# Vitamin D and Respiratory Tract Infections (RTIs): The Impact of Vitamin D on the Risk and Severity of Upper RTIs and the Role of Vitamin D in Influenza Vaccine Immunogenicity in Children

By

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## ABSTRACT

Recent evidence suggests that vitamin D may be important for immune function. Canadian studies have reported varying prevalences of low levels of vitamin D. Whether these low vitamin D levels are associated with susceptibility to respiratory tract infections (RTIs) and infection severity remains unclear given the inconsistent association in recent studies. Influenza virus as a cause of RTI is of particular interest given its prevalence, morbidity and economic burden. Vaccination is a key strategy in prevention, but little is known about the effect of vitamin D on influenza vaccine response.

A prospective cohort study of children 3 to 15 years old living in Hutterite communities in Alberta, Saskatchewan and Manitoba was conducted to assess the prevalence and predictors of low vitamin D levels and evaluate the association between vitamin D and the incidence and severity of laboratory proven respiratory tract infections. In those who received influenza vaccination, the relationship between vitamin D and influenza vaccine immunogenicity was examined.

A total of 743 children were included in the study. The median serum 25-hydroxyvitamin D level (25[OH]D) was 62.0 nmol/L (interquartile range 51.0, 74.0). Levels lower than 50 nmol/L were present in 152 children (20.5%) and lower than 75 nmol/L in 565 children (76%). Lower serum 25(OH)D levels were associated with increased risk of RTI. No association was found between serum

25(OH)D level and disease severity. There was also no relationship found between serum 25(OH)D level and seroprotection or seroconversion from inactivated influenza vaccine.

In conclusion, low serum 25(OH)D levels are a significant problem in Canadian Hutterite communities. Furthermore, low serum 25(OH)D levels were associated with increased risk of proven upper RTIs. Studies evaluating the role of vitamin D supplementation to reduce the burden of disease are warranted, and strategies to improve vitamin D status in rural communities in Canada are needed.

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# PART I: INTRODUCTION

## Vitamin D Background

Vitamin D is a fat-soluble vitamin obtained from either diet (food or supplements) or synthesized by skin, with skin as the predominant source.[1] Ultraviolet B light is absorbed by the skin and 7-dehydrocholesterol is converted to previtamin D3, which is then converted to vitamin D3. Vitamin D3 is metabolized in the liver to 25-hydroxyvitamin D (25[OH]D) which is an inactive compound, but is considered the best marker for individual vitamin D status.[1] 1,25-dihydroxyvitamin D is the active metabolite, produced by 1-α-hydroxylase in the kidneys.

Recently, the optimal serum level of 25(OH)D has become an area of debate. Current recommendations from the American Academy of Pediatrics (AAP) suggest that for children, serum concentrations of 25(OH)D should be at least 50 nmol/L,[2] a level at which the risk of rickets is considered to be low.[3] The Canadian Paediatric Society (CPS) has chosen a higher cut-off of 75 nmol/L,[4] based on the levels at which parathyroid hormone (PTH) production and calcium resorption from the bone are minimized and intestinal calcium absorption is stabilized. Unfortunately, little is known about the optimal serum 25(OH)D levels for extra-skeletal health and there are no prospective trials to define the optimal

25(OH)D level for functions outside of skeletal development, specifically immune function.

Despite available recommendations surrounding serum levels of 25(OH)D and supplementation, evidence suggests that a significant proportion of children living in Canada have low serum 25(OH)D levels, with more than 50% of children having levels lower than the CPS recommendation in most studies (Table 1).[5-10] However, these studies have been conducted primarily in urban settings and have limitations including small samples sizes[5,6,8] and hospital-based identification of patients.[9]

## Vitamin D and the Immune System

The essential role of vitamin D in bone health and calcium homeostasis is well documented. However, vitamin D is now known to be involved in many cellular processes and receptors are found in most cells in the body, suggesting that its importance extends beyond traditionally understood roles.[11] As such, the benefits of vitamin D to other organ systems has become an important area of study with recent advances describing its association with cardiovascular health,[12] autoimmune disease[13] and cancer.[14]

There is also an increasing body of evidence that vitamin D is an important immune system regulator. Vitamin D receptors are found on immune

cells including antigen-presenting cells (e.g. macrophages), T-cells and B-cells. Therefore, these cells have the ability to synthesize and respond to the active form of vitamin D (1,25-dihydroxyvitamin D). They also have a different regulatory system in that the enzyme that produces the active form of vitamin D in macrophages (extra-renal 1$\alpha$-hydroxylase), is not regulated by PTH.[15] Instead, the enzyme activity appears to be regulated by local 25-hydroxyvitamin D levels or induced by cytokines (INF $\gamma$, IL-1, TNF $\alpha$). This suggests that the local levels of the active form of vitamin D (1,25-dihydroxyvitamin D) may differ from systemic levels.

The potential mechanisms of immune system modulation by vitamin D have been recently reviewed.[16] There is evidence that vitamin D can modulate both the innate and adaptive immune responses by mechanisms that will be briefly outlined below.

*Innate Immune Response*

The innate immune system recognizes pathogens through pathogen recognition receptors (PRRs), receptors that interact with components specific to a microbe (pathogen associated molecular patterns or PAMPs).[17] Pathogen recognition receptors can be divided into two groups, secreted or circulating proteins / peptides (e.g. antimicrobial peptides, lectins) and membrane bound receptors (e.g. Toll-like receptors, Nod-like receptors).[18] Vitamin D has been

shown to influence both. With respect to antimicrobial peptides, 1-25-dihydroxyvitamin D has been shown to regulate gene expression for both human beta defensin 2 (or skin antimicrobial peptide 1)[19] and cathelicidin (or LL-37).[20] Similarly, several toll-like receptors have been linked Vitamin D receptor stimulation. For example, CD-14, a co-receptor for TLR4 can be induced by 1,25-dihydroxyvitamin D.[21] In addition to PRRs, vitamin D also influences the cells of the innate immune system directly, as evidenced by documented decreased phagocyte function in children with rickets and reversal of this dysfunction when treated with vitamin D. [22,23]

*Adaptive Immune Response*

Vitamin D has been shown to inhibit dentritic cell maturation and T cell proliferation.[24] In studies, 1,25-dihydroxyvitamin D suppresses Th-1 proliferation and results in increased expression of Th-2 associated cytokines, thereby shifting the adaptive immune system towards Th-2 response.[25-27] Therefore, vitamin D may have the capacity to suppress Th-1 driven autoimmune diseases and deficiency could theoretically increase the risk.[28] Furthermore, 1,25-dihydroxyvitamin D inhibits proliferation and induction of apoptosis of activated B cells, which may contribute to the pathogenesis of various B-cell autoimmune disorders.[25] Observational studies have shown associations between deficiency and Type I Diabetes,[29] Crohn's disease[30], SLE[13] and Multiple Sclerosis.[31]

## Vitamin D and Viral Respiratory Tract Infections

Viral upper respiratory tract infections (RTIs) are very common worldwide. Despite their usually benign nature, the burden of disease is significant in terms of morbidity and economic loss.[32] Viral respiratory infections may also be associated with mortality in certain patient populations.[33,34] Unfortunately, treatment remains unsatisfactory and is often focused on symptom relief. As a result, prevention remains a key strategy in reducing the burden of these infections.

With the recent links between vitamin D and immune function, there has been increasing interest in the role of vitamin D in respiratory infections. It has been postulated that lower vitamin D levels may explain the seasonal variation in influenza and other viral infections.[35] In vitro studies have provided further evidence to support the potential role of vitamin D. Hansdoittir *et al.* showed that primary lung epithelial cells express high baseline levels of 1α-hydroxylase and low levels of 24-hydroxylase resulting in active vitamin D production in the lungs.[36] This active vitamin D leads to increased expression of vitamin D regulated genes including CD-14 (co-receptor for TLRs) and cathelicidin, important components of the innate immune system. In addition, studies have shown that in RSV-infected human airway epithelial cells, vitamin D induces Ikβα, an NF- kβ inhibitor, in airway epithelium and decreases RSV induction of inflammatory genes.[37]

Another proposed mechanism relates to the potential antiviral effects of antimicrobial peptides induced by vitamin D. Cathelicidin (LL-37) has been shown to have anti-viral activity against herpes simplex virus-1 (HSV-1),[38] adenovirus,[39] HIV[40] and human papilloma virus.[41] Finally, polymorphisms in the vitamin D receptor have been linked to susceptibility and severity of several infections including *Mycobacterium tuberculosis*,[42] *Mycobacterium leprae*[43] and acute lower respiratory tract infection.[44]

Several epidemiologic studies have shown an association between rickets and lower respiratory tract disease or pneumonia.[45-47] More recently, case-control studies have shown an association between lower serum 25-hydroxyvitamin D levels and lower respiratory tract infection in children in India,[48] Bangladesh[49] and Turkey.[50] This association has not been found in studies in Canadian children less than 5 years of age.[51,52] The effect of serum 25-hydroxyvitamin D on upper respiratory tract infections in children has not been studied. However, studies in adults have shown that lower vitamin D levels are associated with increased respiratory infections (self reported)[53-55] and absence from work due to respiratory symptoms.[53,56] The observational studies are summarized in Table 2.[48-61]

Randomized controlled trials (RCTs) examining effects of vitamin D supplementation have delivered mixed results (Table 3). Adult RCTs have failed to show a reduction in self-reported respiratory tract infections with vitamin D

supplementation using doses ranging from 400 IU per day to 200,000 IU per month.[57-59,61,62] Two of these studies also failed to demonstrate a reduction in severity of illness.[57,61]

Pediatric RCTs have had more promising results. In a trial in Japan, school children randomized to 1200 IU of vitamin D compared to placebo had a significantly decreased risk of developing laboratory-proven Influenza A (RR 0.58, 95% CI 0.34, 0.99).[63] Similarly, a decrease in parent-reported respiratory tract infections was noted in children in Mongolia given vitamin D-fortified milk (300 IU of vitamin $D_3$).[60] In this study, children had very low serum 25(OH)D levels initially. Unfortunately, no trials in children have looked at illness severity.

## Limitations of Previous Research

These studies have several key limitations. In the observational studies dealing with upper respiratory tract infection as the outcome, the focus was on specific patient populations (e.g. military base recruits, medical employees) and lacked pediatric data. In addition, the outcome in these trials was self-reported infection, which was often retrospectively obtained. The observational trials in children have used lower respiratory tract infection as the outcome and have been case-control designs identifying cases and controls in the hospital setting. They have been small studies with a low number of cases.

None of the observational studies have had confirmed diagnoses with which to examine susceptibility to specific viruses. In addition, serum 25(OH)D level cut-offs used have varied.

In the randomized controlled trials of vitamin D supplementation, studies have often failed to evaluate serum 25(OH)D levels prior to supplementation and have not focused on laboratory proven infection as the outcome. Therefore, in the adult trials showing no benefit from vitamin D supplementation, it is not known if the lack of benefit could be due to adequate serum 25(OH)D levels at the start of the study.

## Vitamin D and Influenza Vaccine Immunogenicity

Influenza is the cause of annual seasonal epidemics estimated to affect 5% to 10% of the population, posing an important public health burden.[64] Vaccination with an influenza vaccine can reduce morbidity and mortality associated with influenza. Therefore, vaccination is a key strategy to prevent illness and transmission.

Protection obtained from influenza vaccination can vary widely depending on the vaccine match with circulating strains of influenza and the individual immune response.[65] The response is often reduced in immunocompromised

patients, elderly patients, patients with chronic conditions and other individuals because of immunological changes of which many have yet to be fully elucidated.[66] Therefore, strategies to boost the effectiveness and duration of immunity after vaccination are of specific interest. Although strategies such an increase in vaccine dose, intradermal injection, and use of adjuvants have been shown to increase immunogenicity,[67-69] little known about the effect of vitamins on influenza vaccine antibody response.

Unfortunately, the link between vitamin D levels and vaccine responsiveness remains theoretical and has received little focus in existing studies. Evidence to date includes mouse models which demonstrate that mucosal and systemic antibody responses to influenza are enhanced when the vaccine is co-administered with calcitriol (1,25-dihydroxyvitamin D).[70,71] However, a study in healthy adults failed to demonstrate a benefit from calcitriol intramuscular co-administration with the vaccine.[72] Furthermore, a post-hoc analysis of a prospective influenza vaccine trial in HIV positive individuals did not find a difference in vaccine responsiveness between those receiving routine vitamin D supplementation and those not on supplementation.[73] These studies were limited by their lack of baseline serum 25(OH)D levels, making it uncertain as to whether individuals had low serum 25(OH)D levels at baseline prior to supplementation. More recently, a small study of 35 patients with prostate cancer found an association between baseline vitamin D level and influenza vaccine response.[74]

The pediatric age group is an important potential target group to optimize vaccine response given their potential role in transmission to high-risk groups.[75] As described previously, this is also a group where vitamin D deficiency can be common, especially in North America.[7,8,76]

# PART II: AIMS AND IMPLICATIONS

## Aims of the Project

This project had several objectives. The first aim was to describe the vitamin D status in Hutterite children in Canada and to identify potential determinants of vitamin D status. In addition, we wanted to determine whether variation in vitamin D levels was associated with individual, household or community characteristics, in order to evaluate whether environmental factors (community, household) or individual factors (e.g. genetics) contributed more to vitamin D status.

The second aim was to determine if serum 25(OH)D levels, used as a marker for individual vitamin D status, were associated with subsequent risk of laboratory-proven viral upper respiratory tract infections in children. Furthermore, in children who developed a proven respiratory tract infection, the objective was to assess whether there was an association between serum 25(OH)D levels and severity of respiratory tract infection, as defined by duration of symptoms and development of complications.

Finally, for those children receiving influenza vaccine, the objective was to determine if there was an association between serum 25(OH)D level and inactivated trivalent influenza vaccine immunogenicity in children.

## Research Questions

1) In children, what variables are associated with serum 25(OH)D level and is the variation explained primarily at the individual, family or community level?
2) In children, is there an association between serum 25(OH)D level and risk of proven respiratory tract infection?
3) In children who develop a proven respiratory tract infection, is there an association between serum 25(OH)D level and disease severity?
4) In children given trivalent inactivated influenza vaccine, is there an association between serum 25(OH)D level and vaccine immunogenicity?

## Implications

There are several important implications of addressing the above research questions. With respect to Vitamin D levels, several studies in Canada have documented low serum 25(OH)D levels and predictors in a variety of urban settings (Table 1). However, factors associated with serum 25(OH)D levels have not been evaluated in rural settings where the predictors may be different.

Therefore, this project may allow for identification of new high-risk groups to target with supplementation. In addition, by assessing the proportion of variation in serum 25(OH)D level explained at the individual, family and community level, it may give further insight into the potential sources of variability in serum 25(OH)D levels.

With respect to infection risk, the need for a safe and effective agent to prevent respiratory tract infections cannot be underemphasized given their associated morbidity and economic cost. Vitamin D has been one proposed agent, but observational studies to date have been conflicting. This is project will provide new information as to whether serum 25(OH)D level is associated with proven viral RTIs, an outcome that has not been assessed in previous studies. As such, it may provide insight into the role of vitamin D in infection and will provide essential information for the development of RTI preventative strategies. Similarly, whether there is an association between serum 25(OH)D level and disease severity may provide information for medical and public health practice.

Finally, strategies to enhance immune response to influenza vaccine are an important area of study, but there is limited information on the effect of vitamins on influenza vaccine immunogenicity. This study will address this research gap and assess the association between serum 25(OH)D level and vaccine immunogenicity in children, a potentially important group to target.

# PART III: METHODS

## The Data set

The dataset available consists of data from a Canadian Institutes of Health Research (CIHR) and the National Institute for Allergy and Infectious Diseases funded cluster randomized controlled trial in Hutterite communities in Alberta, Saskatchewan and Manitoba. The goal of this trial was to assess the effect of influenza vaccination of children on infection rates in the communities. Details and results of this RCT have been previously published.[75]

## Population and Study Details

Children aged 3 to 15 years from 46 Hutterite communities (colonies) were included in the trial. Hutterite communities are rural, self-governing and self-sufficient communal groups of Anabaptists. Children were randomly assigned by colony to receive either inactivated seasonal influenza vaccine (A/Brisbane/59/2007[H1N1]-like virus, A/Brisbane/10/2007 [H3N2]-like virus, B/Florida/4/2006-like virus; Vaxigrip) or hepatitis A vaccine (Avaxim-Pediatric, Sanofi Pasteur).

Participants were followed for signs and symptoms of respiratory tract infection over the influenza season, defined by the start date (1 laboratory-

confirmed influenza case in 2 consecutive weeks) and stop date (no laboratory-confirmed influenza cases for 2 consecutive weeks). This period was from December 28, 2008 to June 23, 2009.

Follow-up involved twice weekly assessments by research nurses using a standardized checklist of self-reported (or parent reported) signs and symptoms including fever ($\geq 38^{o}C$), cough, runny nose, sore throat, headache, muscle aches, chills, fatigue, ear ache or infection and sinus congestion. One household representative completed the forms for all family members and reports were verified at site visits conducted by the research nurses, including the onset and resolution dates of each symptom. Research nurses also followed up on missing and incomplete data. A nasopharyngeal swab was taken if a study participant developed two or more of the previously mentioned symptoms. Specimens were tested using the Center for Disease Control and Prevention Human Influenza Virus Real-Time PCR Detection and Characterization Panel[77] and confirmed with reverse transcriptase polymerase chain reaction (RT-PCR).

In addition to symptom data, information was collected on age, sex and presence of underlying conditions, including asthma. Data was also collected on complications including hospitalization, ICU admission, physician proven pneumonia and otitis media.

Blood specimens for serum 25(OH)D levels were collected at baseline. Serum from venous blood was frozen at -80$^{o}$C until batched analysis was performed according to the manufacturer's instructions using the DiaSorin LIAISON® chemiluminescence assay. Blood specimens for influenza antibody titers were collected at baseline and 3-5 weeks after vaccination. The paired samples were then tested using hemagglutination inhibition (HAI) using turkey erythrocytes and reference antigens for A/Brisbane/59/2007[H1N1]-like virus, A/Brisbane/10/2007 [H3N2]-like virus and B/Brisbane/60/2008-like virus.[78]

## Study Design and Statistical Analysis

The following sections outline the study design and statistical analysis used for each question.

### i. Prevalence and Predictors of Low Serum 25(OH)D Levels

To address this question, a cross-sectional analysis was done. Covariables of interest were age, sex, presence of underlying conditions, season and colony latitude. Age was analyzed as a both a continuous variable and categorized into less than 5 years, 5 to 9 years and 10 to 15 years. The effect of seasonality was based on the date of the vitamin D level sample and categorized into Winter (November to March) and Other (April to October) to be consistent with previous

studies in Canada[7] and based on the National Health and Nutrition Examination Survey (NHANES).[79]

*Outcome:*

The primary outcome was prevalence of vitamin D insufficiency defined by both AAP (< 50 nmol/L) and CPS (< 75 nmol/L) guidelines. Vitamin D concentration was measured using the serum 25(OH)D level.

