century.[26] This likely resulted in higher affinity antibodies in older adults which prevented severity from pH1N1. The increase in hospitalization and mortality were alarming and drew reason for concern in at risk populations, especially the obese and immunocompromised.

2.6 Cross Reactive Immunity

Cross-reactivity is measured by the way in which the body's immune system can combat antigens which appear similar.[21] Antibody binding sites, also known as epitopes, consist of about 15 amino acid sequences, 5 of them being responsible for binding of the antibody. The paratope, which is the part of the antibody molecule that binds to the epitope, consists of about 50 variable amino acids. A paratope can bind to unrelated epitopes, and an epitope can bind to unrelated paratopes.[21] Similar structures such as shape and charge can aid in unrelated paratopes and epitopes binding together. This relationship is the basis for cross reactive antibody immunity.

Concerning the influenza virus, the determination of epitopes which are presented on the virus's surface is determined by the hemagluttanin and neuraminidase variation presented. H3N1 and H3N2 have similar hemagluttanin epitopes while the neuraminidase antigen is different. It is likely that the antibody paratope to N1 can bind and provide protection against the N2 epitopes presented in the differing strain. As mentioned in the previous section, pandemic H1N1 was expected to have no cross reacting antibodies as the epitopes on pH1N1 were completely new to the majority of the population. However, it soon became apparent that older individuals had cross-reacting antibodies from exposures to pH1N1 like viruses, and therefore had pre-existing cross-

reacting antibodies that could protect them from pH1N1 infection as opposed to younger individuals, who did not have cross-reacting antibodies.[25]

CHAPTER 3

METHODS

3.1 Study Design and Participants

Participants were recruited as part of a prospective observational study from the University of North Carolina Family Medicine Center in Chapel Hill, NC. The study is designed to understand the relationship between high BMI and immune response to the influenza TIV vaccine. For this study, participants were selected from adults who received the influenza vaccine during the 2010-2011 flu season, the vaccine did not contain pH1N1 as one of the vaccine strains.

During the enrollment visit, the study nurse obtained informed consent, medical records, weight, height, BMI and baseline blood samples. For this study, a sample of 91 participants were selected, 45 obese individuals (BMI $\geq$30) and 46 healthy weight individuals (BMI 18.5-25). Participants selected were HAI negative.

3.2 Indirect ELISA

In order to determine antibody levels to specific proteins of the influenza virus, enzyme-linked immunoabsorbant assay (ELISA) was utilized. ELISA is useful in detecting the presence of a substance, generally an antigen or antibody. For accurate quantitative results, Indirect ELISA was used for this experiment.[23] First, the antigen is plated in each respective well. Next blocking buffer is added to inhibit binding of antibody to open protein-binding sites. After incubation a primary antibody is added and after an addition incubation, secondary antibody-horse radish peroxidase (HRP) conjugate is added to bind to the primary antibody. Finally, a substrate such as tetramethylbenzidine (TMB) and peroxide is added. Any HRP bound to the secondary antibody will act on the TMB and peroxide to produce a blue byproduct. After a specified amount of time,

the reaction is stopped with the addition of sulfuric acid. The color intensity is proportional to the amount of HRP activity, which in turn is related to the bound antibody. The color intensity was measured at 450nm using a spectrophotometer.[24]

Following the indirect ELISA protocol, first Hemagluttanin (HA), Matrix Protein 1 (M1) and Nucleoprotein (NP) from the 2009 pH1N1 were isolated, diluted with coating buffer (250 mL 1x PBS solution with .397 $NaCO_3$ and .935 HCO_3) and 50 uL were platted to the 96 well plate. HA was concentrated to a 1:1 viral antigen to coating buffer ratio while M1 and NP were concentrated at a 3:1 viral antigen to coating buffer ratio. After a 20-30 hour incubation period at 4ºc, the plates were flicked to remove any remaining antigen solution in wells and then blocked with 200 uL of blocking buffer (3 g dried milk with 100 mL coating buffer) for 1-3 hours at 36ºc. After blocking, plates were washed 6x with 10x PBS tween and then loaded with serum, diluted with loading buffer (3 g dried milk with 100 mL 1x PBS). Respective dilutions for each antigen are listed in Table 3. Respective dilution series selected were optimal for avoiding oversaturation of the serum to protein. ELISA plates with serum dilutions were again incubated for 20-30 hours at 4ºc. Following incubation, plates were again washed 6x with 10x PBS tween, then 50 uL of secondary antibody IgGt-HRP with loading buffer at a 1uL to 1mL ratio was added to each well. This was incubated at 36ºc for 1 hours. After incubation the plates were washed 6x with 10x PBS tween and then 100 uL of substrate, TMB solution, was added and let sit for 20 minutes at room temperature in a dark environment. Following 20 minutes, 100 uL 1 M sulfuric acid was added to each well for desired color change and absorbance readings. The Dynatech Elisa plate reader was used to read each ELISA plate at 450 nm for absorbance values.

pH1N1 Protein	Concentration	Serial dilution series
Hemagglutinin (HA)	1 uL/mL	1:9000 1:27000 1:54000 1:162000
Matrix Protein 1 (M1)	3 uL/mL	1:3000 1:9000 1:27000 1:54000
Nucleoprotein (NP)	3 uL/mL	1:750 1:3000 1:9000 1:27000

Table 3. Concentration of pH1N1 protein plated with respective serum dilution series

3.3 Statistical Analysis

End-point ELISA with subsequent O.D readings were used to analyze individual samples. Excel sheets with average of triplicate concentration readings of for each sample and respective protein were plotted. J.M.P Pro 9.0.0 statistical software (SAS Institute, Cary, NC) was used to analyze and determine significance for the ELISA data set. Additionally GraphPad Prism software was used to graphically display stratified demographic data.

CHAPTER 4

RESULTS

4.1 Demographics of the Study Population

Participants were categorized into 2 BMI based groups: healthy weight (BMI 18.5-25) and obese (BMI ≥ 30). Forty-six healthy weight participants and forty-five obese participants were found in each group that were HAI negative (N=91). The complete demographics for the study population are presented in **Table 4**.

		Healthy Weight	**Obese**
Sample Size		46	45
BMI	Mean ± SE	23.3 ± 0.2	35.4 ± 0.7
	Range	19.5 – 25.4^	30 - 46.9
Age	Mean ± SE	45.2 ± 1.8	47.3 ± 1.6
	Range	23 - 60	20 - 60
Gender	Male	19	17
	Female	27	28
Race	White	36	16
	African-American	8	26
	Other	3	1

Table 4. Demographic summary of BMI, age, gender and race in subject population.
^7 participants' in the Healthy Weight BMI group had BMI's of greater than or equal to 25, but were kept in the study as healthy weight.

In addition to the 91 participants selected for this study, two infected patients and two pediatric patient serum were also tested for baseline values of antibody against NP, M1 and HA. Both pre-vaccination serum samples were from 2 year olds who were HAI negative for pH1N1 and H3N2, these samples were obtained from the Pediatric Continuity Care Clinic. Two convalescent serum samples from individuals who were positive for pH1N1 were obtained from BEI resources. One serum sample had a high antibody response, while the other had low antibody response; these infected samples were distinguished as Infected Hi and Infected Low. BMI was not known for pediatric or infected samples.

All participants in the study, excluding the positive control BEI samples, were tested for any pre-existing pH1N1 antibodies. This was accomplished by running Hemagglutination Inhibition Test (HAI). **Figure 1** displays the results of the HAI test for each group of patients, both healthy weight and obese. The HAI results clearly show no pre-existing antibodies before vaccination, when serum samples were taken and used for purposes of this experiment. In addition, there was no significant difference found between the post vaccine antibodies between the healthy weight and obese patients.