*Statistical Analysis:*

Descriptive statistics were used to examine the study sample characteristics. Continuous variables were reported using the mean and standard deviation for normally distributed variables and the median and interquartile range (IQR) for non-normally distributed data. Number and percentage were reported for dichotomous outcomes. Univariable analyses were conducted using the Spearman correlation coefficient for continuous variables and chi-square or Fisher's exact test for dichotomous variables. Vitamin D levels were analyzed as both a continuous variable and dichotomized based on the AAP (< 50 nmol/L) and CPS (< 75 nmol/L) recommendations. In the multivariable model, generalized estimating equations (GEE) were used to adjust for the clustering of data at the colony level. The model was created using a step-wise backwards elimination method retaining variables with p values < 0.10. It was decided *a priori* to adjust for age and sex, and so these factors were not subjected to

significance testing. The continuous dependent variable (serum 25(OH)D level) was log-transformed to correct for positive skewness. Model fit was tested by plotting the standardized residuals against the standardized predicted values to assess the homogeneity of variance.

The covariables from the GEE model were then used to develop a mixed effects model to analyze the variation at the colony, household and individual levels. All analyses were conducted using SPSS version 20 (SPSS Inc., Chicago, IL).

### ii. Serum 25(OH)D Level and Risk of Respiratory Tract infections

This was a prospective cohort study. Covariables of interest were age, sex, presence of underlying conditions, asthma, receipt of influenza vaccination and serum 25(OH)D levels.

*Outcome:*

The primary outcome was laboratory confirmed viral infection defined by a positive nasopharyngeal swab polymerase chain reaction (PCR) result. Copan flocked nasopharyngeal swabs (Copan Italia, Brescia, Italy) were collected in universal transport medium (UTM, Copan Italia). Specimens were first tested for

Influenza A (including pH1N1) and B using the Centers for Disease Control and Prevention Human Influenza Virus Real-time RT PCR Detection and Characterization Panel.[77] Negative specimens were then tested again for Influenza (A, B) in addition to Coronavirus (229E, NL63, OC43), Enterovirus (including Rhinovirus), Parainfluenza (1-4), RSV (A, B), and Human Metapneumovirus by Luminex multiplex PCR.

*Statistical Analysis:*

All covariables, including serum 25(OH)D level, were first evaluated using univariable logistic regression. Vitamin D levels were analyzed as both a continuous variable (log transformed to correct positive skew) and dichotomized based on the AAP (< 50 nmol/L) and CPS (< 75 nmol/L) recommendations. Age was also analyzed both as a continuous variable and categorized into three groups (< 5 years, 5 – 9 years, 10 – 15 years). Variables with a *p* value < 0.1 were considered for inclusion in the multivariable model and the final model was determined using a step-wise backwards elimination method. It was decided *a priori* to adjust the final model for age and sex and to include age and serum 25(OH)D as continuous variables. Generalized estimating equations (GEE) were used to account for clustering at the colony level.

Survival analysis was also done to assess the relationship between time to respiratory infection and explanatory variables. Univariable analyses were

conducted to obtain unadjusted hazard ratios and the significance determined using the log rank test. A Cox proportional hazards model was used to estimate adjusted hazard ratios and the model was adjusted for clustering at the colony level. The proportional hazards assumption was evaluated using Shoënfeld's global residual test, graphically using Shoënfeld residuals and log-log curves and examination of variables for time dependence. Overall model fit was assessed using Cox-Snell residuals and deviance residual plots.

A recurrent events analysis was conducted to examine the relationship between predictors and rate of occurrence of respiratory tract infection. A counting process model was used under the assumption that each event was identical. The model was adjusted for clustering at the colony level.

All estimates are presented with 95% confidence intervals. A $p$ value < 0.05 was considered significant. SPSS version 20 (SPSS Inc, Chicago, IL) was used for logistic regression and GEE and Stata Statistical Software release 12 (StataCorp. 2011. College Station, TX: StataCorp LP) for the survival analysis.

### iii. Serum 25(OH)D Levels and Respiratory Infection Severity

This was a prospective cohort study. Only children and adolescents with serum 25(OH)D levels and proven viral respiratory tract infection were included

in the analysis. Of the 743 children with serum 25(OH)D levels included in the trial, 229 had a proven viral respiratory tract infection.

*Outcome:*

The primary outcome of interest was time to symptom resolution (in days) with the start date defined by the presence of two or more of fever (≥ 38$^{o}$C), cough, runny nose, sore throat, headache, muscle aches, chills, or sinus congestion starting within one week of proven viral RTI and end date defined by 48 hours of one or less symptom. If participants had more than one respiratory infection, the number of days of symptoms associated with the first infection was used. Secondary outcomes were presence of fever and time to fever resolution, presence of fever and cough combined and time to resolution of these symptoms, school absenteeism and complications including hospitalization and physician proven pneumonia and otitis media.

*Statistical Analysis:*

To describe participant characteristics, continuous variables were reported with mean and standard deviation for normally distributed data and median and IQR for non-normally distributed data. Survival analysis was done to assess the relationship between time to symptom resolution and explanatory variables. Logistic regression was used to assess the relationship for

dichotomous outcomes (presence of fever, presence of fever and cough, school absenteeism, hospitalization, pneumonia, otitis media). All models were adjusted for clustering at the colony level.

All covariables, including serum 25(OH)D level, were first evaluated using the univariable Kaplan-Meier method (for time to symptom resolution) and univariable logistic regression (for dichotomous outcomes). Vitamin D levels were analyzed as both a continuous variable (log transformed to correct positive skew) and dichotomized based on the AAP (< 50 nmol/L) and CPS (< 75 nmol/L) recommendations. Age was also analyzed both as a continuous variable and categorized into three groups (< 5 years, 5 – 9 years, 10 – 15 years). Variables with a $p$ value < 0.1 were considered for inclusion in the multivariable model and the final model was determined using a step-wise backwards elimination method. It was decided *a priori* to adjust the final model for age and sex and to include age and serum 25(OH)D as continuous variables. For the survival analysis, a Cox proportional hazards model was used to estimate adjusted hazard ratios. The proportional hazards assumption was evaluated using the Schoënfeld global test. Overall model fit was assessed using Cox-Snell residuals for the Cox PH model and by plotting the deviance residuals against the predicted values for the logistic regression model.

All estimates are presented with 95% confidence intervals. A *p* value < 0.05 was considered significant. SPSS version 20 (SPSS Inc, Chicago, IL) was used to conduct the GEE logistic regression analyses and Stata Statistical Software release 12 (StataCorp 2011, College Station, TX: StataCorp LP) for the survival analysis.

### iv. Serum 25(OH)D Level and Influenza Vaccine Immunogenicity

This was a prospective cohort study. Only children and adolescents who received influenza vaccination and had vitamin D levels were included in the analysis. Of the 743 children and adolescents with serum 25(OH)D levels included in the trial, 391 (53%) were randomized to receive influenza vaccination.

*Vaccination Details*

The trivalent inactivated influenza vaccine (Vaxigrip; Sanofi Pasteur, Lyon France) contained purified surface antigen from the strains recommended for the 2008-2009 influenza season by the World Health Organization for the Northern Hemisphere: A/Brisbane/59/2007[H1N1]-like virus, A/Brisbane/10/2007 [H3N2]-like virus and B/Florida/4/2006-like virus. The 0.5 mL dose of vaccine included 15 μg of hemagluttinin antigen per recommended strain. Vaccine administration start dates ranged from October 30, 2008 for communities in Alberta to November 13, 2008 for communities in Manitoba. Participants

received 0.5 mL of the influenza vaccine intramuscularly. Children less than 9 years of age with no history of previous influenza vaccine received a second dose of vaccine 4 weeks after the first dose, in keeping with influenza vaccination recommendations.

*Outcomes:*

The primary outcome was immunogenicity, using criteria that included seroprotection (post-vaccination titer ≥ 40) and seroconversion (post-vaccination titer ≥ 40 for participants with pre-vaccine titer <10 or four-fold rise in post-vaccination titer for those with a pre-vaccine titer ≥ 10), as defined by the Food and Drug Association (FDA)[80] and the Committee for Proprietary Medicinal Products (CPMP).[81] Other outcomes measured included change in antibody titer (four-fold change and fold increase in geometric mean titers [GMT]) and antibody level post vaccination ($log_2$-transformed). Fold increase was calculated by the ratio of post-vaccination to pre-vaccination titer.

*Statistical Analysis:*

Baseline characteristics were described using mean and standard deviation for normally distributed data and median and IQR for non-normal distributions. Characteristics were compared between those who had serology and those missing serology using the independent t-test or Wilcoxon rank sum

test for continuous variables and chi-square or Fisher's exact test for dichotomous outcomes.

For each outcome, analyses were conducted on the antibody titers to each influenza A antigen individually (A/Brisbane/59/2007[H1N1]-like virus and A/Brisbane/10/2007[H3N2]-like virus). B/Brisbane/60/2008-like virus antibody titers were also analyzed despite the different vaccine antigen (B/Florida/4/2006-like virus) to assess for potential cross-protection between lineages.

Logistic regression was used to examine serum 25(OH)D level as a predictor of dichotomous outcomes (seroprotection, seroconversion and four-fold change in antibody titers). All relevant covariables (age, sex, presence of at least one comorbidity, serum 25(OH)D level) were first evaluated using univariable logistic regression. Vitamin D levels were analyzed as both a continuous variable (log transformed to correct positive skew) and dichotomized based on the AAP (< 50 nmol/L) and CPS (< 75 nmol/L) recommendations. Variables with a *p* value < 0.1 were considered for inclusion in the multivariable model and the final model was determined using a step-wise backwards elimination method. It was decided *a priori* to adjust the final model for age and sex and to include serum 25(OH)D as a continuous variable.

Linear regression was used to examine the relationship between covariables and post vaccination HAI titers. Titers were $\log_2$-transformed using $\text{titer}_{(\text{transformed})} = \log_2(\text{titer}/5)$ resulting in the following: 0 = no HAI activity, 1= 1:10, 2 = 1:20, 3 = 1:40 etc. Covariables were analyzed and included in the final model as outlined for the logistic regression model. Generalized estimating equations (GEE) were used to account for clustering at the colony level for both regression analyses.

Geometric mean titers (GMT) were calculated at baseline and post vaccination and compared between subjects grouped by serum 25(OH)D levels based on the AAP (< 50 nmol/L) and CPS (< 75 nmol/L) recommendations. Fold increase in GMT was calculated in each group (ratio of GMT pre and post vaccination) and the arithmetic mean of the log of the fold increase was compared using the independent t-test.

All estimates are presented with 95% confidence intervals. A $p$ value < 0.05 was considered significant. SPSS version 20 (SPSS Inc, Chicago, IL) was used to conduct the analyses.

# PART IV: RESULTS

## Participant Characteristics

A total of 1186 children age 3 to 15 years were included in the study. Vitamin D levels were available on 743 (63%) subjects from 43 colonies and 304 households. Almost all vitamin D levels were sampled from October to December (n=724, 97%). Subject characteristics are presented in Table 4. The mean age was 9.3 years (standard deviation (SD) 4.3 years); 52.5% were female. Underlying conditions were present in 2.4% (n=18); the most common condition was asthma (n=11). All participants were of the same ethnicity. The colony latitudes ranged from 49.2$^{o}$N to 54.8$^{o}$N with a median of 50.0$^{o}$N.

The median serum 25(OH)D level was 62.0 (IQR 51.0, 74.0). Four participants (0.5%) had serum 25(OH)D levels lower than 25 nmol/L. Serum 25(OH)D levels were lower than 50 nmol/L in 152 children (20.5%) and lower than 75 nmol/L in 565 children (76.0%).

### i. Predictors of Serum 25(OH)D Levels

In univariable analyses, age, sex and latitude were associated with serum 25(OH)D level. Age ($r=-0.22$ $p<0.001$) and latitude ($r=-0.26$, $p< 0.001$) were negatively correlated with serum 25(OH)D level (Table 5). Participants aged 10

to 15 years were at higher risk for serum 25(OH)D levels lower than 50 nmol/L (OR 2.11, 95% CI 1.07, 4.16, p=0.012) and lower than 75 nmol/L (OR 3.38, 95% CI 2.00, 5.80, p<0.001) compared to participants age 3 to 4. Male sex was associated with higher serum 25(OH)D levels. There was no interaction between age and sex.

In the multivariable analysis accounting for clustering of data, age and latitude remained important predictors of vitamin D level (Table 6). Sex was no longer an independent predictor of serum 25(OH)D level or levels lower than 50 nmol/L, but remained an important predictor for levels lower than 75 nmol/L with females at increased risk (aOR 1.69, 95% CI 1.07, 2.65, p=0.024). There was no interaction between age and sex.

The multi-level model showed that approximately 19% of the variation in log 25(OH)D levels was associated with households and 9% with colonies (Table 7). Most of the variation (72%) was associated with individuals, even after adjusting for age, sex and latitude.

*Assumption Testing and Model Fit*

Given the continuous dependent variable (serum 25(OH)D level) used for this analysis, a linear regression approach to modeling was chosen. Ordinary linear regression has several assumptions, one of which requires that

observations are conditionally independent. Given the clustering of data at the colony level, this assumption of independence was violated. Several approaches can be used to model correlated data including marginal models (e.g. GEE), conditional models (e.g. mixed effects models) and dummy coding. Marginal models do not model between cluster variation and therefore do not include a random effect that can be estimated. The generalized estimating equations (GEE) method, a form of marginal model, estimates within-cluster correlation in order to re-estimate regression parameters and calculate standard errors. In contrast to ordinary linear regression, it relaxes the assumptions of independence of observations and the linear relationship between variable and outcome.[82]

GEE was used for the multivariable model to evaluate whether there was an association between covariables and log serum 25(OH)D level. The *Quasi-likelihood under independence criteria* (QIC) was used to define the appropriate correlation matrix. The covariance matrix was specified using the robust estimator (Huber/White/Sandwich estimate). This estimator is a form of corrected model-based estimate that provides a consistent approximation of the covariance even when the working correlation matrix is not chosen correctly. Model fit was evaluated by plotting the Pearson residuals against the standardized predicted values to assess the homogeneity of variance (Figure 1). There appeared to be a random scatter around the horizontal line at residual = 0 with no suggestion of a systematic trend.

### ii. Serum 25(OH)D Levels and Risk of Respiratory Tract infections

Participants were followed between December 22, 2008 and June 23, 2009 for a median of 156 days (range 17 to 176 days). Ten participants (1%) withdrew from the study in the first month. A total of 229 (31%) participants developed at least one proven respiratory tract infection. The most common infection was influenza (n=101, 14%), followed by enterovirus/rhinovirus (n=81, 11%), RSV (n=35, 5%), parainfluenza (n=33, 4%), coronavirus (n=26, 3%), HMPV or MPV (n=7, 1%). Co-infection occurred in 6 participants. Repeated infections occurred in 46 participants; 44 participants (6%) had two infections and 2 participants (0.5%) had three infections.

In univariable analyses, age, sex, influenza vaccination (vs. hepatitis A) and serum 25(OH)D level were associated with respiratory tract infection (Table 8). In multivariable analysis accounting for clustering at the colony level, age and serum 25(OH)D level remained independently associated with RTI (Table 9). Children less than 5 years were at highest risk of RTI compared to those 5 to 9 years (aOR 2.25, 95% CI 1.34, 3.78, p=0.002) and 10 to 15 years (aOR 3.08, 95% CI 1.66, 5.73, p<0.001).

Serum 25(OH)D level was associated with protection against respiratory tract infection. For every 1-unit increase in log 25(OH)D level (corresponding to

a 2.72-fold increase in serum 25(OH)D level), the odds of respiratory tract infection decreased by 60% (aOR 0.42, 95% CI 0.24, 0.73, p=0.002). When levels were dichotomized based on AAP and CPS recommendations, levels lower than 50 nmol/L (vs. ≥ 50 nmol/L)(aOR 1.92, 95% CI 1.24, 2.98, p=0.004) and levels lower than 75 nmol/L (vs. ≥ 75 nmol/L)(aOR 1.61, 95% CI 1.10, 2.36, p=0.015) were both associated with increased risk. There were no interactions found between serum 25(OH)D level and either age or sex.

In time to event analysis, the rate of infection decreased in participants with higher serum 25(OH)D levels (aHR 0.52, 95% CI 0.35, 0.79, p=0.002). Participants with serum 25(OH)D levels lower than 50 nmol/L (vs. ≥ 50 nmol/L) had an increased hazard (aHR 1.67, 95% CI 1.16, 2.40, p=0.006), as did those with levels lower than 75 nmol/L (aHR 1.51, 95%CI 1.10, 2.07, p=0.011) compared to greater than or equal to 75 nmol/L (Figures 2 and 3).

Taking into account the multiple events for some participants, age and log serum 25(OH)D levels were associated with increased rate of occurrence of respiratory tract infection (Table 10). Younger age conferred an increased risk with a hazard of 1.08 for every year decrease in age (aHR 1.08, 95% CI 1.02, 1.14, p=0.010). Log serum 25(OH)D level was also an important predictor both as a continuous variable (aHR 0.57, 95% CI 0.39, 0.83, p=0.003) and when dichotomized based on levels lower than 50 nmol/L (aHR 1.74, 95% CI 1.21, 2.49, p=0.003) and lower than 75 nmol/L (aHR 1.54, 95% CI 1.13, 2.10, p=0.007).

On analysis of individual viruses, there was an association between serum 25(OH)D levels and parainfluenza virus infection with levels analyzed as both a continuous variable (per 1 unit change in log value, OR 0.19, 95% CI 0.08, 0.48, p<0.001) and dichotomized based on levels lower than 50 nmol/L (OR 3.55, 95% CI 2.21, 5.71, p<0.001) and levels lower than 75 nmol/L (OR 2.58, 95% CI 1.00, 6.66, p=0.050) (Table 11). There was also a trend toward an association between serum 25(OH)D levels lower than 50 nmol/L and RSV infection (OR 2.61, 95% CI 0.98, 6.92, p=0.054). No associations with other viruses were found adjusting for age and sex.

*Assumption Testing and Model Fit*

For the time to event analysis, the PH assumption was evaluated using three methods. First, overall goodness of fit was evaluated using the Schoënfeld's method. The p value for the global test was 0.4483 suggesting that the PH assumption was reasonable. Further testing was done plotting the scaled Schoënfeld residuals for the continuous variables (log 25(OH)D level and age) against time (Figures 4 and 5). The straight line indicates that the PH assumption is reasonable. Secondly, log-log curves were used to evaluate the dichotomous variables (sex, vitamin D cut-offs) adjusted for other variables (Figures 6, 7, 8). The log-log curves for sex appeared to cross, suggesting that the PH assumption may be violated. However, when the observed plot versus the expected plot was analyzed (Figure 9), the PH assumption seemed reasonable. Finally, the variables were assessed for time dependence using the "tvc" function and both

the Wald test and likelihood ratio statistic to compare the model with and without time dependent variables. None of the time dependent variables were significant and therefore the PH assumption appeared to be valid.