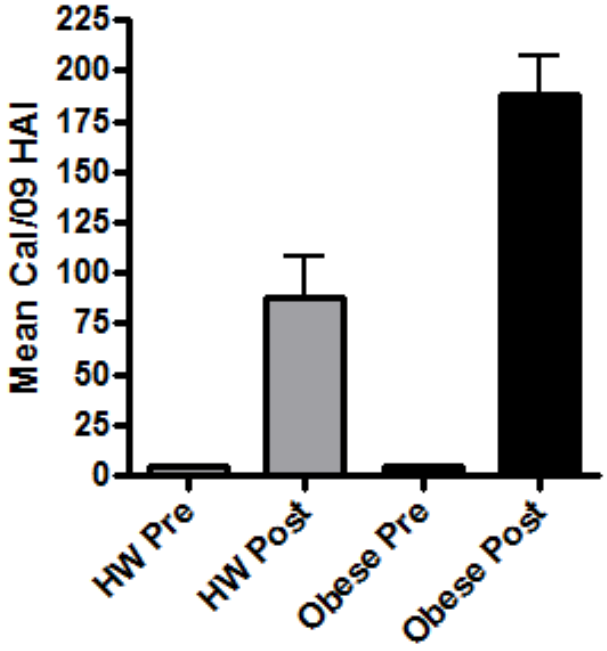

Figure 1. Results of HAI for pre/post-vaccine in healthy weight (HW) and obese patients.

4.2 Convalescent and Pediatric serum antibodies per influenza protein

Convalescent and pediatric serum samples were run with the same respective serum dilution series as healthy weight and obese for hemagglutinin (HA), matrix protein 1 (M1) and nucleoprotein (NP). The pediatric trend lines represents a naïve response to pH1N1 as these samples likely do not have any pre-existing antibodies to any influenza strain, and thereby functions as a negative control. Additionally the infected trend lines represent a protective level against pH1N1, as these samples were exposed to pH1N1 and as a result have developed protective antibodies against the influenza strain, and function as a positive control. The O.D values and concentrations were logarithmically plotted for graphical interpretation. Figures 2-4 display the resulting O.D values vs. concentration for positive and negative controls of each influenza protein.

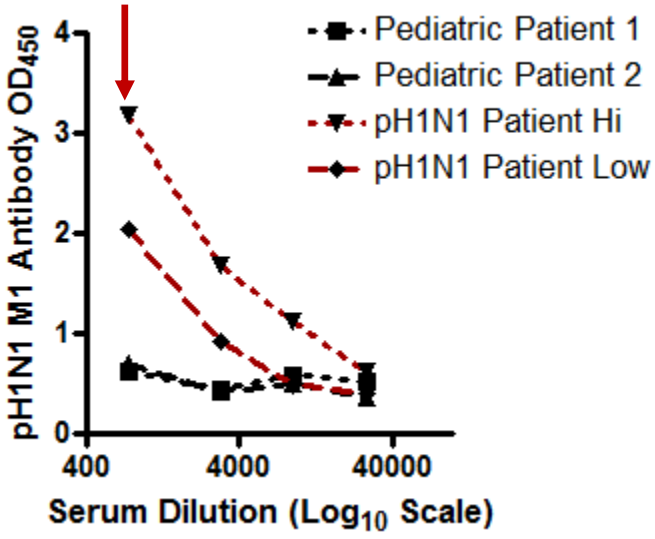

Figure 2. M1 OD values for serial dilution series in infected and pediatric samples.

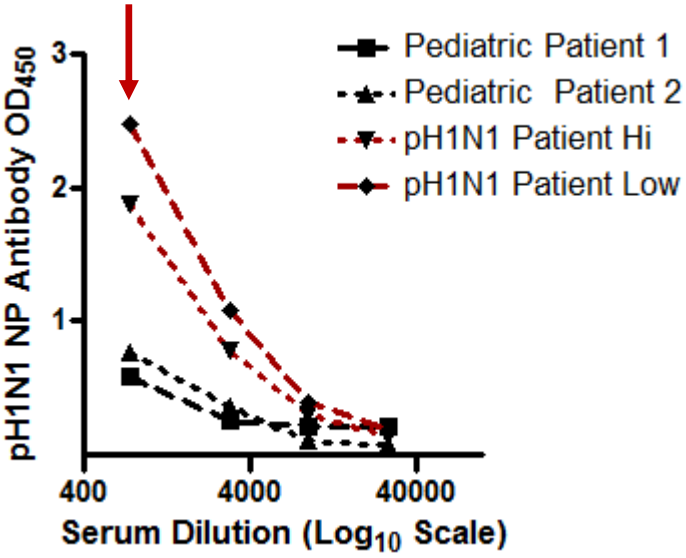

Figure 3. NP OD values for serial dilution series in infected and pediatric samples.

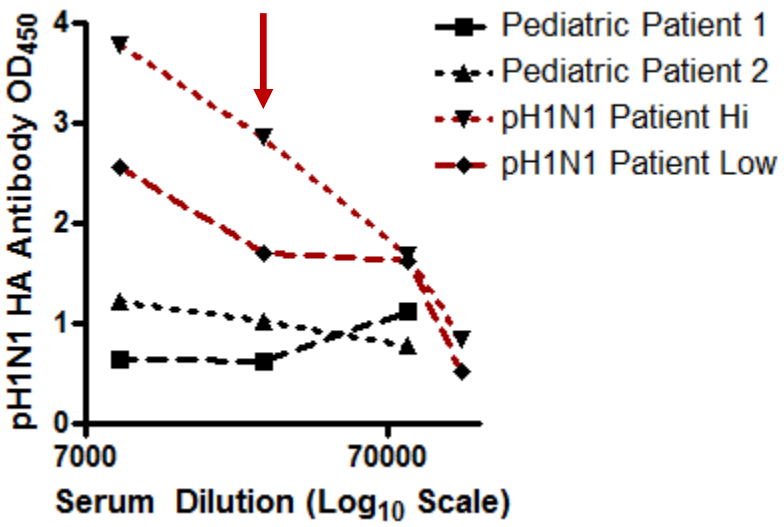

Figure 4. HA OD values for serial dilution series in infected and pediatric samples.

The infected and pediatric samples gave baseline results for what can be distinguished as minimal to no cross-reactive antibody protection, as displayed in the pediatric serum sample trend line, and protective antibodies to pH1N1 as displayed by the infected serum samples. The red arrow in each respective graph represents the concentration selected for average OD values of healthy weight and obese serum samples. These concentrations were selected because the greatest difference between infected and pediatric OD values were seen at this level. This dilution has the greatest sensitivity to cross reactive antibody response. These dilutions are specifically 1:750 (NP), 1:3000 (M1) and 1:27000 (HA). The HA concentration selected was not the first dilution because the OD values of the infected Hi patient was at maximal concentration reading, therefore a range which had a range above infected Hi was selected.

4.3 Healthy Weight and Obese antibody response

As mentioned in the section above, the dilutions selected for the study samples were determined by the infected and pediatric OD separation. The average M1 antibody OD values at dilution factor 1:3000 was calculated for healthy weight and obese patients. Figure 5 below displays a graphical representation of this data.

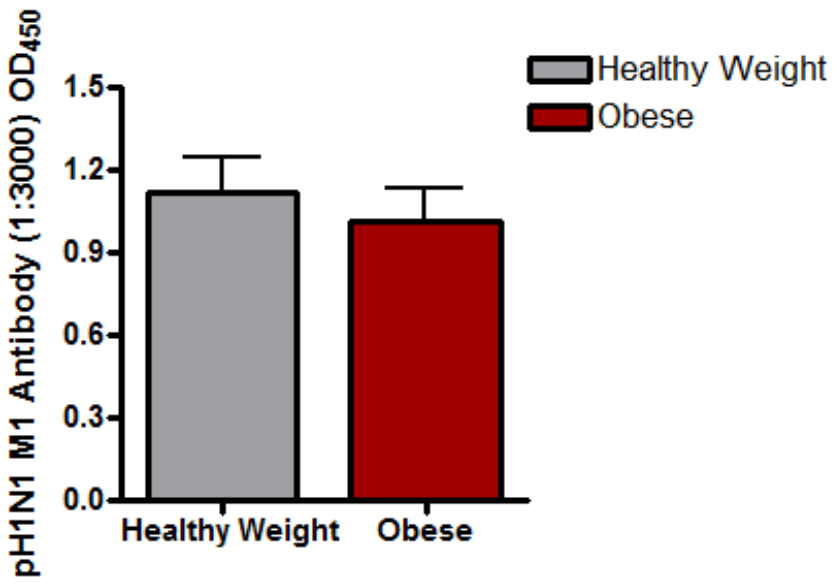

Figure 5. M1 mean antibody OD value at a 1:3000 dilution factor.