To assess model fit, Cox-Snell residuals were estimated and the Nelson-Aalen cumulative hazard estimator for CS residuals was plotted (Figure 10). The overall fit was reasonable with some expected variability around the 45-degree line toward the right hand tail. In addition, deviance residuals were checked and plotted against participant ID. This graph showed that residuals were distributed between -2 and 3 with no specific pattern to suggest deviation from PH assumption (Figure 11).

### iii. Serum 25(OH)D Level and Respiratory Infection Severity

The baseline characteristics of 229 participants who developed a respiratory tract infection are presented in Table 12. The mean age was 8.7 years (SD 3.5); 58.1% were female. Comorbidities were uncommon (n=6, 2.6%). The median serum 25(OH)D level was 61.0 nmol/L (IQR 49.0, 70.0). Serum 25(OH)D levels were less than the AAP recommendations (≤ 50 nmol/L) in 62 (27%) participants and less than the CPS recommendations (≤ 75 nmol/L) in 184 (80%) participants.

Outcomes are summarized in Table 13. The majority of children (89%) had more than one day of two or more symptoms; the mean duration was 2.9 days (SD 2.0). In univariable analyses, none of the covariables, including serum 25(OH)D level, were associated with the time to symptom resolution (Table 14).

Only 21% (n=46) of children had fever and the mean duration was 0.4 days (SD 0.9). None of the covariables were associated with increased odds of having fever. However, for every one-year increase in age, the time to symptom resolution decreased (HR 1.12, 95% CI 1.04, 1.21, p=0.003). Children less than 5 years of age had longer duration of fever than children over 10 years of age (HR 0.45, 95% CI 0.23, 0.87, p=0.018). Age remained a significant predictor in multivariable analysis (Table 15). There was no significant association between serum 25(OH)D level and time to fever resolution. Similarly, age was significantly associated with duration of fever and cough combined (HR 1.07, 95% CI 1.01, 1.13, p=0.015) and odds of developing fever and cough (OR 0.91, 95% CI 0.84, 0.98, p=0.014). There was a trend toward increased odds of fever and cough in those with serum 25(OH)D levels less than 50 nmol/L (OR 1.80, 95% CI 0.98, 3.34, p=0.063), however this did not reach statistical significance.

No participants were admitted to hospital for respiratory virus infection and very few had infectious complications including pneumonia (n=1, 0.1%) and otitis media (n=6, 0.8%). However, 85 participants (11%) missed school for an

average of 1 day (SD 0.23). As age increased, there was a trend towards increased odds of missing school (OR 1.07, 95% CI 0.99, 1.16, p=0.09) (Table 16). Children less than 5 years of age were significantly less likely to miss school (OR 0.16, 95% CI 0.03, 0.86, p=0.03) compared to children over 10 years of age, which may be related to age of school attendance. Serum 25(OH)D level was not associated with absenteeism from school due to respiratory tract infection.

*Assumption Testing and Model Fit*

The PH assumption for the time to symptom resolution analysis was evaluated using Schoënfeld's global test. The p value for the global test was 0.7073 suggesting that the PH assumption was reasonable. The overall model fit was reasonable as determined by estimating the Cox-Snell residuals and plotting the Nelson-Aalen cumulative hazard estimator for CS residuals (Figure 12).

In order to assess the power of this analysis to detect an association, the hazard ratio estimates for serum 25(OH)D level (as a continuous variable and dichotomized) and their respective confidence intervals were examined. In addition, the minimal hazard ratios that could be detected with various study powers were calculated based on the available sample size (Table 17). A total of 202 participants from 31 colonies (average size =17) were included in the analysis. After adjusting for clustering using an inflation factor, the effective sample size was calculated to be 78. The minimal value of hazard ratio that could be detected with 80% power and this sample size was 2.78 (or 0.36). Therefore,

given the smaller hazard ratio estimate for Log serum 25(OH)D level from our analysis (HR 0.87, 95% CI 0.57, 1.33, p=0.532), it cannot be determined whether the lack of significance was related to insufficient power or truly a lack of association.

Model fit for the GEE logistic regression analysis was assessed by plotting the Pearson residuals against the predicted values (Figure 13).

### iv. Serum 25(OH)D Level and Influenza Vaccine Immunogenicity

Baseline characteristics of the 391 vaccinated children are presented in Table 18. Serum 25(OH)D levels were taken between October 16, 2008 and January 29, 2009. Most sera was drawn between October and December 2008 (97%); no samples were taken after the post-vaccination titers. Of the 391 children/adolescents, 221 (57%) had post-vaccination serology and could be included in the immunogenicity analyses. There were no significant differences between groups based on availability of serology aside from sex (Table 18). Post-vaccination titers were drawn a median of 11 weeks after the initial titer (IQR 6.9, 21.3).

Total event rates for the outcomes are summarized in table 19. Post vaccination titers were greater than or equal to 40 (seroprotection) for A/Brisbane/10/2007[H3N2] in 159 (71.9%) participants,

A/Brisbane/59/2007[H1N1] in 138 (62.4%) and both antigens in 127 (57.5%) participants. Seroprotection against B/Brisbane/60/2008 occurred in 79 (35.7%) participants. In univariable analysis, there was no association between age, sex, presence of comorbidities or serum 25(OH)D level and seroprotection against any strain (Table 20).

Seroconversion occurred in 94 (42.5%) children in response to A/Brisbane/10/2007[H3N2], 127 (57.5%) in response to A/Brisbane/59/2007[H1N1] and 66 (29.9%) to B/Brisbane/60/2008. In univariable and multivariable analyses, the presence of a comorbidity resulted in reduced odds of seroconversion (OR 0.17, 95% CI 0.03, 0.88, p=0.034) to A/Brisbane/59/2007[H1N1] but not to the other strains. None of the other covariables, including serum 25(OH)D level, were associated with seroconversion to any of the strains (Table 21). Similarly, there was no association between any of the covariables and presence of a four-fold change in titer or post-vaccination titer level ($log_2$transformed).

The fold increase in GMT was highest for A/Brisbane/59/2007[H1N1] (mean 5.25, 95% CI 3.39, 8.15) compared to A/Brisbane/10/2007[H3N2] (mean 1.39, 95% CI 0.37, 2,15). The fold increase in GMT for B/Brisbane/60/2008 was 2.21 (95%CI 1.75, 2.79). There was no significant difference in fold change in

GMT between groups based on vitamin D level cut-offs of 50 nmol/l and 75 nmol/L (Table 22).

*Assumption Testing and Model Fit*

Model fit for each outcome was assessed by plotting the Pearson residuals against the predicted outcome (Figure 14).

*Sensitivity Analyses*

Given the range in timing of post-vaccination titers (median 11 wks) and to account for possible waning immunity with time, a sensitivity analysis was conducted excluding subjects that had post-vaccination titers more than 3 months after vaccination; this group also excluded the subjects that had late serum 25(OH)D levels. In this analysis, there was no change in the significance of covariables for any of the outcomes. Additionally, the analyses were conducted including the time from last vaccine dose as a variable in the analysis. This did not change the results appreciably.

In a second sensitivity analysis, participants with proven influenza infection were excluded given the probability that the antibody change was related to natural infection not vaccine immunogenicity. There was no significant change in the results.

Finally, participants who may not have had enough time to respond to the vaccine or were not appropriately vaccinated (i.e. only 1 vaccine dose in participants less than 9 years of age) were excluded. There was no significant change in the results.

# PART V: DISCUSSION

## Summary of findings

We found that in children and adolescents living in Hutterite communities in Canada, low serum 25(OH)D levels were common, with levels < 50 nmol/L in 21% of participants and levels < 75 nmol/L in 76% of participants. The majority of serum 25(OH) level measurements were taken in October/November, just after the time when serum 25(OH) levels peak in the Northern Hemisphere.[83] Therefore, it is likely that even more children have levels below the recommendations later in the winter season.

We also found a significant association between vitamin D status and proven viral respiratory tract infections (RTIs) in Hutterite communities in Canada. Lower serum 25(OH)D levels were associated with increased risk of RTI after adjusting for age and sex. Younger age was also associated with increased risk. Serum 25(OH)D level was not significantly associated with the severity of proven upper respiratory tract infections, as measured by the time to resolution of two or more symptoms, time to fever resolution, time to resolution of fever and cough combined and absenteeism from school. No participants were hospitalized and very few developed pneumonia and otitis media. Therefore, it was not possible to assess the impact of serum 25(OH)D level on these outcomes.

Finally, serum 25(OD) level was not significantly associated with immunogenicity as measured by seroprotection (post-vaccination titer ≥ 40) and seroconversion. Serum 25(OH)D level was also not associated with other commonly reported measures of vaccine immunogenicity, including presence of a four-fold rise in antibody titer, the fold change in GMT or post vaccination titer.

## Prevalence and Predictors of Low Serum 25(OH)D levels

Our findings of low vitamin D levels are consistent with those reported for children and adolescents in St. John's Newfoundland and Labrador.[6] Although at a lower latitude (47°N), St. John's receives the most rain of all major cities in Canada, which may account for its lower vitamin D levels.[84] Other studies of the prevalence of insufficiency in children/adolescents in Canada have shown varying results. Studies from Calgary, Alberta[9] and the Canadian Health Measures Survey (CHMS)[7] both found that the prevalence of serum 25(OH)D levels below 75 nmol/L was lower (39% and 58% respectively) than in our study. This difference is not likely related to latitude, as Calgary is at 51°N and the CHMS surveyed 15 sites across Canada of varying latitudes. However, the relatively small variation in latitude in Canada may limit the potential for latitude effects to be detected. Possible explanations for the discrepant results include differences in socioeconomic status[85] and lower dietary vitamin D (secondary to availability of fortified food),[5] both shown to be risk factors for lower levels of serum 25(OH)D.

Older children were found to have lower serum 25(OH)D levels, a finding consistent with results from two studies in Calgary.[5,9] We found children aged 10-15 years to be at the highest risk for insufficiency. Age may be a surrogate for weight, with sequestration of vitamin D in body fat leading to lower serum 25(OH)D levels.[86] Although we were not able to adjust for BMI in the analysis, it is unlikely that this would have contributed in this population, given its low prevalence of obesity[87] and given that other evidence suggests that body fat percentage does not increase systematically in adolescents.[88]

Girls had lower serum 25(OH)D levels compared to boys in univariable analysis, but this effect was not noted after adjustment for age and serum 25(OH)D level. The risk associated with sex in other studies has varied. Rates of vitamin D deficiency have been shown to be high in studies conducted exclusively in female adolescents.[89,90] However, in a study in Edmonton, Alberta,[5] males were found to be at higher risk. This was a smaller study with a different population (children presenting to an urban emergency center), which may account for its discrepant results.

Finally, our study is unique in that we were able to examine the effects of the community, household and individual on variation in 25(OH)D levels. The determinants of vitamin D status are likely multifactorial, including

environmental (e.g. exposure to sunlight) and individual (e.g. age, sex, genetics) factors. Recent evidence suggests that genetics contributes to the variability in serum 25(OH)D levels,[91] although less than expected based on twin studies.[92] We found that most of the variance in serum 25(OH)D levels was explained at the individual level suggesting that other individual level predictors may be important. Given the relatively homogeneous distributions of ethnicity, diet and skin colour in this population, it is possible that genetic variants, such as vitamin D receptor polymorphisms, account for some of the differences.

## Serum 25(OH)D Levels and Risk of Respiratory Tract Infection

This analysis provides new important information on the effect of vitamin D status on proven viral upper respiratory tract infections in children and adolescents. Several observational studies have shown an association between low serum 25-hydroxyvitamin D levels and risk of lower respiratory tract infection in children in developing countries.[48-50] Our study extends these results, suggesting that vitamin D status may also be important for susceptibility to viral upper respiratory tract infections in children and adolescents, a finding consistent with the adult literature.[53-56] These findings have important public health implications given the frequency of viral upper RTIs and their associated morbidity and the prevalence of low vitamin D levels.

We found that serum 25(OH)D level was an important predictor of respiratory tract infection both as a continuous variable (per 1 unit change in log 25(OH)D), and also when dichotomized into levels lower than 50 nmol/L (AAP recommendations) and levels lower than 75 nmol/L (CPS recommendations). Therefore, although maintaining levels greater than 50 nmol/L is important to prevent rickets,[3] higher levels may be needed for optimal immune function. This was suggested in a recent study involving healthy adults, where a serum 25(OH)D concentration of greater than 38 ng/ml (~95 nmol/L) was associated with a reduced incidence of acute viral respiratory tract infection.[53] Further studies in children are needed to determine the optimal level required for immune function.

Our study is unique in that we were able to explore for associations between serum 25(OH)D levels and specific viruses. We found a trend toward an increased risk of RSV infection in children with levels lower than 50 nmol/L. This is consistent with *in vitro* findings that vitamin D induces Ikβα (an NF- kβ inhibitor) in RSV-infected human airway epithelial cells and decreases RSV induction of inflammatory genes.[37] We also found an association between vitamin D and parainfluenza virus, which is in the same *Paramyxovirus* family. Although interesting, these subgroup findings should be interpreted with caution given the small sample sizes and multiple comparisons not specified *a priori*. These results should be considered exploratory and should be interpreted with caution.

Other Canadian studies have failed to show an association between vitamin D status and risk for hospitalization for acute lower respiratory infection (ALRI) in children less than 5 years old.[51,52] Although differences in vitamin D levels were not found in these studies, a higher proportion of participants with ALRI were found to have Vitamin D receptor polymorphisms associated with reduced receptor expression in one study.[44] We did find an association between serum 25(OH)D level and upper RTI in children of this age, which may be related to differences in population (urban vs. rural, genetic differences like vitamin D receptor polymorphisms), study design, serum 25(OH)D measurements or the measured outcome (upper vs. lower RTI).

## Serum 25(OH)D Levels and Severity of Infection

Our findings are consistent with adult trials assessing vitamin D supplementation and respiratory virus severity. Murdoch et al.[61] found no difference in the sum of the Wisconsin Upper Respiratory Symptom Survey [WURSS-24])[93] scores during the first seven days of symptoms in the placebo group compared to those receiving oral vitamin $D_3$ 200,000 IU monthly for 2 months then 100,000 IU per month for 18 months. Li-Ng et al.[57] also found no decrease in the severity of upper respiratory tract infections in participants on vitamin D supplements, measured using a combination of a 5-point severity scale and duration of symptoms. We also did not find evidence for an association between duration of symptoms and serum 25(OH)D levels in children and

adolescents with upper respiratory tract infections. However, the lack of association may be related to sample size and inadequate power. This is further reflected in the large confidence intervals around the estimates.

Furthermore, we were only able to examine duration of symptoms as a measure of severity. We did not have self-reported scores on which to measure participant perceived disease severity. In addition, we were unable to examine the impact if illness on daily life, aside from measuring absenteeism from school. Therefore, serum 25(OH)D levels may be associated with other severity outcomes and this should be the focus of future studies.

### Serum 25(OH)D Levels and Influenza Vaccine Immunogenicity

Our finding of a lack of association between serum 25(OH)D levels and influenza vaccine immunogenicity is consistent with two randomized controlled trials of vitamin D supplementation in influenza vaccinated subjects, one in healthy adults[72] and the other in HIV-infected adults.[73] Both studies found no effect of supplementation on serologic responses. We also did not find an association between serum 25(OH)D levels and any of the commonly used immunogenicity criteria. However, this failure to detect an association may be related to sample size and power.

Our findings are different from a recent study of influenza vaccination in prostate cancer patients (n=35) which found that a replete vitamin D status, defined as the upper quartile of serum 25(OH)D levels, was associated with more frequent serological response (titer ≥ 1:40 at 3 months against any of the 3 strains).[74] They also reported an association between serum 25(OH)D levels and serologic response (p=0.0446), but the magnitude of effect and confidence intervals were not provided. This trial was limited by the definition of serologic response (response to any of the three antigens) and the high serum 25(OH)D levels in the population (median 44.8 ng/mL ≈ 112 nmol/L). Another possible explanation for the divergent results is the different patient population. The effect of serum 25(OH)D level on vaccine immunogenicity may be different in the immunocompromised population, a group which tends to be more hyporesponsive to influenza vaccinations.[66]

Overall, seroprotection and seroconversion proportions for the Influenza A strains were in keeping with the recommended standards.[94] A better seroconversion percentage and fold change in GMT were noted for the A/Brisbane/59/2007[H1N1] strain compared to the A/Brisbane/10/2007[H3N2] strain and this was reflected in the protective efficacy with 8 confirmed cases of H3N2, but no confirmed cases of seasonal H1N1.

With respect to the Influenza B immunogenicity data, another important finding in our study was the rates of seroprotection and seroconversion to B/Brisbane/60/2008-like virus despite vaccination with B/Florida/4/2006-like virus. These strains are of different lineages, B/Victoria and B/Yamagata respectively, and are believed to result in very little cross-protection.[65] However, we observed seroprotection and seroconversion percentages of 35% and 30%, respectively. Only 2 subjects who demonstrated an antibody response had proven influenza B infection, therefore these antibody changes represent either vaccine response or sub-clinical infection. Although these percentages are lower than recommended for vaccine licensure (seroprotection >60%, seroconversion >30%)[94], it does suggest some potential cross-protection if the antibody responses are related to vaccination.

## Limitations

There are several limitations of this study and the analyses. The first is related to the generalizability of the results. As the study was conducted in children and adolescents from Hutterite communities, the results may not be translatable to other populations. However, there were advantages in that the homogeneity of the population allowed us to look at environmental and individual level factors contributing to variation in vitamin D levels. Other limitations associated with the dataset and analyses are outlined below.

***The Dataset***

The data for this study was collected prospectively during the 2008-2009 influenza season in Canada. Therefore, variables that were not collected during this time period could not be included in the analysis even if they were considered potentially important to adjust for. For the predictors of serum 25(OH)D level, data was not available on body mass index (BMI), exposure to sunlight or sources of vitamin D intake and as such, these variables could not be included in the analysis.

Another limitation was with respect to the serum 25(OH)D levels. There was only one serum 25(OH)D measurement for each participant and it was taken at baseline over a range of dates. Therefore, the serum 25(OH)D level may not reflect vitamin D status at the time of infection. However, the majority of serum 25(OH) measurements were taken around the same time in October/November and reflect the vitamin D status at the start of the respiratory infection season. We believe this estimate is more appropriate than estimates taken at the time of illness because the impact of illness on serum 25(OH)D level has not been studied. To deal with the variation in timing of serum 25(OH)D levels, sensitivity analysis was done excluding patients with levels done after the start of the follow-up period and there was no change in the significance of the predictors.