The mean antibody OD value in healthy weight subjects was found to be 1.12 with standard error (SE) of 0.13. The mean antibody OD value in obese subjects was found to be 1.01 with SE of 0.11. Despite the fact that a greater antibody level was found in healthy weight subjects, the difference was not found to be significant between the two groups.

The mean of OD values at 1:750 for all samples of NP were calculated. Figure 6 below shows the bar graph of mean NP OD values in healthy weight and obese subjects.

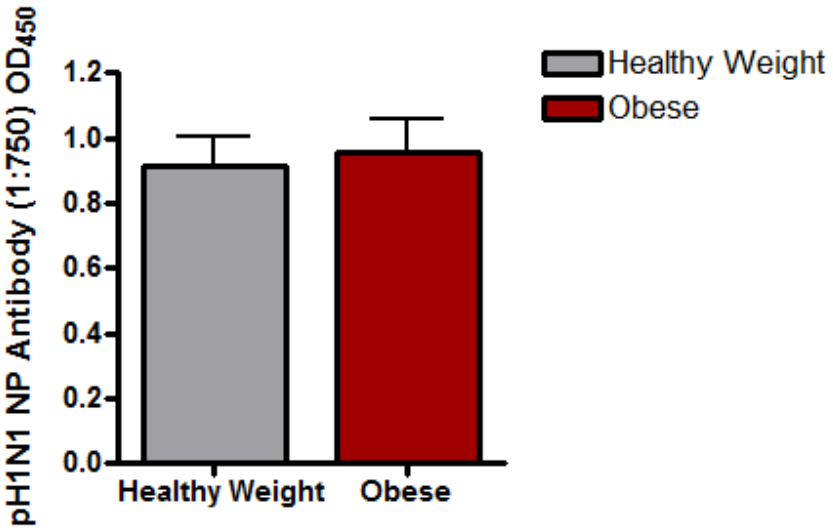

Figure 6. NP mean antibody OD value at a 1:750 dilution factor.

The mean antibody OD value in healthy weight subjects was found to be 0.91 with SE of 0.10. The mean antibody OD value in obese subjects was found to be 0.97 with SE of 0.09. The difference between the two groups was not found to be significant.

Finally, the mean OD values at 1:27000 for all samples for HA were calculated. Figure 7 below shows the bar graph of mean HA OD values in healthy weight and obese subjects. 31 lean samples and 30 obese samples were used (N=71) for this analysis. Figure 7 displays the bar graph of mean +/- SE HA OD values in healthy weight and obese subjects.

Figure 7. HA mean antibody OD value at a 1:27000 dilution factor.

The mean antibody OD for healthy weight subjects was 1.32 with SE of 0.10. The mean antibody OD value for obese patients was 1.28 with a SE of 0.10. The healthy weight subjects did have a higher average antibody level than the obese patients, however the difference found between antibody levels was not significant. Table 5 below summarizes the OD values for each protein conducted in this study.

	Healthy Weight Mean OD ± SE	**Obese** Mean OD ± SE
Hemagglutinin (HA)	1.32 ± 0.10	1.28 ± 0.10
Matrix Protein 1 (M1)	1.12 ± 0.13	1.01 ± 0.11
Nucleoprotein (NP)	0.91 ± 0.10	0.97 ± 0.09