The variability in the timing of the post-vaccination titers was also an important consideration. This was addressed in two separate sensitivity analyses, first by excluding participants with levels drawn more than 3 months after baseline levels/vaccination (n=72, 32%) and secondly, by including the time from last vaccine dose as a variable in the analysis. No significant difference in the results was found in either analysis. Furthermore, antibody titers were only available on study participants who agreed to the follow-up bloodwork (n=221, 57%). This limited sample size impacts the conclusions that can be drawn from the lack of association. Similarly, this is a consideration for the RTI severity analysis where the sample size was limited (n=229) and no association was found.

***Statistical Analysis***

Ten participants withdrew from the study and as a result had incomplete follow-up information. Removal of the participants in sensitivity analyses did not materially change the results.

Finally, there were a large number of statistical analyses for the various questions addressed in this study. Therefore, some associations may have been found by chance alone. This is a concern primarily for the analysis of the association between serum 25(OH)D levels and risk of respiratory virus infection. However, we defined the primary analysis of interest (risk of proven

viral respiratory tract infection) *a priori*. All other analyses (e.g. risk of the individual respiratory viruses) should be considered exploratory and should be confirmed with future studies.

## PART IV: CONCLUSION

Vitamin D insufficiency at levels lower than 50 nmol/L and lower than 75 nmol/L is a significant health problem in rural Hutterite communities in Alberta, Saskatchewan and Manitoba, and these populations should be targeted for supplementation. Older age and living at a higher latitude were factors associated with lower serum 25(OH)D levels. The majority of variation in serum 25(OH)D levels was at the individual level. Additional studies are needed to explore the potential mechanisms leading to lower serum 25(OH)D levels, especially individual level factors (e.g. genetics).

We also found that children and adolescents with lower vitamin D levels were at increased risk for proven viral upper respiratory tract infection. Current recommendations regarding target serum 25(OH)D levels may be too low and further research to define the optimal serum 25(OH)D level for immune function is needed. This study provides evidence in support of future interventional trials examining the efficacy of vitamin D supplementation on viral respiratory tract infections in children and adolescents.

Serum 25(OH)D level was not associated with time to symptom resolution or absenteeism from school in children and adolescents with proven viral respiratory tract infections, however, sample size may have limited our ability to detect a significant difference. Further studies are needed to assess the

relationship between serum 25(OH)D level and other outcomes, including hospitalization and self-reported symptom severity based on validated scores in children.[95]

Finally, serum 25(OH)D level was not associated with influenza vaccine immunogenicity in children and adolescents. Given the important role of children and adolescents in the spread of viral infections and the potential benefits from their immunization to protect other higher risk groups, other strategies should continue be evaluated to enhance the vaccine immune response. The role of serum 25(OH)D level in influenza vaccine immunogenicity in other populations, specifically immunocompromised patients, may warrant further study.

## REFERENCES

**1.** Holick MF. Vitamin D deficiency. *N Engl J Med.* Jul 19 2007;357(3):266-281.

**2.** Wagner CL, Greer FR. Prevention of rickets and vitamin D deficiency in infants, children, and adolescents. *Pediatrics.* Nov 2008;122(5):1142-1152.

**3.** Greer FR. 25-Hydroxyvitamin D: functional outcomes in infants and young children. *Am J Clin Nutr.* Aug 2008;88(2):529S-533S.

**4.** Vitamin D supplementation: Recommendations for Canadian mothers and infants. *Paediatrics & child health.* Sep 2007;12(7):583-598.

**5.** Roth DE, Martz P, Yeo R, Prosser C, Bell M, Jones AB. Are national vitamin D guidelines sufficient to maintain adequate blood levels in children? *Canadian journal of public health. Revue canadienne de sante publique.* Nov-Dec 2005;96(6):443-449.

**6.** Newhook LA, Sloka S, Grant M, Randell E, Kovacs CS, Twells LK. Vitamin D insufficiency common in newborns, children and pregnant women living in Newfoundland and Labrador, Canada. *Maternal & child nutrition.* Apr 2009;5(2):186-191.

**7.** Langlois K, Greene-Finestone L, Little J, Hidiroglou N, Whiting S. Vitamin D status of Canadians as measured in the 2007 to 2009 Canadian Health Measures Survey. *Health reports / Statistics Canada, Canadian Centre for Health Information = Rapports sur la sante / Statistique Canada, Centre canadien d'information sur la sante.* Mar 2010;21(1):47-55.

**8.** Maguire JL, Birken CS, O'Connor DL, et al. Prevalence and predictors of low vitamin D concentrations in urban Canadian toddlers. *Paediatrics & child health.* Feb 2011;16(2):e11-15.

**9.** Stoian CA, Lyon M, Cox RG, Stephure DK, Mah JK. Vitamin D concentrations among healthy children in Calgary, Alberta. *Paediatrics & child health.* Feb 2011;16(2):82-86.

**10.** Mark S, Gray-Donald K, Delvin EE, et al. Low vitamin D status in a representative sample of youth from Quebec, Canada. *Clinical chemistry.* Aug 2008;54(8):1283-1289.

**11.** Bikle D. Nonclassic actions of vitamin D. *J Clin Endocrinol Metab.* Jan 2009;94(1):26-34.

**12.** Wang L, Manson JE, Song Y, Sesso HD. Systematic review: Vitamin D and calcium supplementation in prevention of cardiovascular events. *Annals of internal medicine.* Mar 2 2010;152(5):315-323.

**13.** Adorini L, Penna G. Control of autoimmune diseases by the vitamin D endocrine system. *Nat Clin Pract Rheumatol.* Aug 2008;4(8):404-412.

**14.** Garland CF, Gorham ED, Mohr SB, Garland FC. Vitamin D for cancer prevention: global perspective. *Annals of epidemiology.* Jul 2009;19(7):468-483.

**15.** Wu S, Ren S, Nguyen L, Adams JS, Hewison M. Splice variants of the CYP27b1 gene and the regulation of 1,25-dihydroxyvitamin D3 production. *Endocrinology.* Jul 2007;148(7):3410-3418.

**16.** Beard JA, Bearden A, Striker R. Vitamin D and the anti-viral state. *J Clin Virol.* Mar 2011;50(3):194-200.

**17.** Travis J. Origins. On the origin of the immune system. *Science.* May 1 2009;324(5927):580-582.

**18.** Kumagai Y, Akira S. Identification and functions of pattern-recognition receptors. *J Allergy Clin Immunol.* May 2010;125(5):985-992.

**19.** Wang TT, Nestel FP, Bourdeau V, et al. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J Immunol.* Sep 1 2004;173(5):2909-2912.

**20.** Liu PT, Stenger S, Li H, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science.* Mar 24 2006;311(5768):1770-1773.

**21.** Oberg F, Botling J, Nilsson K. Functional antagonism between vitamin D3 and retinoic acid in the regulation of CD14 and CD23 expression during monocytic differentiation of U-937 cells. *J Immunol.* Apr 15 1993;150(8 Pt 1):3487-3495.

**22.** Stroder J, Kasal P. Evaluation of phagocytosis in rickets. *Acta Paediatr Scand.* May 1970;59(3):288-292.

**23.** Bar-Shavit Z, Noff D, Edelstein S, Meyer M, Shibolet S, Goldman R. 1,25-dihydroxyvitamin D3 and the regulation of macrophage function. *Calcif Tissue Int.* 1981;33(6):673-676.

**24.** van Etten E, Mathieu C. Immunoregulation by 1,25-dihydroxyvitamin D3: basic concepts. *J Steroid Biochem Mol Biol.* Oct 2005;97(1-2):93-101.

**25.** Chen S, Sims GP, Chen XX, Gu YY, Lipsky PE. Modulatory effects of 1,25-dihydroxyvitamin D3 on human B cell differentiation. *J Immunol.* Aug 1 2007;179(3):1634-1647.

**26.** Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, O'Garra A. 1alpha,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. *J Immunol.* Nov 1 2001;167(9):4974-4980.

**27.** Lemire JM, Adams JS, Kermani-Arab V, Bakke AC, Sakai R, Jordan SC. 1,25-Dihydroxyvitamin D3 suppresses human T helper/inducer lymphocyte activity in vitro. *J Immunol.* May 1985;134(5):3032-3035.

**28.** Ponsonby AL, McMichael A, van der Mei I. Ultraviolet radiation and autoimmune disease: insights from epidemiological research. *Toxicology.* Dec 27 2002;181-182:71-78.

**29.** Mathieu C, Gysemans C, Giulietti A, Bouillon R. Vitamin D and diabetes. *Diabetologia.* Jul 2005;48(7):1247-1257.

**30.** Suibhne TN, Cox G, Healy M, O'Morain C, O'Sullivan M. Vitamin D deficiency in Crohn's disease: prevalence, risk factors and supplement use in an outpatient setting. *Journal of Crohn's & colitis.* Mar 2012;6(2):182-188.

**31.** Munger KL, Levin LI, Hollis BW, Howard NS, Ascherio A. Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis. *JAMA.* Dec 20 2006;296(23):2832-2838.

**32.** Fendrick AM, Monto AS, Nightengale B, Sarnes M. The economic burden of non-influenza-related viral respiratory tract infection in the United States. *Arch Intern Med.* Feb 24 2003;163(4):487-494.

**33.** Quach C, Piche-Walker L, Platt R, Moore D. Risk factors associated with severe influenza infections in childhood: implication for vaccine strategy. *Pediatrics.* Sep 2003;112(3 Pt 1):e197-201.

**34.** Mandell GL, Bennett JE, Dolin R. *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases* Churchhill Livingstone, An Imprint of Elsevier; 2010.

**35.** Cannell JJ, Vieth R, Umhau JC, et al. Epidemic influenza and vitamin D. *Epidemiol Infect.* Dec 2006;134(6):1129-1140.

**36.** Hansdottir S, Monick MM, Hinde SL, Lovan N, Look DC, Hunninghake GW. Respiratory epithelial cells convert inactive vitamin D to its active form: potential effects on host defense. *J Immunol.* Nov 15 2008;181(10):7090-7099.

**37.** Hansdottir S, Monick MM, Lovan N, Powers L, Gerke A, Hunninghake GW. Vitamin D decreases respiratory syncytial virus induction of NF-kappaB-linked chemokines and

cytokines in airway epithelium while maintaining the antiviral state. *J Immunol.* Jan 15 2010;184(2):965-974.

**38.** Gordon YJ, Huang LC, Romanowski EG, Yates KA, Proske RJ, McDermott AM. Human cathelicidin (LL-37), a multifunctional peptide, is expressed by ocular surface epithelia and has potent antibacterial and antiviral activity. *Curr Eye Res.* May 2005;30(5):385-394.

**39.** Gordon YJ, Romanowski EG, Shanks RM, Yates KA, Hinsley H, Pereira HA. CAP37-derived antimicrobial peptides have in vitro antiviral activity against adenovirus and herpes simplex virus type 1. *Curr Eye Res.* Mar 2009;34(3):241-249.

**40.** Bergman P, Walter-Jallow L, Broliden K, Agerberth B, Soderlund J. The antimicrobial peptide LL-37 inhibits HIV-1 replication. *Current HIV research.* Jul 2007;5(4):410-415.

**41.** Howell MD, Jones JF, Kisich KO, Streib JE, Gallo RL, Leung DY. Selective killing of vaccinia virus by LL-37: implications for eczema vaccinatum. *J Immunol.* Feb 1 2004;172(3):1763-1767.

**42.** Roth DE, Soto G, Arenas F, et al. Association between vitamin D receptor gene polymorphisms and response to treatment of pulmonary tuberculosis. *J Infect Dis.* Sep 1 2004;190(5):920-927.

**43.** Fitness J, Tosh K, Hill AV. Genetics of susceptibility to leprosy. *Genes Immun.* Dec 2002;3(8):441-453.

**44.** Roth DE, Jones AB, Prosser C, Robinson JL, Vohra S. Vitamin D receptor polymorphisms and the risk of acute lower respiratory tract infection in early childhood. *J Infect Dis.* Mar 1 2008;197(5):676-680.

**45.** Muhe L, Lulseged S, Mason KE, Simoes EA. Case-control study of the role of nutritional rickets in the risk of developing pneumonia in Ethiopian children. *Lancet.* Jun 21 1997;349(9068):1801-1804.

**46.** Najada AS, Habashneh MS, Khader M. The frequency of nutritional rickets among hospitalized infants and its relation to respiratory diseases. *J Trop Pediatr.* Dec 2004;50(6):364-368.

**47.** Siddiqui TS, Rai MI. Presentation and predisposing factors of nutritional rickets in children of Hazara Division. *Journal of Ayub Medical College, Abbottabad : JAMC.* Jul-Sep 2005;17(3):29-32.

**48.** Wayse V, Yousafzai A, Mogale K, Filteau S. Association of subclinical vitamin D deficiency with severe acute lower respiratory infection in Indian children under 5 y. *Eur J Clin Nutr.* Apr 2004;58(4):563-567.

**49.** Roth DE, Shah R, Black RE, Baqui AH. Vitamin D status and acute lower respiratory infection in early childhood in Sylhet, Bangladesh. *Acta Paediatr.* Mar 2010;99(3):389-393.

**50.** Karatekin G, Kaya A, Salihoglu O, Balci H, Nuhoglu A. Association of subclinical vitamin D deficiency in newborns with acute lower respiratory infection and their mothers. *Eur J Clin Nutr.* Apr 2009;63(4):473-477.

**51.** Roth DE, Jones AB, Prosser C, Robinson JL, Vohra S. Vitamin D status is not associated with the risk of hospitalization for acute bronchiolitis in early childhood. *Eur J Clin Nutr.* Feb 2009;63(2):297-299.

**52.** McNally JD, Leis K, Matheson LA, Karuananyake C, Sankaran K, Rosenberg AM. Vitamin D deficiency in young children with severe acute lower respiratory infection. *Pediatr Pulmonol.* Oct 2009;44(10):981-988.

**53.** Sabetta JR, DePetrillo P, Cipriani RJ, Smardin J, Burns LA, Landry ML. Serum 25-hydroxyvitamin d and the incidence of acute viral respiratory tract infections in healthy adults. *PLoS One.* 2010;5(6):e11088.

**54.** Ginde AA, Mansbach JM, Camargo CA, Jr. Association between serum 25-hydroxyvitamin D level and upper respiratory tract infection in the Third National Health and Nutrition Examination Survey. *Arch Intern Med.* Feb 23 2009;169(4):384-390.

**55.** Berry DJ, Hesketh K, Power C, Hypponen E. Vitamin D status has a linear association with seasonal infections and lung function in British adults. *Br J Nutr.* Nov 2011;106(9):1433-1440.

**56.** Laaksi I, Ruohola JP, Tuohimaa P, et al. An association of serum vitamin D concentrations < 40 nmol/L with acute respiratory tract infection in young Finnish men. *Am J Clin Nutr.* Sep 2007;86(3):714-717.

**57.** Li-Ng M, Aloia JF, Pollack S, et al. A randomized controlled trial of vitamin D3 supplementation for the prevention of symptomatic upper respiratory tract infections. *Epidemiol Infect.* Oct 2009;137(10):1396-1404.

**58.** Avenell A, Cook JA, Maclennan GS, Macpherson GC. Vitamin D supplementation to prevent infections: a sub-study of a randomised placebo-controlled trial in older people (RECORD trial, ISRCTN 51647438). *Age Ageing.* Sep 2007;36(5):574-577.

**59.** Laaksi I, Ruohola JP, Mattila V, Auvinen A, Ylikomi T, Pihlajamaki H. Vitamin D supplementation for the prevention of acute respiratory tract infection: a randomized, double-blinded trial among young Finnish men. *J Infect Dis.* Sep 1 2010;202(5):809-814.

**60.** Camargo CA, Jr., Ganmaa D, Frazier AL, et al. Randomized trial of vitamin d supplementation and risk of acute respiratory infection in mongolia. *Pediatrics.* Sep 2012;130(3):e561-567.

**61.** Murdoch DR, Slow S, Chambers ST, et al. Effect of vitamin D3 supplementation on upper respiratory tract infections in healthy adults: the VIDARIS randomized controlled trial. *JAMA.* Oct 3 2012;308(13):1333-1339.

**62.** Jorde R, Witham M, Janssens W, et al. Vitamin D supplementation did not prevent influenza-like illness as diagnosed retrospectively by questionnaires in subjects participating in randomized clinical trials. *Scandinavian journal of infectious diseases.* Feb 2012;44(2):126-132.

**63.** Urashima M, Segawa T, Okazaki M, Kurihara M, Wada Y, Ida H. Randomized trial of vitamin D supplementation to prevent seasonal influenza A in schoolchildren. *Am J Clin Nutr.* May 2010;91(5):1255-1260.

**64.** Nicholson KG, Wood JM, Zambon M. Influenza. *Lancet.* Nov 22 2003;362(9397):1733-1745.

**65.** Barr IG, McCauley J, Cox N, et al. Epidemiological, antigenic and genetic characteristics of seasonal influenza A(H1N1), A(H3N2) and B influenza viruses: basis for the WHO recommendation on the composition of influenza vaccines for use in the 2009-2010 Northern Hemisphere season. *Vaccine.* Feb 3 2010;28(5):1156-1167.

**66.** Ahmed AH, Nicholson KG. The Efficacy of Influenza Vaccine. *Reviews in Medical Microbiology* 1996;7(1):23-30.

**67.** Jackson LA, Chen WH, Stapleton JT, et al. Immunogenicity and safety of varying dosages of a monovalent 2009 H1N1 influenza vaccine given with and without AS03 adjuvant system in healthy adults and older persons. *J Infect Dis.* Sep 15 2012;206(6):811-820.

**68.** Falsey AR, Treanor JJ, Tornieporth N, Capellan J, Gorse GJ. Randomized, double-blind controlled phase 3 trial comparing the immunogenicity of high-dose and standard-

dose influenza vaccine in adults 65 years of age and older. *J Infect Dis.* Jul 15 2009;200(2):172-180.

**69.** Holland D, Booy R, De Looze F, et al. Intradermal influenza vaccine administered using a new microinjection system produces superior immunogenicity in elderly adults: a randomized controlled trial. *J Infect Dis.* Sep 1 2008;198(5):650-658.

**70.** Daynes RA, Enioutina EY, Butler S, Mu HH, McGee ZA, Araneo BA. Induction of common mucosal immunity by hormonally immunomodulated peripheral immunization. *Infect Immun.* Apr 1996;64(4):1100-1109.

**71.** Daynes RA, Araneo BA. The development of effective vaccine adjuvants employing natural regulators of T-cell lymphokine production in vivo. *Annals of the New York Academy of Sciences.* Aug 15 1994;730:144-161.

**72.** Kriesel JD, Spruance J. Calcitriol (1,25-dihydroxy-vitamin D3) coadministered with influenza vaccine does not enhance humoral immunity in human volunteers. *Vaccine.* Apr 9 1999;17(15-16):1883-1888.

**73.** Cooper C, Thorne A. Vitamin D supplementation does not increase immunogenicity of seasonal influenza vaccine in HIV-infected adults. *HIV clinical trials.* Sep-Oct 2011;12(5):275-276.

**74.** Chadha MK, Fakih M, Muindi J, et al. Effect of 25-hydroxyvitamin D status on serological response to influenza vaccine in prostate cancer patients. *Prostate.* Mar 1 2011;71(4):368-372.

**75.** Loeb M, Russell ML, Moss L, et al. Effect of influenza vaccination of children on infection rates in Hutterite communities: a randomized trial. *JAMA.* Mar 10 2010;303(10):943-950.

**76.** Kumar J, Muntner P, Kaskel FJ, Hailpern SM, Melamed ML. Prevalence and associations of 25-hydroxyvitamin D deficiency in US children: NHANES 2001-2004. *Pediatrics.* Sep 2009;124(3):e362-370.

**77.** Dawood FS, Jain S, Finelli L, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *N Engl J Med.* Jun 18 2009;360(25):2605-2615.

**78.** Canada PHAo. Flu Watch May 31, 2009 to June 6, 2009. http://www.phac-aspc-gc.ca/fluwatch/08-09/w22_09/index-eng.php. Accessed October 4, 2009.

**79.** Looker AC, Pfeiffer CM, Lacher DA, Schleicher RL, Picciano MF, Yetley EA. Serum 25-hydroxyvitamin D status of the US population: 1988-1994 compared with 2000-2004. *Am J Clin Nutr.* Dec 2008;88(6):1519-1527.

**80.** FDA. Clinical Data Needed to Support the Licensure of Pandemic Influenza Vaccines. 2007; http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm091985.pdf. Accessed October 25, 2012.

**81.** CPMP. Note for guidance on harmonisation of requirements for influenza vaccines 1997; http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003945.pdf. Accessed October 25, 2012.

**82.** Hanley JA, Negassa A, Edwardes MD, Forrester JE. Statistical analysis of correlated data using generalized estimating equations: an orientation. *American journal of epidemiology.* Feb 15 2003;157(4):364-375.

**83.** Hypponen E, Power C. Hypovitaminosis D in British adults at age 45 y: nationwide cohort study of dietary and lifestyle predictors. *Am J Clin Nutr.* Mar 2007;85(3):860-868.

**84.** Canada S. Weather Conditions in Capitals and Major Cities 2007; http://www.statcan.gc.ca/tables-tableaux/sum-som/l01/cst01/phys08a-eng.htm. Accessed July 3, 2012.

**85.** Weng FL, Shults J, Leonard MB, Stallings VA, Zemel BS. Risk factors for low serum 25-hydroxyvitamin D concentrations in otherwise healthy children and adolescents. *Am J Clin Nutr.* Jul 2007;86(1):150-158.

**86.** Wortsman J, Matsuoka LY, Chen TC, Lu Z, Holick MF. Decreased bioavailability of vitamin D in obesity. *Am J Clin Nutr.* Sep 2000;72(3):690-693.

**87.** Wey CL, Beare T, Biskeborn K, Binkley T, Arneson L, Specker B. High bone density in young Hutterite children. *Bone.* Mar 2009;44(3):454-460.

**88.** Mihalopoulos NL, Holubkov R, Young P, Dai S, Labarthe DR. Expected changes in clinical measures of adiposity during puberty. *The Journal of adolescent health : official publication of the Society for Adolescent Medicine.* Oct 2010;47(4):360-366.

**89.** Das G, Crocombe S, McGrath M, Berry JL, Mughal MZ. Hypovitaminosis D among healthy adolescent girls attending an inner city school. *Archives of disease in childhood.* Jul 2006;91(7):569-572.

**90.** El-Hajj Fuleihan G, Nabulsi M, Choucair M, et al. Hypovitaminosis D in healthy schoolchildren. *Pediatrics.* Apr 2001;107(4):E53.

**91.** Wang TJ, Zhang F, Richards JB, et al. Common genetic determinants of vitamin D insufficiency: a genome-wide association study. *Lancet.* Jul 17 2010;376(9736):180-188.

**92.** Shea MK, Benjamin EJ, Dupuis J, et al. Genetic and non-genetic correlates of vitamins K and D. *Eur J Clin Nutr.* Apr 2009;63(4):458-464.

**93.** Barrett B, Brown R, Mundt M, et al. The Wisconsin Upper Respiratory Symptom Survey is responsive, reliable, and valid. *Journal of clinical epidemiology.* Jun 2005;58(6):609-617.

**94.** Hannoun C, Megas F, Piercy J. Immunogenicity and protective efficacy of influenza vaccination. *Virus research.* Jul 2004;103(1-2):133-138.

**95.** Jacobs B, Young NL, Dick PT, et al. Canadian Acute Respiratory Illness and Flu Scale (CARIFS): development of a valid measure for childhood respiratory infections. *Journal of clinical epidemiology.* Aug 2000;53(8):793-799.

**96.** Porojnicu AC, Moroti-Constantinescu R, Laslau A, et al. Vitamin D status in healthy Romanian caregivers and risk of respiratory infections. *Public health nutrition.* Mar 14 2012:1-6.

**97.** Manaseki-Holland S, Maroof Z, Bruce J, et al. Effect on the incidence of pneumonia of vitamin D supplementation by quarterly bolus dose to infants in Kabul: a randomised controlled superiority trial. *Lancet.* Apr 14 2012;379(9824):1419-1427.

## Table 1: Canadian Studies of Vitamin D levels in Children

| Author | Population | N | Female N (%) | Location | Latitude | Time Period | Mean Vitamin D Level (nmol/L) | Number (%) Deficiency | | | Variables Associated with serum 25-hydroxyvitamin D Level |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | < 25 nmol/L | < 50 nmol/L | < 75 nmol/L | |
| **Roth**[5] | Age 2 – 16 yrs presenting to the emergency department | 68 | 29 (43) | Edmonton, Alberta | 52°N | April/03 | 47.2 (95%CI 44 – 51) | 4 (6) | 23 (34)* | NA | Vitamin D intake, age and male sex |
| **Newhook**[6] | Age 0 – 14 yrs presenting to hospital | 48 | 21 (44) | St. John's Newfoundland and Labrador | 47 °N | Sept/05-Mar/06 | 60.2 | 0 | 17 (35) | 37 (77) | NA |
| **Langlois**[7] | Survey at 15 sites across Canada Pediatric Component: Age 6 - 19 | 1848 | 906 (49) | Canadian health measures survey | NA | 2007 – 2009 | Age 6 – 11: 75 (95% CI 70 – 80)<br><br>Age 12 – 19: 68.1 (95% CI 64 – 72) | NA | NA | 1076 (58) | NA |
| **Maguire**[8] | Healthy children 24 – 30 months Community pediatric office | 91 | 43 (47) | Toronto, Ontario | 43°N | Nov 2007 – May 2008 | Median: 60 (Range 20 – 126) | 1 (1) | 29 (32) | 75 (82) | Younger age, cow's milk consumption, BMI |
| **Stoian**[9] | Healthy children 2 – 13 years presenting to hospital for elective surgery | 1442 | 580 (40) | Calgary, Alberta | NA | Jan – Dec 2006 | 86.1 ± 35.1 (Range 10 – 323) | 29 (2) | NA | 568 (39) | Age, nonwhite ethnicity, season, BMI, dietary intake |
| **Mark**[10] | Healthy Children Age 9, 13 and 16 | 1753 | 871 (50) | Quebec | 45 – 48 °N | Jan – May 1999 | Range 11.4 – 115.9 | 114 (6.5) | NA | 1712 (98) | Age, increased BMI (girls) |

Abbreviations: NA = not available
* less than 40 nmol/L

## Table 2. Observational Studies Evaluating the Association between Vitamin D and Respiratory Tract Infections (RTI)

| Author | Study Type | Population | Number | Country | Study Period | Outcome | Association | Results |
|---|---|---|---|---|---|---|---|---|
| **Berry**[55] | CS | 1958 birth cohort Age > 45 | 6789 | UK | Sept/02 – April/04 | Self reported URTI in the 3 weeks prior to level | Yes | 10 nmol/L increase in 25(OH)D associated with 7% lower risk of infection |
| **Ginde**[54] | CS | > 12 years | 18,883 | US | Oct/98 – Oct/94 | Self reported recent URTI | Yes | Lower prevalence of respiratory infections with higher 25(OH)D levels |
| **Laaksi**[56] | Cohort | Finnish Men | 800 | Finland (60 – 70$^{o}$N) | Vitamin D measured July 2002 | Number of days of absence from duty due to URTI in 6 months following level | Yes | Men with serum 25(OH)D levels < 40 nmol/L had significantly more days of absence (p=0.0004) |
| **Sabetta**[53] | Cohort | Healthy adults | 198 | Connecticut, US | Sept 2009 – Feb 2010 | Respiratory infection (self reported and lab proven) | Yes | Vitamin D concentrations >= 38 ng/ml (95 nmol/l) were associated with a two fold reduction in risk of RTI and reduction in percentage of days ill. |
| **Porojnicu**[96] | Cohort | Medical employees at hospital | 110 | Bucharest, Romania (45 $^{o}$N ) | Dec/07 – Jan/08 | Self reported URTI | No | No correlation between 25(OH)D level and occurrence of any respiratory tract infection |
| **Wayse**[48] | Case-Control | Children < 5 yrs | 150 | India, Indapur | May-June 2002 | LRTI | Yes | Adequate serum 25(OH)D > 22.5 nmol/l and exclusive breast feeding in 1$^{st}$ 4 months were protective |
| **Karatekin**[50] | Case- Control | Newborns (< 1 month) | 25 | Turkey (41$^{o}$N) | Jan/04 – April/04 | LRTI | Yes | Mean serum 25(OH)D levels were lower in those with ALRTI than controls |
| **McNally**[52] | Case- Control | Children < 5 yrs | 197 | Canada (Saskatoon) | Nov/07 – May/08 | LRTI (bronchiolitis or pneumonia) | No | No difference in mean vitamin D level between cases and controls. |
| **Roth**[49] | Case- Control | Children 1 – 18 | 50 | Bangladesh | Jan/08 – Feb/09 | LRTI | Yes | Mean serum 25(OH)D level significantly lower in cases |
| **Roth**[51] | Case- Control | Children 1 – 2 years | 129 | Edmonton, Canada | Jan – March 2005/2006 | LRTI | No | No difference in mean serum 25(OH)D level between cases and controls |

Abbreviations: CS – cross sectional, URTI – Upper Respiratory Tract Infection, LRTI – Lower respiratory tract infection, 25(OH)D – 25-hydroxyvitamin D

**Table 3. Randomized Controlled Trials Evaluating Vitamin D Prophylaxis for the Prevention of Respiratory Tract Infections**

| Author | Population | N | Country | Study Period | Outcome | Association | Results |
|---|---|---|---|---|---|---|---|
| **Avenell**[58] | Age > 70 years | 3444 | UK | March 2002 | Self reported infection (18 months after randomization) | No | Per-protocol analysis – trend toward benefit of vitamin D (p=NS) OR infection 0.80 (0.64 – 1.01, p=0.06) |
| **Li-Ng**[57] | Healthy Adults Randomized to 2000 IU or none | 162 | US (New York) 40.7$^{o}$N | Dec/06 – March/07 | Self reported URTI | No | No difference in URIs between the vitamin D group and placebo (p=0.57) |
| **Urashima**[63] | School children randomized to 1200 IU or placebo | 334 | Japan | Dec/08 – March/09 | Influenza A infection (NP antigen testing) | Yes | RR of developing influenza (RR), 0.58; 95% CI: 0.34, 0.99; P = 0.04 |
| **Manaseki-Holland**[97] | Children age 1-11 years | 3046 | Kabul, Afghanistan | Nov 4/08 – May/09 | LRTI - pneumonia CXR confirmed | No | No difference in incidence of pneumonia between groups |
| **Jorde**[62] | Participants from 10 intervention clinical trials Age > 18 | 569 | Norway, Austria, USA, Scotland, Denmark, Belgium | Sept/09 – April/10 | Self reported ILI based on survey responses | No | ILI reported 38% Vitamin D group (n=289) and 42% in placebo group (n=280). P=NS |
| **Laaksi**[59] | Finnish Men (Age 18-28) | 164 | Finland (60 – 70$^{o}$N) | Oct/06 – March/07 | Number of days absent from duty due to RTI | No | No difference in number of days absent from work. Proportion of participants with no days absent higher in Vitamin D arm (p=0.045) |
| **Camargo**[60] | Children with vitamin D deficiency from grade 3 and 4 | 744 | Mongolia | Jan – March | Number of parent reported RTI over the 3 months | Yes | Children in Vitamin D group had fewer parent reported RTI (p=0.047) |
| **Murdoch**[61] | Healthy adults | 322 | New Zealand | Feb/10 – Nov/11 | Number of URTI episodes | No | No difference in the number of URTI episodes. No difference in severity using WURSS-21. |

Abbreviations: RCT – randomized controlled trial, URTI – Upper Respiratory Tract Infection, LRTI – Lower respiratory tract infection, 25(OH)D – 25-hydroxyvitamin D

**Table 4. Baseline Characteristics of Children with Serum 25(OH)D levels**

| | Vitamin D levels available* (n=743) |
|---|---|
| **Age, Mean (SD)** | 9.3 (3.4) |
| **Female** | 390 (52.5) |
| **Comorbidities** | |
| **>=1 Comorbidity**** | 18 (2.4) |
| **Asthma** | 11 (1.5) |
| | |
| **Vitamin D Level (nmol/L), Median (IQR)** | 62.0 (51.0, 74.0) |
| **Vitamin D Deficiency** | |
| **< 25 nmol/L** | 4 (0.5%) |
| **AAP < 50 nmol/L** | 152 (20.5%) |
| **CPS < 75 nmol/L** | 565 (76%) |

*Categorical variables presented as number (%)
** Comorbidity: heart/lung disease (including asthma), blood disorder, swallowing/choking disorder, ASA use, chronic metabolic condition, kidney/liver disease, immunodeficiency
Abbreviations: 25(OH)D – 25-hydroxyvitamin D, SD – standard deviation, IQR – interquartile range

**Table 5. Predictors of Serum 25(OH)D levels; Univariable Analyses**

| Variable | Serum 25(OH)D Level (nmol/L) | | Serum 25(OH)D by threshold* | |
|---|---|---|---|---|
| | Spearman rank coefficient, p | B coefficient, p | OR (95% CI) | |
| | | | < 50 nmol/L | < 75 nmol/L |
| **Age (years)** | -0.222, p < 0.001 | -1.39, p <0.001 | 1.09 (1.03 – 1.15) | 1.14 (1.08 – 1.20) |
| 3 – 4 years | Reference | | | |
| 5 – 9 years | | -5.06, p=0.04 | 1.00 (0.49, 2.04) | 1.36 (0.80, 2.29) |
| 10 – 15 years | | -13.82, p<0.001 | 2.11 (1.07, 4.16) | 3.38 (2.00, 5.80) |
| **Male Sex** | NA | 3.13, p=0.031 | 0.81 (0.57 – 1.2) | 0.61 (0.44 – 0.86) |
| **Comorbidities** | NA | 3.85, p=0.413 | 1.98 (0.73 – 5.37) | 0.49 (0.19 – 1.27) |
| **Latitude** | -0.255, p < 0.001 | -3.72, p<0.001 | 1.52 (1.33 – 1.74) | 1.35 (1.15 – 1.58) |
| **Season** | NA | 1.41, p=0.329 | 1.34 (0.94, 1.92) | 0.79 (0.56, 1.11) |

*American Academy of Pediatrics (AAP) cut-off of <50 nmol/L and Canadian Paediatric Society (CPS) cut-off of < 75 nmol/L
Abbreviations: NA = not applicable (predictor variable dichotomous), OR = odds ratio

**Table 6. Predictors of Serum 25(OH)D Level; Multivariable Analysis**

| Variable | Beta Coefficient * | P Value | Vitamin D Level OR (95% CI) | |
|---|---|---|---|---|
| | | | < 50 nmol/L | < 75 nmol/L |
| **Age (yrs)** | -0.022 | <0.001 | 1.12 (1.05, 1.20) | 1.16 (1.09, 1.23) |
| **Female** | -0.048 | 0.083 | 1.28 (0.83, 1.97) | 1.69 (1.07, 2.65) |
| **Latitude** | -0.064 | <0.001 | 1.60 (1.31, 1.94) | 1.43 (1.17, 1.74) |

* Outcome variable: log-25(OH)D level

**Table 7. Multi-level Model (Outcome Log 25(OH)D Level)**

| | Intercept Only | 1 Fixed Predictor | 2 Fixed Predictors | 3 Fixed predictors |
|---|---|---|---|---|
| **Fixed Component** | | | | |
| Intercept | 4.11 | 4.33 | 4.31 | 7.27 |
| Age | | -0.023 | -0.023 | -0.023 |
| Sex | | | 0.041 | 0.042 |
| Latitude | | | | -0.058 |
| **Random Component** | | | | |
| Residual variance | 0.0656 | 0.0603 | 0.0600 | 0.0601 |
| Variance (household) | 0.0175 | 0.0165 | 0.0164 | 0.0161 |
| Variance (colony) | 0.0111 | 0.0132 | 0.0132 | 0.0075 |
| Deviance | 270.38 | 217.34 | 213.13 | 198.45 |

**Table 8. Predictors of Proven Viral Respiratory Tract Infection; Univariable Analyses**

| Variable | OR (95% CI) | p-value | HR (95% CI) | p-value |
|---|---|---|---|---|
| **Age** | 0.92 (0.88, 0.96) | <0.001 | 0.93 (0.90, 0.97) | 0.001 |
| **Male** | 0.72 (0.53, 0.99) | 0.042 | 0.73 (0.56, 0.96) | 0.022 |
| **>=1 comorbidity** | 1.13 (0.42, 3.04) | 0.815 | 1.07 (0.47, 2.40) | 0.877 |
| **Asthma** | 0.84(0.22, 3.19) | 0.840 | 0.81 (0.26, 2.52) | 0.714 |
| **Vaccination** | | | | |
| **Influenza** | 0.66 (0.48, 0.90) | 0.009 | 0.74 (0.57, 0.97) | 0.026 |
| **Vitamin D level*** | 0.52 (0.31, 0.86) | 0.012 | 0.61 (0.41, 0.91) | 0.017 |
| **Vitamin D deficiency** | | | | |
| **< 25 nmol/L** | 0.75 (0.08, 7.22) | 0.801 | 0.72 (0.10, 5.10) | 0.738 |
| **< 50 nmol/L** | 1.75 (1.21, 2.53) | 0.003 | 1.54 (1.15, 2.06) | 0.004 |
| **< 75 nmol/L** | 1.43 (0.98, 2.09) | 0.067 | 1.35 (0.97, 1.89) | 0.074 |

Abbreviations: OR – odds ratio, CI – confidence interval, HR – hazard ratio
*Using log serum 25(OH)D level