Table 5. Mean OD and SE for HA, M1, NP in Healthy Weight and Obese subjects

CHAPTER 5

DISCUSSION

5.1 Cross Reactive Antibodies to Influenza

Obesity has recently become a major global health concern as the worldwide population of those who are obese has increased to over 1 billion overweight adults. This increase in the rate of obesity has resulted in heightened research interest in understanding how obesity contributes to wide range of diseases, including type 2 diabetes, cardiovascular disease and cancer.[1,7] Of particular interest for this thesis project, obesity was recognized as an independent risk factor for increased influenza morbidity and mortality during the 2009 influenza pandemic.[14] In a typical flu season, anywhere from 5% to 20% of US citizens will contract the flu virus.[8]

Recent studies suggest that obesity is a risk factor for severity of influenza virus, especially during the 2009 pandemic H1N1 outbreak. During the 2009 influenza pandemic, it was found that while overall severity to pH1N1 was mild, obese individuals had higher rates of hospitalization and death than healthy weight individuals. It was hypothesized that the general decreased severity to the pandemic was a result of pre-existing, cross-reactive immunity to the virus. It is known that antibodies have the ability to bind to conserved epitopes on different viral antigens, however the amount has never been quantified. This is particularly important for the influenza virus, as viral antigens change annually; however there are multiple conserved proteins found within the virus. It is likely these conserved proteins in the virus will have cross-reactive antibodies that can bind and function to increase immune response. Previous vaccination or infection to a certain strain of influenza may render protection to different strains of the virus through cross reactive antibodies. The purpose of this study was to determine if the impaired immunity seen in obese humans stemmed from less pre-existing, non-neutralizing, cross-reactive antibodies.

Results from this study show that obese individuals do not have less pre-existing, non-neutralizing, cross-reactive antibodies than health weight individuals to pH1N1. Although no significant differences were found in cross-reacting antibody responses between healthy weight and obese subjects, there were some interesting trends identified. The M1 and HA antibody levels in healthy weight individuals was higher than that of obese individuals. It is possible with increased sample size, the data between the two groups would be significant.

5.2 Limitations

There were several limitations in this study. First, the relatively small sample size could address the lack of significance found for any of the pandemic protein antibody levels. The sample size should be increased with the possibility of finding significance in some of the findings. It is possible that with increased sample size the increased antibody levels seen in M1 and HA in healthy weight subjects would be significant. It would be ideal to increase the sample size to close to 100 in each group, effectively doubling the sample size of the initial study.

A recent study demonstrated that perhaps body fat percentage is a better indicator of obesity compared with BMI.[35] While BMI is a good indicator of relative weight to height and will indicate obesity in most patients, it is also possible that individuals with relatively high BMI's, to the extent where they are >30 and considered obese may actually be muscular individuals. Muscle mass is 18% more dense than fat.[34] This is often why athletes have high BMI's but are actually very fit and have very little associated obesogenic effects. It is possible that results were skewed due to patients being identified as obese, however their BMI was not associated with a large amount of fat. However, this is unlikely, given our study population, as most adults were non-athletic middle-aged individuals.

Finally, determining OD values based on specific dilution may not have been the correct method of approach to determine antibody response against influenza. Individual antibody response to an infection may not be determined by a specific OD value, but rather how many antibodies the specific individual requires for protection. For means of this study, the greatest difference between infected and pediatric serum dilutions was selected; however the OD value associated with the healthy weight and obese values may not be representative of their actual protection against influenza. Rather than averaging specific OD values for a dilution factor, the dilution series should be analyzed to determine when measurable antibody levels stay within 10% of the previous dilution factor to determine no longer measurable amounts of antibody.[36]

5.3 Future studies

As mentioned in the limitation section, a titer of the dilution series should be approached to determine antibody level to specific proteins of the influenza virus. This will give a more justified approach to understanding how obese and healthy weight individuals react to the influenza virus. Given the serial dilution series completed for this project, the data could be re-analyzed to determine if a higher associated titer is found with healthy weight individuals, suggesting that despite the initial antibody level is not as high, the antibodies do provide protection for the individual and will titer out further than obese subjects.