**Table 9. Association between Serum 25(OH)D level and Proven Viral Respiratory Tract Infection, adjusted for Age and Sex**

| | Adjusted OR | P-value | Adjusted HR | P-Value |
|---|---|---|---|---|
| **Age** (Per 1 year increase) | 0.90 (0.84, 0.97) | 0.004 | 0.92 (0.87, 0.97) | 0.005 |
| **Male** | 0.74 (0.49, 1.10) | 0.139 | 0.74 (0.52, 1.04) | 0.082 |
| **Log 25-hydroxyvitamin D level (nmol/L)** | 0.42 (0.24, 0.73) | 0.002 | 0.52 (0.35, 0.79) | 0.002 |

Abbreviations: OR – odds ratio, CI – confidence interval, HR – Hazard ratio

**Table 10. Serum 25(OH)D level as a Predictor of Recurrent Respiratory Tract Infections, adjusted for Age and Sex**

| | Adjusted HR | P-value |
|---|---|---|
| **Age** | 0.93 (0.88, 0.98) | 0.010 |
| **Male** | 0.81 (0.60, 1.09) | 0.150 |
| **Serum 25-hydroxyvitamin D level (nmol/L)*** | | |
| **Per 1 unit change in log** | 0.57 (0.39, 0.83) | 0.003 |
| **Level** | | |
| < 50 nmol/L | 1.74 (1.21, 2.49) | 0.003 |
| < 75 nmol/L | 1.54 (1.13, 2.10) | 0.007 |

*Analyzed as both a continuous variable and dichotomized

**Table 11. Association between Serum 25(OH)D levels and Individual Respiratory Viruses adjusted for Age and Sex**

| | **Log Serum 25(OH)D level** | | **Serum 25(OH)D Level < 50 nmol/L** | | **Serum 25(OH)D Level < 75 nmol/L** | |
|---|---|---|---|---|---|---|
| | OR (95%CI) | p-value | OR (95%CI) | p-value | OR (95%CI) | p-value |
| **Influenza (A or B)** n=101 | 0.54 (0.25, 1.20) | 0.131 | 1.13 (0.62, 2.07) | 0.682 | 1.51 (0.85, 2.66) | 0.158 |
| **Coronavirus** n=26 | 1.37 (0.51, 3.70) | 0.536 | 1.10 (0.45, 2.66) | 0.840 | 1.53 (0.55, 4.24) | 0.415 |
| **Enterovirus/Rhinovirus** n=81 | 0.62 (0.34, 1.14) | 0.122 | 1.56 (0.97, 2.51) | 0.069 | 1.22 (0.74, 2.03) | 0.435 |
| **RSV** n=35 | 0.45 (0.12, 1.46) | 0.183 | 2.61 (0.98, 6.93) | 0.054 | 2.36 (0.80, 6.98) | 0.120 |
| **Parainfluenza** n=33 | 0.19 (0.08, 0.48) | <0.001 | 3.55 (2.21, 5.71) | <0.001 | 2.58 (1.00, 6.66) | 0.050 |

**Table 12. Baseline Characteristics of Participants who Developed a Proven Respiratory Tract Infection (n=229)**

| | **Value*** |
|---|---|
| **Age,** Mean (SD) | 8.7 (3.5) |
| **Male** | 96 (41.9) |
| **Comorbidities** | |
| **>=1 Comorbidity**** | 6 (2.6%) |
| **Asthma** | 3 (1.3%) |
| | |
| **Vitamin D Level (nmol/L),** Median (IQR) | 61.0 (49.0, 70.0) |
| **Vitamin D Deficiency** | |
| **< 25 nmol/L** | 1 (0.4%) |
| **AAP < 50 nmol/L** | 62 (27.1%) |
| **CPS < 75 nmol/L** | 184 (80.3%) |

*Categorical variables presented as number (%)
** Comorbidity: heart/lung disease (including asthma, blood disorder, swallowing/choking disorder, ASA use, chronic metabolic condition, kidney/liver disease, immunodeficiency
Abbreviations: SD – standard deviation, IQR – interquartile range

**Table 13. Summary of Severity Outcomes**

| | **Value*** |
|---|---|
| **Outcomes** | |
| Mean number of days of ≥ 2 symptoms | 2.9 (2.0) |
| Mean number of days of fever and cough | 0.4 (0.9) |
| Mean number of days of fever | 0.5 (1.1) |
| Hospitalization | 0 |
| Pneumonia | 1 (0.1%) |
| Otitis Media | 6 (0.8%) |
| Absenteeism (missed school) | 85 (11%) |

*Categorical variables presented as number (%), continuous variables presented as mean (standard deviation) unless otherwise mentioned

**Table 14. Unadjusted and Adjusted Associations between Covariables and Time to Symptom Resolution (≥2 symptoms)**

| Variable | Univariable Analysis | | Multivariable Analysis | |
|---|---|---|---|---|
| | **HR (95% CI)*** | **p-value** | **HR (95% CI)** | **p-value** |
| **Age** (continuous) | 1.00 (0.97, 1.03) | 0.884 | 0.99 (0.97, 1.03) | 0.946 |
| < 5 years | 1.01 (0.78, 1.30) | 0.937 | | |
| 5 – 9 years | 0.99 (0.76, 1.27) | 0.911 | | |
| > 10 years | Reference | | | |
| **Male** | 1.15 (0.90, 1.47) | 0.273 | 1.16 (0.89, 1.50) | 0.271 |
| **Comorbidities** | 1.22 (0.87, 1.72) | 0.244 | | |
| **Log serum 25(OH)D level** | 0.87 (0.57, 1.33) | 0.532 | 0.86 (0.55, 1.33) | 0.486 |
| **Vitamin D deficiency** | | | | |
| **Level < 50 nmol/L** | 0.94 (0.73, 1.21) | 0.638 | | |
| **Level < 75 nmol/L** | 0.98 (0.67, 1.45) | 0.931 | | |

***Outcome = time to symptom resolution, hazard ratio > 1 reflects shorter duration of symptoms and hazard ratio < 1 reflects longer duration of symptoms

**Table 15. Unadjusted and Adjusted Associations between Covariables and Time to Fever Resolution**

| Variable | Univariable Analysis | | Multivariable Analysis | |
|---|---|---|---|---|
| | **HR (95% CI)** | **p-value** | **HR (95% CI)** | **p-value** |
| **Age** (continuous) | 1.12 (1.04, 1.21) | 0.003 | 1.12 (1.05 1.20) | 0.001 |
| < 5 years | 0.45 (0.23, 0.87) | 0.018 | | |
| 5 – 9 years | 0.62 (0.38, 1.02) | 0.060 | | |
| > 10 years | Reference | | | |
| **Male** | 1.26 (0.92, 1.72) | 0.153 | 1.35 (0.86, 2.13) | 0.190 |
| **Comorbidities** | 3.83 (2.05, 7.15) | <0.001 | 3.17 (1.82, 5.52) | <0.001 |
| **Log serum 25(OH)D level** | 0.89 (0.47, 1.67) | 0.718 | 0.88 (0.54, 1.44) | 0.614 |
| **Vitamin D deficiency** | | | | |
| **Level < 50 nmol/L** | 1.14 (0.69, 1.89) | 0.606 | | |
| **Level < 75 nmol/L** | 0.79 (0.53, 1.16) | 0.225 | | |

**Table 16. Unadjusted and Adjusted Associations between Covariables and Absenteeism**

| **Variable** | **Univariable Analysis** | | **Multivariable Analysis** | |
|---|---|---|---|---|
| | OR (95% CI) | p-value | OR (95% CI) | p-value |
| **Age** (continuous) | 1.07 (0.99, 1.16) | 0.093 | 1.07 (0.98, 1.17) | 0.148 |
| < 5 years | 0.16 (0.03, 0.86) | 0.033 | | |
| 5 – 9 years | 0.96 (0.58, 1.59) | 0.884 | | |
| > 10 years | Reference | | | |
| **Male** | 0.89 (0.52, 1.50) | 0.667 | 0.90 (0.53, 1.53) | 0.690 |
| **Comorbidities** | 4.76 (0.97, 23.45) | 0.055 | | |
| **Influenza vaccine group** | 0.61 (0.23, 1.62) | 0.325 | | |
| **Log serum 25(OH)D level** | 0.68 (0.22, 2.07) | 0.497 | 0.79 (0.23, 2.76) | 0.714 |
| **Vitamin D deficiency** | | | | |
| **Level < 50 nmol/L** | 1.01 (0.48, 2.12) | 0.990 | | |
| **Level < 75 nmol/L** | 1.46 (0.70, 3.06) | 0.318 | | |

**Table 17. Calculated Minimum Hazard Ratio for Serum 25(OH)D level and Dichotomized Variables based on Different Levels of Power using the Available Sample Size (n=78)***

| **Continuous Variable (Log Serum 25(OH)D)** | | | | | | |
|---|---|---|---|---|---|---|
| **Power (%)** | 50 | 75 | 80 | 85 | 90 | 95 |
| **Effect Size / Minimum Hazard Ratio** | 2.05 | 2.62 | 2.78 | 2.99 | 3.27 | 3.73 |
| **Dichotomous Variables** | | | | | | |
| **Power (%)** | 50 | 75 | 80 | 85 | 90 | 95 |
| **Effect Size / Minimum Hazard Ratio** | 1.58 | 1.88 | 1.97 | 2.08 | 2.23 | 2.49 |

*Sample size calculated based on actual sample size = 202, adjusted by inflation factor (2.63) for clustering based an average cluster size = 17 and an intercluster correlation (ICC) = 0.1019

**Table 18. Baseline Characteristics of Vaccinated Participants**

| | **Overall (n=391)** | **Serology Available (n=221)** | **No Serology (n=170)** | **p-value** |
|---|---|---|---|---|
| **Age,** Mean (SD, Range) | 9.26 (3.39, 3-15) | 9.16 (3.28, 3–15) | 9.39 (3.42, 3–15) | 0.506 |
| **Female** | 222 (58.6%) | 115 (52.0%) | 107 (62.9%) | 0.031 |
| **Comorbidities** | | | | |
| **>=1 Comorbidity** | 12 (3.1%) | 10 (4.5%) | 2 (1.2%) | 0.057 |
| **Asthma** | 8 (2.0%) | 7 (3.2%) | 1 (0.6%) | 0.072 |
| | | | | |
| **Vitamin D Level (nmol/L),** Median (IQR) | 61.0 (51.0, 72.0) | 61.0 (50.0, 71.0) | 62.0 (53.0, 74.0) | 0.181 |
| **Vitamin D Deficiency** | | | | |
| **< 25 nmol/L** | 3 (0.8 %) | 2 (0.9%) | 1 (0.6%) | 0.598 |
| **AAP < 50 nmol/L** | 82 (21%) | 54 (24.4%) | 28 (16.5%) | 0.055 |
| **CPS < 75 nmol/L** | 305 (78%) | 176 (79.6%) | 129 (75.9%) | 0.374 |

*Categorical variables presented as number (%)
**Independent t-test or Wilcoxon rank sum test for continuous variables, chi-square or Fisher's exact test for dichotomous variables
Abbreviations: SD – standard deviation, IQR – interquartile range

**Table 19. Summary of Outcomes by Influenza Strain**

| | Number (percentage) n=221 |
|---|---|
| **Number with Pre-Vaccination titer >=1:40** | |
| A/Brisbane/10/2007[H3N2] | 157 (71.0) |
| A/Brisbane/59/2007[H1N1] | 93 (42.1) |
| B/Brisbane/60/2008 | 33 (14.9) |
| **Seroprotection (Number with Post-Vaccination titer >=40)** | |
| A/Brisbane/10/2007[H3N2] | 159 (71.9) |
| A/Brisbane/59/2007[H1N1] | 138 (62.4) |
| Both A strains | 127 (57.5) |
| B/Brisbane/60/2008 | 79 (35.7) |
| **Seroconversion (Pre-titer < 10 and post >=40)** | |
| A/Brisbane/10/2007[H3N2] | 50 (22.6) |
| A/Brisbane/59/2007[H1N1] | 76 (34.4) |
| Both A strains | 24 (10.9) |
| B/Brisbane/60/2008 | 49 (22.2) |
| **Seroconversion (Pre titer >= 10 and four-fold change)** | |
| A/Brisbane/10/2007[H3N2] | 44 (19.9) |
| A/Brisbane/59/2007[H1N1] | 23 (23.1) |
| Both A strains | 15 (6.8) |
| B/Brisbane/60/2008 | 17 (7.7) |
| **Seroconversion (Pre-titer < 10 and post >=40 OR Pre titer >= 10 and four-fold change)** | |
| A/Brisbane/10/2007[H3N2] | 94 (42.5) |
| A/Brisbane/59/2007[H1N1] | 127 (57.5) |
| Both A strains | 77 (34.8) |
| B/Brisbane/60/2008 | 66 (29.9) |
| **Four-Fold Change in Titer** | |
| A/Brisbane/10/2007[H3N2] | 96 (43.4) |
| A/Brisbane/59/2007[H1N1] | 128 (57.9) |
| Both A strains | 79 (35.7) |
| B/Brisbane/60/2008 | 73 (33) |

**Table 20. Predictors of Seroprotection after Influenza Vaccination by Influenza Strain (Univariable Analyses)**

| | **Seroprotection (Post Vaccine Antibody Titer ≥ 1:40)** | | | | | |
|---|---|---|---|---|---|---|
| | **A/Brisbane/10/2007 [H3N2]** | | **A/Brisbane/59/2007 [H1N1]** | | **B/Brisbane/60/2008** | |
| | **OR (95% CI)** | p-value | **OR (95% CI)** | p-value | **OR (95% CI)** | p-value |
| **Age (per 1 year change)** | 0.94 (0.86 1.02) | 0.128 | 0.96 (0.87, 1.06) | 0.389 | 0.99 (0.86, 1.15) | 0.930 |
| **< 5 years** | 0.58 (0.27, 1.25) | 0.166 | 0.61 (0.20, 1.82) | 0.606 | 0.92 (0.26, 3.23) | 0.893 |
| **5-9 years** | 0.63 (0.37, 1.07) | 0.088 | 0.67 (0.34, 1.28) | 0.665 | 1.23 (0.60, 2.50) | 0.571 |
| **> 9 years** | Reference | | | | | |
| **Male** | 1.17 (0.55, 2.49) | 0.685 | 0.57 (0.29, 1.12) | 0.104 | 0.93 (0.44, 1.98) | 0.855 |
| **Comorbidity** | 1.59 (0.49, 5.20) | 0.443 | 0.59 (0.26, 1.33) | 0.202 | 0.19 (0.03, 1.42) | 0.189 |
| **Log serum 25(OH)D** | 1.52 (0.69, 3.34) | 0.295 | 1.42 (0.52, 3.91) | 0.495 | 0.85 (0.29, 2.54) | 0.771 |
| **Vitamin D Deficiency** | | | | | | |
| **AAP < 50** | 0.72 (0.48, 1.06) | 0.097 | 0.84 (0.44, 1.59) | 0.587 | 0.97 (0.41, 2.31) | 0.942 |
| **CPS < 75** | 0.92 (0.50, 1.70) | 0.781 | 0.79 (0.34, 1.83) | 0.589 | 0.80 (0.47, 1.34) | 0.392 |

**Table 21. Predictors of Seroconversion after Influenza Vaccination by Influenza Strain (Univariable Analyses)**

| | **Seroconversion*** | | | | | |
|---|---|---|---|---|---|---|
| | **A/Brisbane/10/2007 [H3N2]** | | **A/Brisbane/59/2007 [H1N1]** | | **B/Brisbane/60/2008** | |
| | **OR (95% CI)** | p-value | **OR (95% CI)** | p-value | **OR (95% CI)** | p-value |
| **Age (per 1 year change)** | 0.99 (0.89, 1.12) | 0.933 | 0.97 (0.88, 1.07) | 0.502 | 0.97 (0.86, 1.09) | 0.632 |
| **< 5 years** | 0.68 (0.27, 1.76) | 0.684 | 0.61 (0.18, 2.04) | 0.419 | 0.71 (0.21, 2.41) | 0.233 |
| **5-9 years** | 1.16 (0.60, 2.26) | 0.666 | 0.69 (0.37, 1.31) | 0.261 | 1.43 (0.80, 2.56) | 0.233 |
| **> 9 years** | Reference | | | | | |
| **Male** | 1.34 (0.68, 2.62) | 0.396 | 0.60 (0.29, 1.23) | 0.164 | 0.95 (0.45, 1.99) | 0.881 |
| **Comorbidity** | 0.90 (0.39, 2.04) | 0.794 | 0.17 (0.03, 0.88) | 0.034 | ** | |
| **Log serum 25(OH)D** | 1.02 (0.54, 1.92) | 0.951 | 0.98 (0.44, 2.15) | 0.952 | 0.78 (0.21, 2.92) | 0.706 |
| **Vitamin D Deficiency** | | | | | | |
| **AAP < 50** | 1.00 (0.60, 1.67) | 0.990 | 0.82 (0.54, 1.24) | 0.346 | 1.11 (0.42, 2.90) | 0.837 |
| **CPS < 75** | 1.14 (0.63, 2.07) | 0.668 | 1.38 (0.87, 2.20) | 0.173 | 0.93 (0.48, 1.81) | 0.827 |

*post-vaccination titer ≥ 1:40 for participants with pre-vaccine titer <1:10 and four-fold rise in post-vaccination titer for those with a pre-vaccine titer ≥ 1:10
**unable to estimate because of Hessian matrix singularity

**Table 23. Geometric Mean Titers (baseline, post-vaccination and fold change) by Influenza Strain and Comparison based on Serum 25-hydroxyvitamin D Level**

| | Overall | Serum 25(OH)D Level | | | Serum 25(OH)D Level | | |
|---|---|---|---|---|---|---|---|
| | | <50 nmol/L | >=50 nmol/L | p-value* | < 75 nmol/L | >=75 nmol/L | p-value* |
| **A/Brisbane/10/2007[H3N2]** | | | | | | | |
| Baseline | 93.9 (70.2, 125.5) | 114.6 (62.6, 209.8) | 88.0 (63.0,122.9) | 0.443 | 91.5 (65.0, 128.6) | 103.9 (61.3, 176.2) | 0.727 |
| Post | 130.9 (96.3, 178.0) | 123.8 (63.1, 242.8) | 133.3 (94.2, 188.7) | 0.839 | 133.0 (94.1, 187.9) | 123.1 (61.3, 247.2) | 0.843 |
| **A/Brisbane/59/2007[H1N1]** | | | | | | | |
| Baseline | 22.8 (18.2, 28.6) | 20.8 (12.6, 34.4) | 23.5 (18.2, 30.4) | 0.647 | 19.9 (15.5, 25.6) | 38.8 (22.7, 66.2) | 0.020 |
| Post | 119.9 (84.4, 170.3) | 116.1 (53.9, 250.2) | 121.2 (81.4, 180.2) | 0.918 | 112.2 (75.5, 166.8) | 155.1 (71.0, 339.0) | 0.465 |
| **B/Brisbane/60/2008** | | | | | | | |
| Baseline | 8.9 (7.6, 10.4) | 7.3 (5.6, 9.6) | 9.5 (7.9, 11.4) | 0.169 | 8.4 (7.2, 0.9) | 11.0 (7.1, 16.9) | 0.181 |
| Post | 19.7 (15.5, 25.2) | 18.9 (11.7, 30.8) | 20.0 (15.0, 26.6) | 0.859 | 19.1 (14.4, 25.2) | 22.6 (13.4, 38.3) | 0.581 |
| **Fold Increase GMT** | | | | | | | |
| H3N2 | 1.39 (0.91, 2.15) | 1.08 (0.43, 2.74) | 1.51 (0.93, 2.48) | 0.509 | 1.45 (0.88, 2.41) | 1.18 (0.52, 2.68) | 0.708 |
| H1N1 | 5.25 (3.39, 8.15) | 5.58 (2.05, 15.23) | 5.15 (3.16, 8.40) | 0.877 | 5.63 (3.41, 9.30) | 4.00 (1.58, 10.12) | 0.537 |
| Influenza B | 2.21 (1.75, 2.79) | 2.59 (1.69, 3.96) | 2.10 (1.59, 2.77) | 0.452 | 2.25 (1.73, 2.94) | 2.06 (1.26, 3.41) | 0.764 |

* t-test comparing means of log titers between groups

**Figure 1. Scatter Plot of Standardized Pearson Residuals against the Predicted Value of the Log 25(OH)D Level**

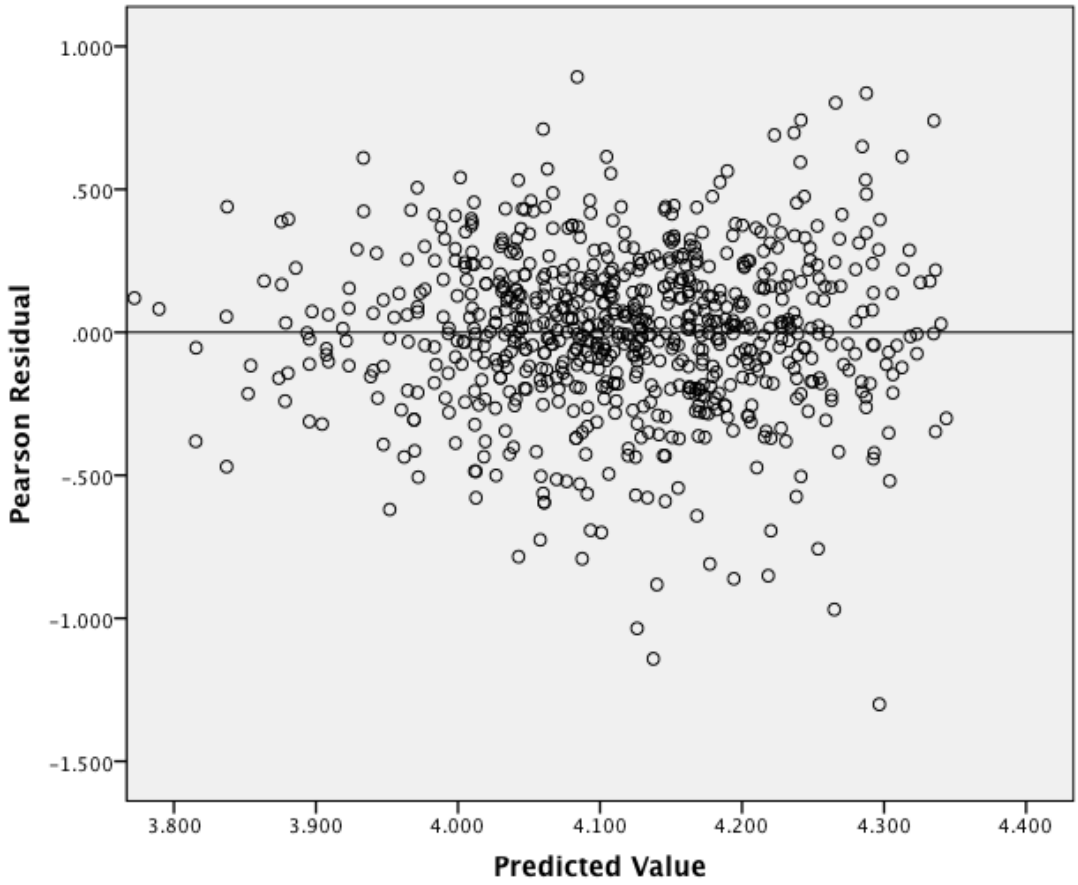


**Figure 2. Time to Proven Viral Infection by Serum 25(OH)D level ( < 50 nmol/L vs. ≥ 50 nmol/L) adjusted for Age and Sex**

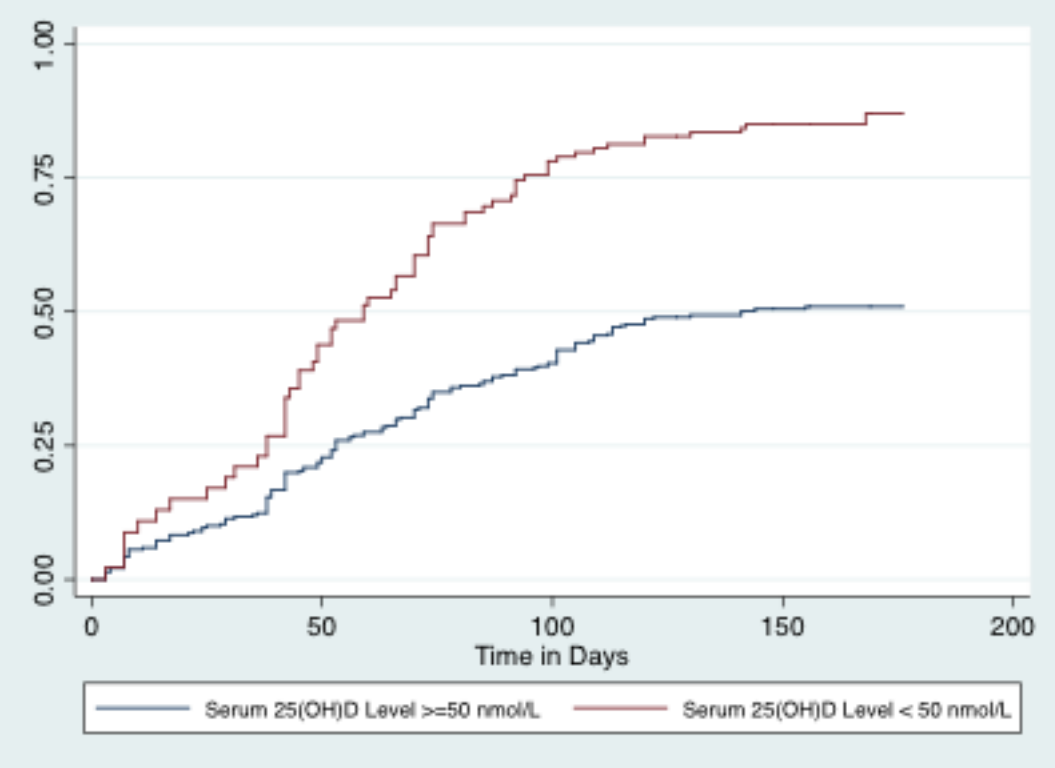


**Figure 3. Time to Proven Viral Infection by Serum 25(OH)D level ( < 75 nmol/L vs. ≥ 75 nmol/L) adjusted for Age and Sex**