In addition, due to the lack of the significance found for antibody response, it is possible that the mechanism for increased susceptibility in obese individuals compared to healthy weight individuals to pH1N1 infection does not involve cross-reacting antibodies. Perhaps differences in the cellular immune response are more important for protection. Of note, our lab has previously demonstrated that obese individuals have impaired CD4+ and CD8+ T cells responses to influenza

virus stimulation. Taken together, our study demonstrated that cross-reacting antibodies could be detected in the serum of healthy weight and obese individuals. In addition, we did not detect any statistical differences between healthy weight and obese individuals with respect to cross-reacting antibody levels. More research is required to understand why obesity puts individuals at greater risk for pH1N1 severity.

REFERENCES:

1. *What Are the Health Risks of Overweight and Obesity? - NHLBI, NIH*. (n.d.). Retrieved March 31, 2014, from http://www.nhlbi.nih.gov/health/health-topics/topics/obe/risks.html
2. *Healthy Weight: Assessing Your Weight: BMI: About Adult BMI | DNPAO | CDC*. (n.d.). Retrieved March 31, 2014, from http://www.cdc.gov/healthyweight/assessing/bmi/adult_bmi/
3. *CDC - Influenza (Flu) Viruses | Seasonal Influenza (Flu)*. (n.d.). Retrieved March 31, 2014, from http://www.cdc.gov/flu/about/viruses/
4. *WHO | Influenza*. (n.d.). Retrieved March 31, 2014, from http://www.who.int/topics/influenza/en/
5. *CDC - Estimating Seasonal Influenza-Associated Deaths in the United States: CDC Study Confirms Variability of Flu | Seasonal Influenza (Flu)*. (n.d.). Retrieved March 24, 2014, from http://www.cdc.gov/flu/about/disease/us_flu-related_deaths.htm
6. Van kerkhove MD, Vandemaele KA, Shinde V, et al. Risk factors for severe outcomes following 2009 influenza A (H1N1) infection: a global pooled analysis. PLoS Med. 2011;8(7):e1001053.
7. Oliveira AG, Araujo TG, Carvalho BM, et al. Acute exercise induces a phenotypic switch in adipose tissue macrophage polarization in diet-induced obese rats. Obesity (Silver Spring). 2013;21(12):2545-56.
8. *CDC - Types of Influenza Viruses | Seasonal Influenza (Flu)*. (n.d.). Retrieved March 25, 2014, from http://www.cdc.gov/flu/about/viruses/types.htm

9. *Vaccines: Vac-Gen/How Vaccines Prevent Disease*. (n.d.). Retrieved March 25, 2014, from http://www.cdc.gov/vaccines/vac-gen/howvpd.htm
10. *WHO | Seasonal influenza and influenza A(H1N1)*. (n.d.). Retrieved March 26, 2014, from http://www.who.int/ith/diseases/si_iAh1n1/en/
11. *Prevention and Control of Seasonal Influenza with Vaccines*. (n.d.). Retrieved March 1, 2014, http://www.cdc.gov/mmwr/preview/mmwrhtml/rr6207a1.htm?s_cid=rr6207a1_w#PersonsAtRiskMedicalComplicationsAttributableSevereInfluenza
12. *Structure of influenza virus*. (n.d.). Retrieved March 27, 2014, from http://www.virology.ws/2009/04/30/structure-of-influenza-virus/
13. Greenbaum et al. PNAS. 2011.
14. Samji T. Influenza A: understanding the viral life cycle. Yale J Biol Med. 2009;82(4):153-9.
15. Notice to Clinicians: Early Reports of pH1N1-Associated Illnesses for the 2013-14 Influenza Season. (n.d.). *HAN Archive*. Retrieved April 1, 2014, from http://emergency.cdc.gov/han/han00359.asp
16. 2009 H1N1 Flu ("Swine Flu") and You. (n.d.). *Centers for Disease Control and Prevention*. Retrieved March 31, 2014, from http://www.cdc.gov/h1n1flu/qa.htm
17. Black S, Eskola J, Siegrist CA, et al. Importance of background rates of disease in assessment of vaccine safety during mass immunisation with pandemic H1N1 influenza vaccines. Lancet 2009;374(9707):2115–22.
18. *H1N1 Dominates A Dangerous Flu Season In US - Health News - redOrbit*. (n.d.). Retrieved March 13, 2014, from

http://www.redorbit.com/news/health/1113038974/h1n1-dominates-dangerous-flu-season-us-010514/514/