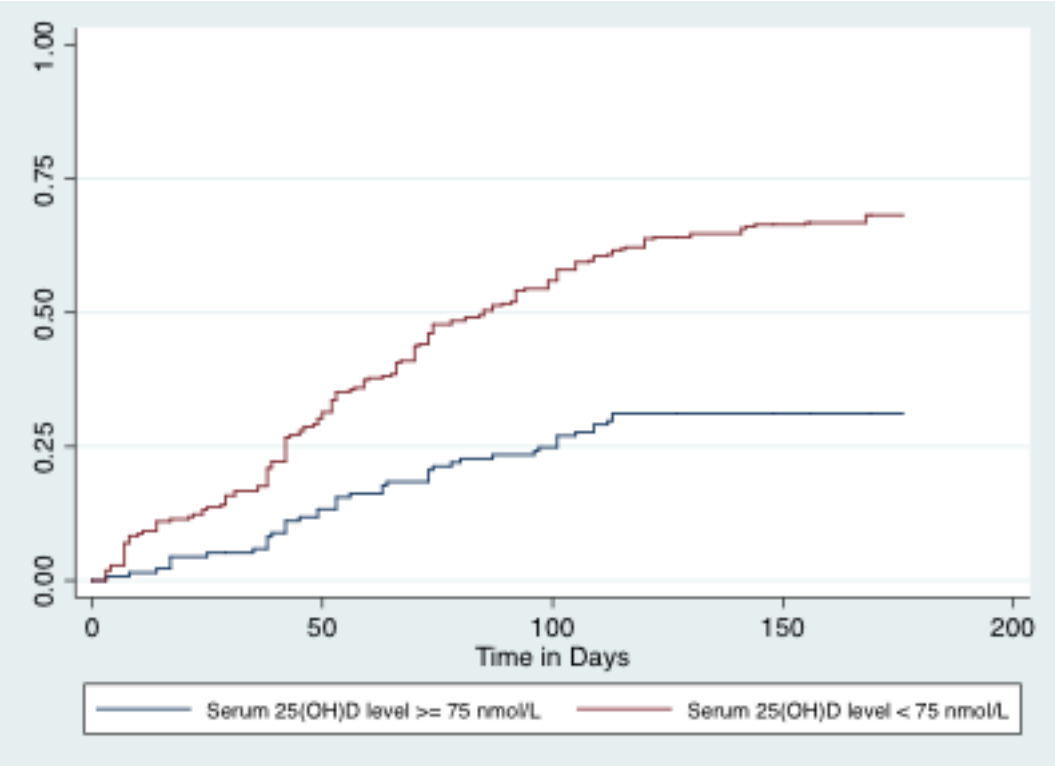


**Figure 4. Scatter plot Scaled Schoënfeld Residuals for Log 25(OH)D against Time (Days)**

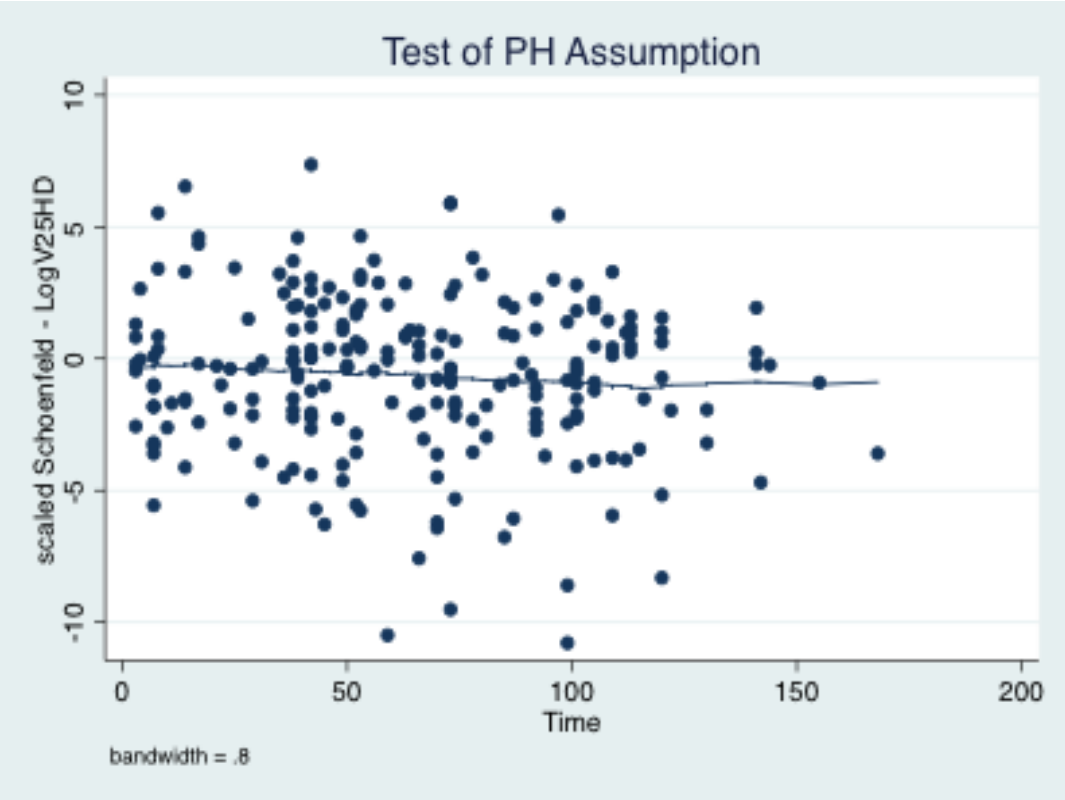


**Figure 5. Scatter plot Scaled Schoënfeld Residuals for Age against Time (Days)**

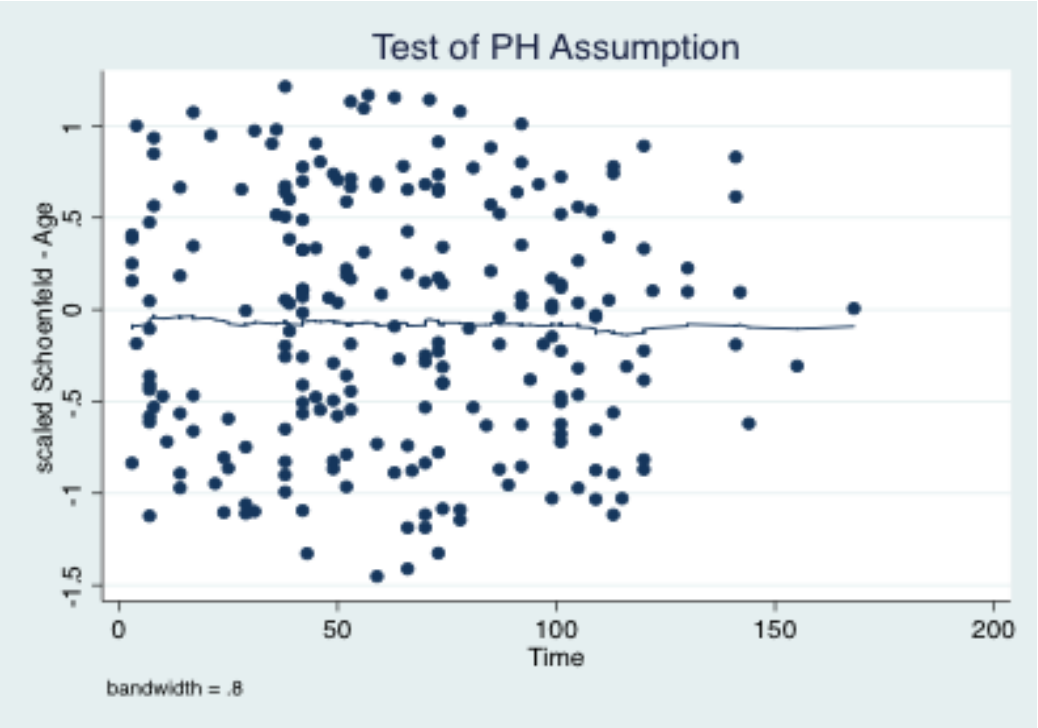


**Figure 6. Log-Log curves by Sex**

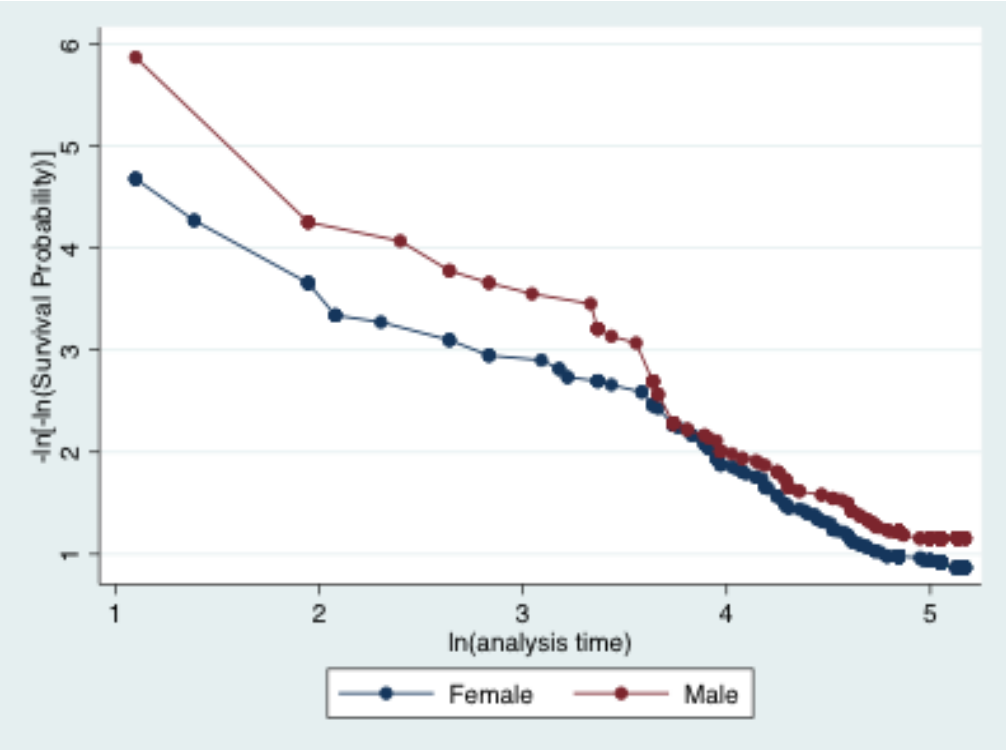


**Figure 7. Log-Log Curves by Serum 25(OH)D Level < 50 nmol/L and >=50 nmol/L**

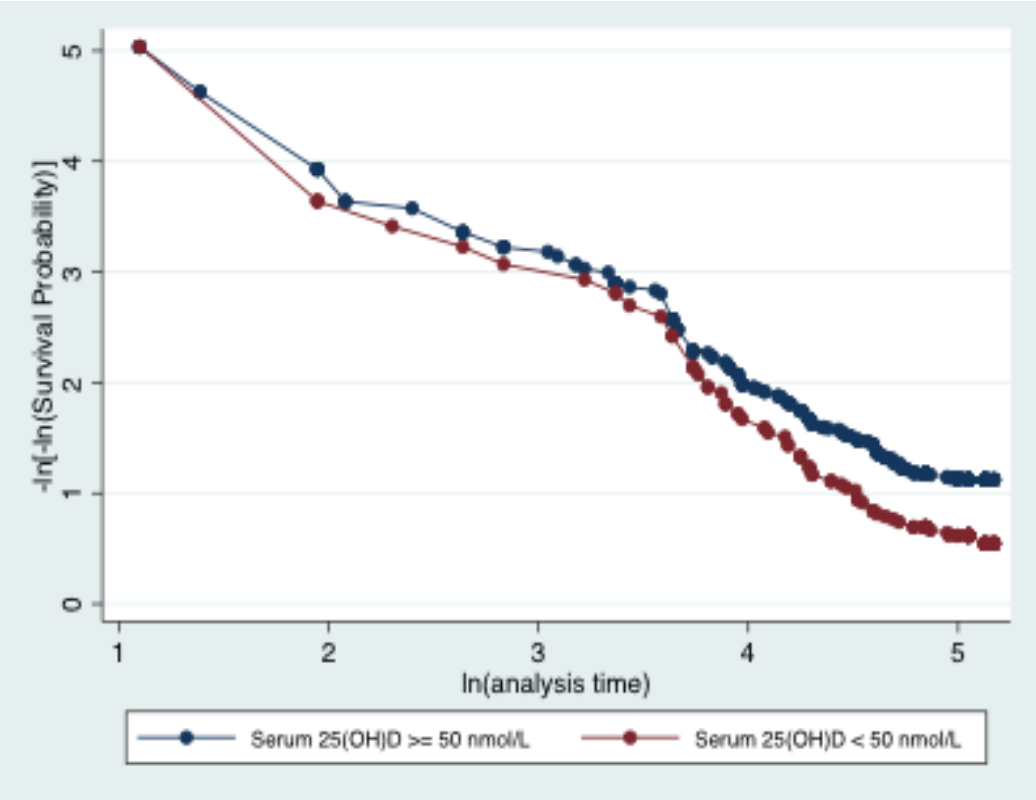


**Figure 8. Log-Log Curves by Serum 25(OH)D Level < 75 nmol/L and >=75 nmol/L**

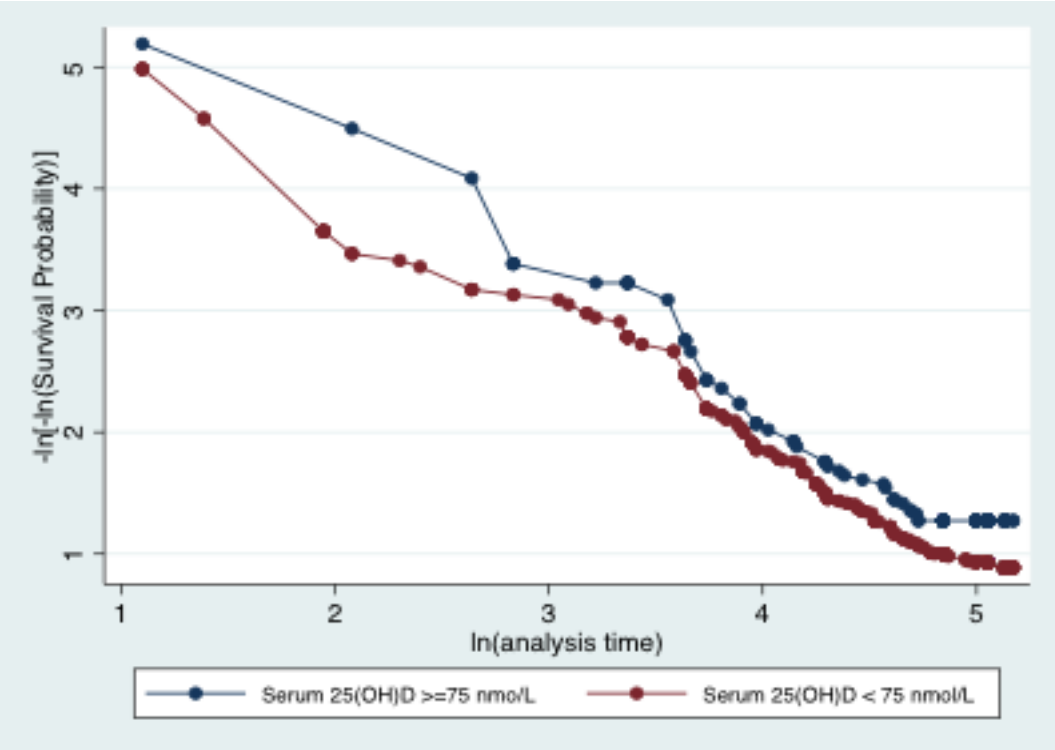


**Figure 9. Observed vs. Expected Survival Probability Graphs by Sex**

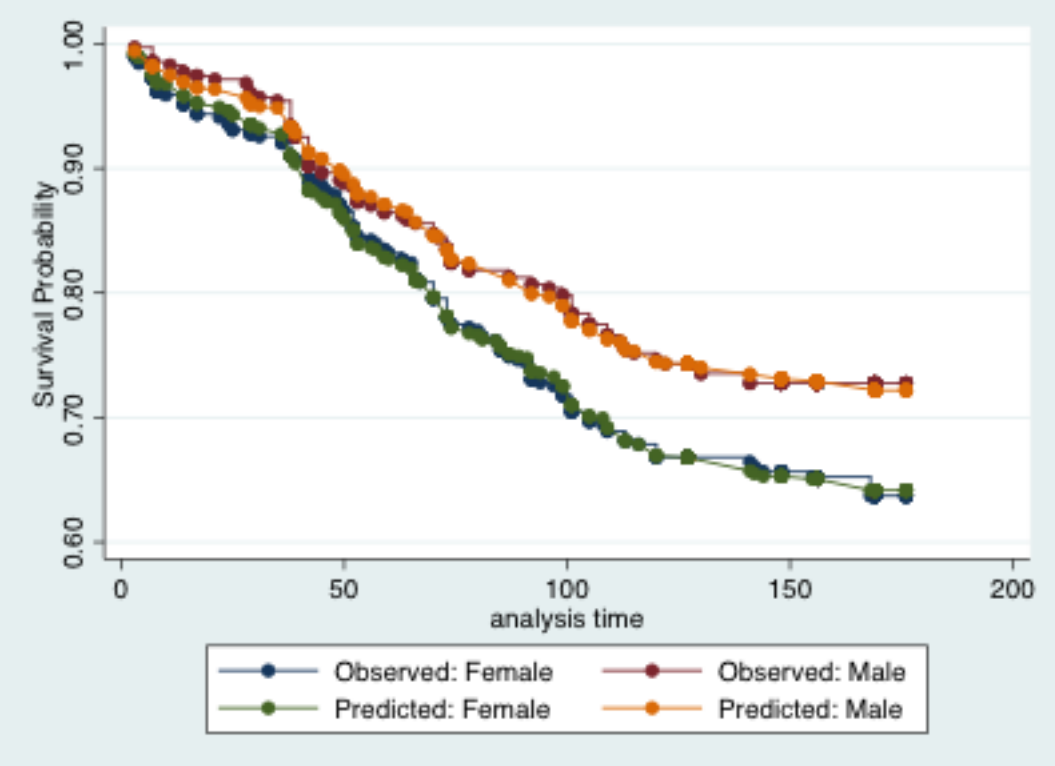


**Figure 10. Nelson-Aalen Cumulative Hazard estimator for Cox-Snell Residuals**

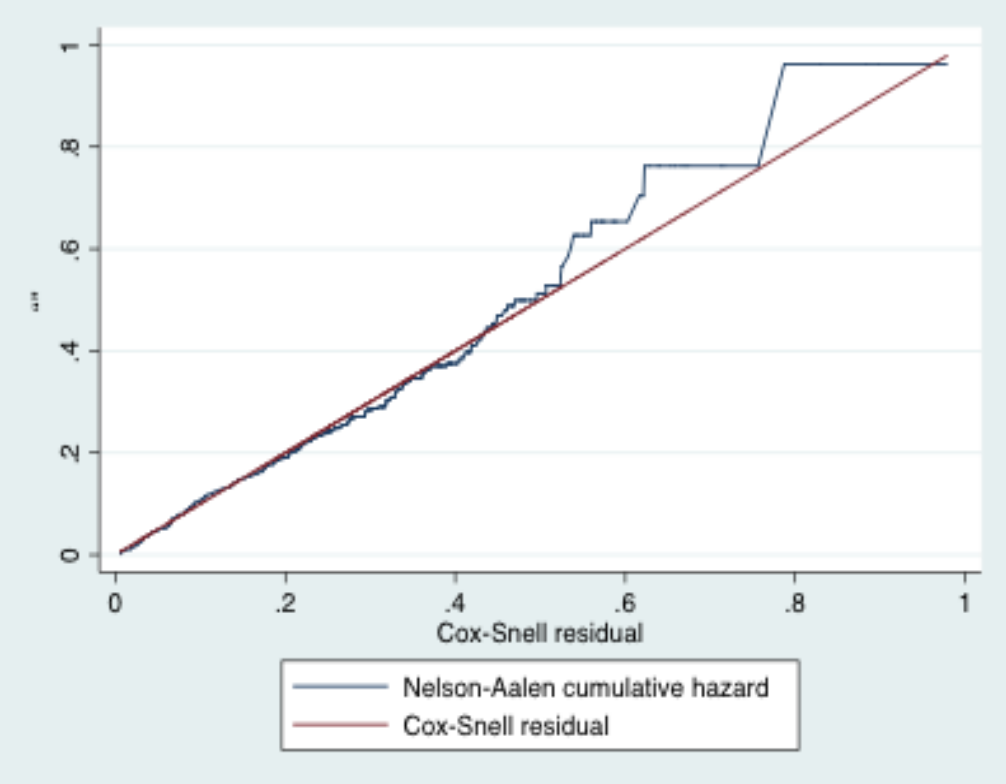


**Figure 11. Scatter Plot of Deviance Residuals by Participant ID Number**

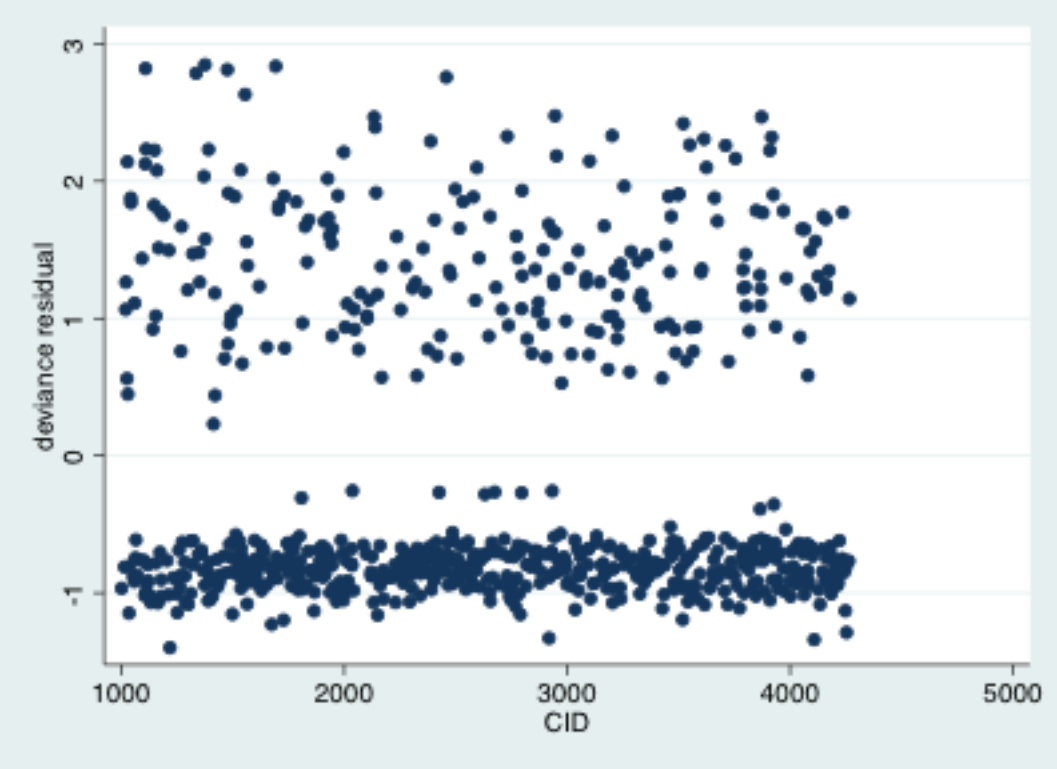


**Figure 12. Nelson-Aalen Cumulative Hazard estimator for Cox-Snell Residuals for Time to Resolution of Symptoms**

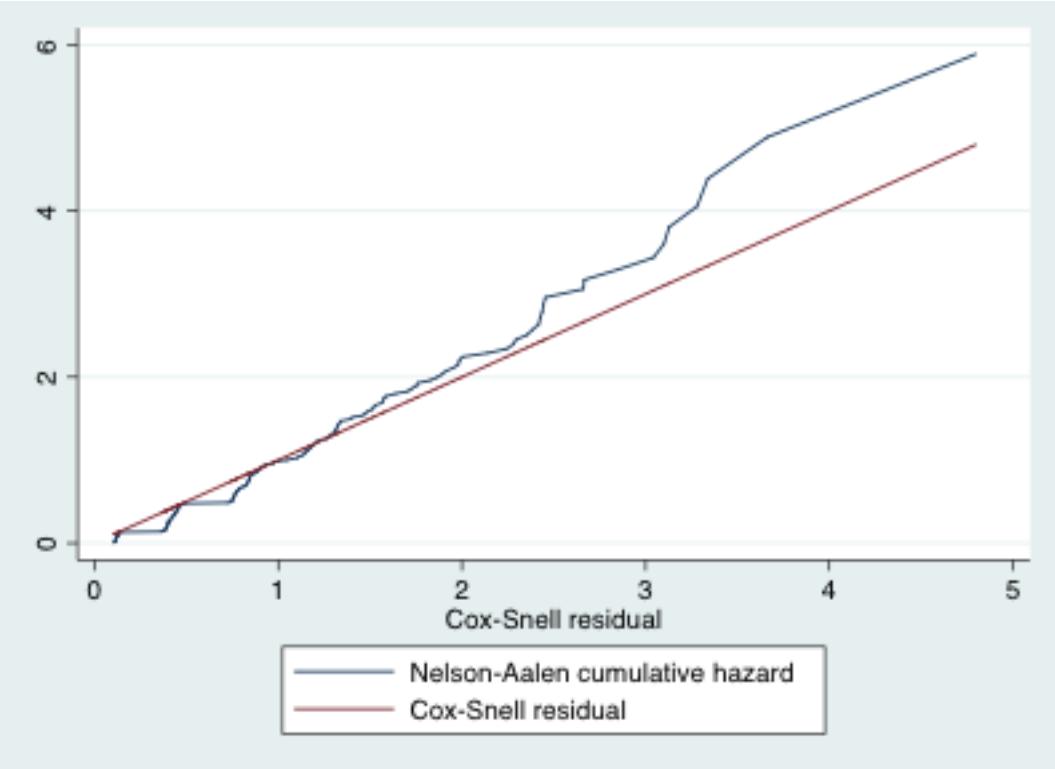
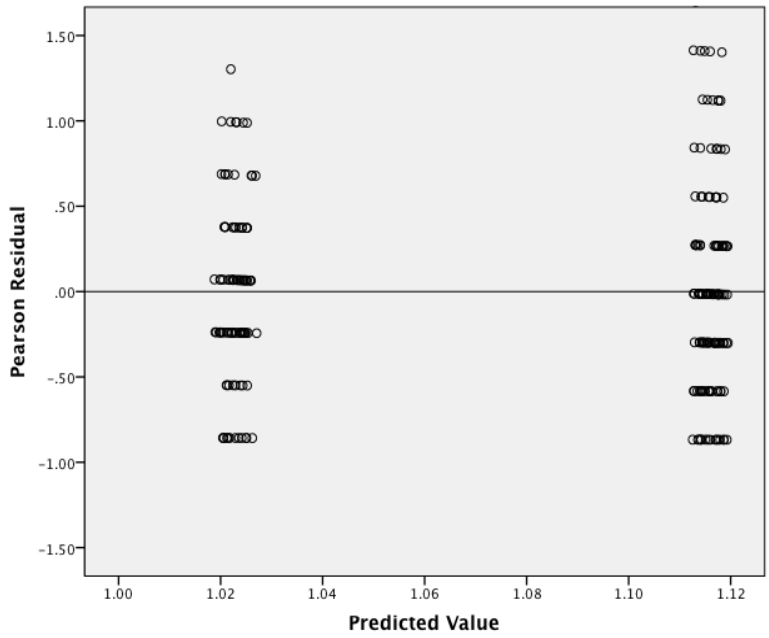


**Figure 13. Scatter Plots of Pearson Residuals by Predicted Value and Log 25(OH)D level**



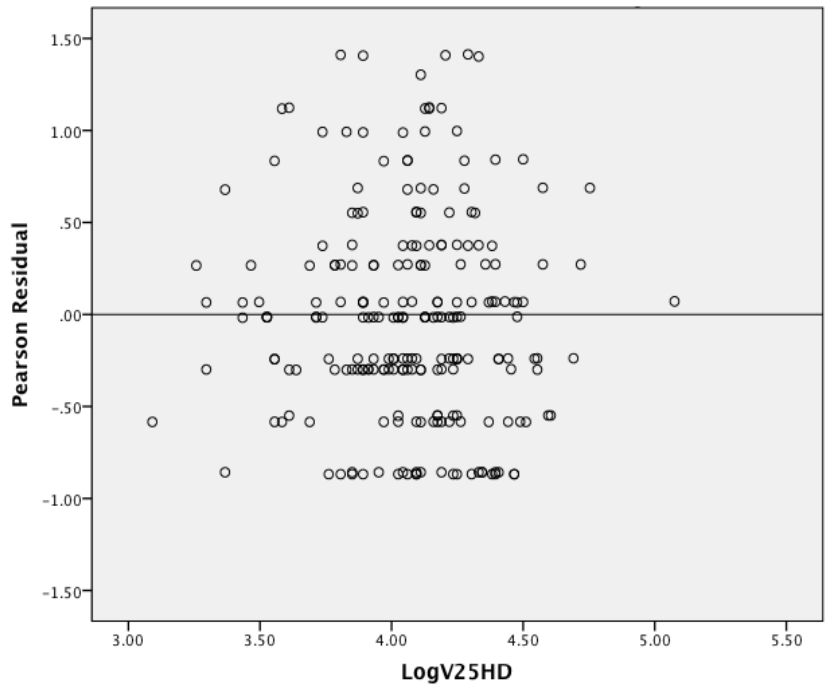



**Figure 14. Scatter Plot of Pearson Residuals by Predicted Value**
**(Outcome Influenza A/Brisbane/10/2007 [H3N2] Seroprotection: Antibody Titer >= 1:40)**