19. *Pandemic H1N1 2009 Influenza*, Janurary 2013. Available from http://www.ncirs.edu.au/immunistation/fact-sheets/pH1N1-factsheet.pdf (Accessed March 2014)
20. *CDC H1N1 Flu | In The News*. (n.d.). Retrieved March 23, 2014, from http://www.cdc.gov/h1n1flu/in_the_news/updated_cdc_estimates.htm
21. Haji-ghassemi O, Müller-loennies S, Saldova R, et al. Groove-type recognition of Chlamydiaceae-specific lipopolysaccharide antigen by a family of antibodies possessing an unusual variable heavy chain N-linked glycan. J Biol Chem. 2014;
22. Antibodies Cross-reactive to Influenza A (H3N2) Variant Virus and Impact of 2010-11 Seasonal influenza vaccine on cross-reactive antibodies – United States. (2012, April 13). Centers for Disease Control and Prevention. Retrieved April 1, 2014 from http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6114a1.htm
23. *Indirect ELISA protocol.* January 2013 Available from: http://www.abcam.com/ps/pdf/protocols/indirect%20ELISA%20protocol.pdf (March 2014).
24. *Indirect ELISA Protocol - Leinco Technologies*. (n.d.). Retrieved March 25, 2014, from http://www.leinco.com/indirect_elisa/
25. *Elderly better protected against H1N1 flu, scientists find - Chicago Tribune*. (n.d.). Retrieved March 30, 2014, from http://articles.chicagotribune.com/2011-08-31/news/ct-x-health-briefs-0831-20110831_1_seasonal-flu-h1n1-soft-bedding

26. *WHO | Obesity and overweight*. (n.d.). Retrieved March 25, 2014, from http://www.who.int/mediacentre/factsheets/fs311/en/

27. *Obesity and Overweight for Professionals: Adult: Causes - DNPAO - CDC*. (n.d.). Retrieved March 25, 2014, from http://www.cdc.gov/obesity/adult/causes/index.html

28. Finkelstein EA, Khavjou OA, Thompson H, et al. Obesity and severe obesity forecasts through 2030. Am J Prev Med. 2012;42(6):563-70.

29. Milner, J. J., & Beck, M. A. (2012). The impact of obesity on the immune response to infection. *Proceedings of the Nutrition Society*, *71*(02), 298-306.

30. De heredia FP, Gómez-martínez S, Marcos A. Obesity, inflammation and the immune system. Proc Nutr Soc. 2012;71(2):332-8.

31. Kanneganti, T. D., & Dixit, V. D. (2012). Immunological complications of obesity. *Nature immunology*, *13*(8), 707-712.

32. Muñoz M, Mazure RA, Culebras JM. [Obesity and the immune system]. Nutr Hosp. 2004;19(6):319-24.

33. Mikhail N. Matrosovich,, Tatyana Y. Matrosovich, Thomas Gray, Human and avian influenza viruses target different cell types in cultures of human airway epithelium PNAS 2004 101 (13) 4620-4624; published ahead of print March 15, 2004,doi:10.1073/pnas.0308001101

34. *One Pound of Fat Versus One Pound of Muscle: Clearing up the Misconceptions @ Bamboo Core Fitness*. (n.d.). Retrieved April 2, 2014, from http://bamboocorefitness.com/one-pound-of-fat-versus-one-pound-of-muscle-clearing-up-the-misconception/

35. Perelman School of Medicine at the University of Pennsylvania. (2013, August 22). BMI not accurate enough: Obesity/mortality paradox demonstrates urgent need for more refined metabolic measures. ScienceDaily. Retrieved April 12, 2014 fromwww.sciencedaily.com/releases/2013/08/130822141948.htm

36. LaMere M., Moquin A, Lee E., et al. *Regulation of Antinucleoprotein IgG by systemic vaccination and its effect on Influenza Virus Clearance.* Journal of Virology May 2011 p. 5027-5035. Accessed April 12, 2014.