BIOCHEMICAL STATUS OF VITAMIN D AND RELATED BIOMARKERS AS PREDICTORS OF SEVERE INFLUENZA INFECTION IN CHILDREN

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Biochemical Status of Vitamin D and Related Biomarkers as Predictors of Severe Influenza Infection in Children

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2016

ABSTRACT

Eighty-two influenza-positive children were recruited prospectively from the inpatient units of four Canadian pediatric referral centers. Serum 25-hydroxyvitamin D [25(OH)D] levels were inversely associated with influenza severity ($B = -0.43$, $p$-value = 0.03). Participants whose serum 25(OH)D levels fell below 50 nmol/L had significantly higher maximum Composite Severity Index ® (maxCSI) scores than those with serum levels $\geq 50$ nmol/L (medians of 56 vs. 37, $p$-value = 0.01). Serum LL-37 levels were also found to be inversely associated with maxCSI scores among participants ($B = -0.78$, $p$-value = 0.03). Only serum 25(OH)D levels remained a significant predictor of influenza severity when incorporated into a multivariable model controlling for patient sex, age, zBMI, maternal education, vaccination status and influenza type ($B = -0.50$, $SE = 0.20$, $p$-value = 0.04). Results from this study indicate that serum 25(OH)D levels <50 nmol/L may be associated with the development of severe disease in influenza-positive children.
ACKNOWLEDGEMENTS

Thank you first and foremost to my two graduate supervisors, Dr. Deborah O’Connor and Dr. Dat Tran. I am grateful for all they have taught me, directly or by example. Thank you to Dr. O’Connor for providing an ideal balance of support and criticism over the course of my degree. Without her encouragement and the positive learning environment that she is renowned for creating, I would not have succeeded. Thank you to Dr. Tran for providing me with the opportunity to work with the FluGene cohort. It was a privilege to work with both of you.

Thank you to the incredible women in the Department of Nutritional Sciences who were my colleagues, but also my role models. Kayla Furlong was an inspiration to work with, and from her I learned what can be achieved with a strong work ethic. Lesley Plumptre was a wonderful mentor, offering steady support and guidance. Dawn Ng and Susanne Aufreiter provided dependable advice and regularly took time from their schedules to help me with a huge array of tasks. Lastly, thank you to Shelley Vanderhout, Marie-Elsssa Morency, Veronik Connan, Anne Fard and Allison Daniels for their emotional support and for sharing their journeys through graduate studies with me!

I would also like to thank the Department of Nutritional Sciences as a whole for providing a wonderful sense of community from the moment I began this degree. I feel fortunate to have been a part of this department and to have had the opportunity to share my work with so many of my professional idols. I would also like to specifically thank Louisa Kung for her patience and dependability. She is the source of all answers!

Thank you to the children and their families who took the time to participate in our project. I would also like to acknowledge the support of the Canadian Institutes of Health Research and the Canadian Foundation for Dietetic Research for the funding to complete my project. In addition, the services provided by Laboratory Services at Mount Sinai Hospital cannot go unacknowledged. Thank you to Hilde Vandenberghe and Michelle Rodrigues for their tireless efforts to keep my thesis defense timeline on track.

Lastly I would like to thank everyone at the SickKids Centre for Global Child Health for inspiring me. Thank you to Dr. Stanley Zlotkin, Dr. Ashley Aimone and Dr. Suzanne Attia for providing career advice and guidance. As a whole, the research being conducted in The Centre never fails to remind me to think on a global scale and aspire to advocate for disadvantaged populations worldwide.
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<th>Full Form</th>
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<tr>
<td>AI</td>
<td>Adequate Intake</td>
</tr>
<tr>
<td>AMP</td>
<td>Antimicrobial peptide</td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchoalveolar Lavage Fluid</td>
</tr>
<tr>
<td>BD2</td>
<td>Beta-defensin 2</td>
</tr>
<tr>
<td>BD3</td>
<td>Beta-defensin 3</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>CHMS</td>
<td>Canadian Health Measures Survey</td>
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<tr>
<td>DEQAS</td>
<td>Vitamin D External Quality Assessment Scheme</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive Care Unit</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IOM</td>
<td>Institute of Medicine</td>
</tr>
<tr>
<td>IU</td>
<td>International Units</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>LL-37</td>
<td>Human cathelicidin LL-37</td>
</tr>
<tr>
<td>LOS</td>
<td>Length of Hospital Stay</td>
</tr>
<tr>
<td>NACI</td>
<td>National Advisory Committee on Immunization</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized Controlled Trial</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended Dietary Allowance</td>
</tr>
<tr>
<td>RR</td>
<td>Relative Risk</td>
</tr>
<tr>
<td>RTI</td>
<td>Respiratory Tract Infection</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VBP</td>
<td>Vitamin D Binding Protein</td>
</tr>
<tr>
<td>VDRE</td>
<td>Vitamin D Response Elements</td>
</tr>
<tr>
<td>1,25(OH)D</td>
<td>1,25-hydroxyvitamin D</td>
</tr>
<tr>
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<td>25-hydroxyvitamin D</td>
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| Influenza-like illness | An illness presenting with symptoms similar to an influenza infection, but that has not been confirmed through laboratory testing |
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STUDENT CONTRIBUTIONS

- Processed, catalogued and stored patient blood samples at The Hospital for Sick Children, Toronto, ON
- Coordinated follow-up phone calls and collected patient information from patients’ parents and guardians
- Assembled research supply kits for the collection of biological samples
- Orchestrated the shipment of research supplies to 12 patient recruitment sites
- Participated in the training of research staff at 12 patient recruitment sites
- Managed study documentation such as consent forms, case report forms and electronic databases
- Participated in the writing and submission of three grant proposals to various agencies
  - Ontario Lung Association (OLA): Unsuccessful
  - Canadian Institutes of Health Research (CIHR) Operating Grant: Successful
  - Canadian Foundation for Dietetic Research (CFDR): Successful
- Participated in the creation of material transfer agreements for the analysis of serum samples at two separate laboratories
- Established a organizational plan for two -80º freezers which involved cataloguing 255 freezer boxes, containing a total of approximately 19,000 cryovials
- Combined data from three databases by re-coding variables
- Performed statistical analysis using SAS 9.4
- Wrote and submitted abstract for Infectious Disease Week 2016
- Wrote thesis manuscript
CHAPTER ONE – INTRODUCTION

1.1 Preface

The study of human nutrition is thought to have begun in the mid-1700s, far later than other biological sciences such as anatomy and physiology.\textsuperscript{1,2} In relative terms, nutritional science is young. Nevertheless, many researchers in the 1940s were said to have declared the study of nutritional science “complete” following the discovery of the vitamins and minerals.\textsuperscript{3} In present day we know this is not the case. For instance, a vast number of non-nutrient compounds, including many phytochemicals, remain to be studied. Although the nutritional science community has a strong understanding of the essential nutrients and their roles, even well-characterized compounds have been found to be involved in many more biological processes than originally speculated. Vitamin D is a prime example.

Since its discovery in 1922, vitamin D has been primarily associated with bone health outcomes.\textsuperscript{4} Accordingly, vitamin D was thought to be involved solely in the prevention and healing of rickets and osteomalacia for the majority of the 20\textsuperscript{th} century.\textsuperscript{4,5} It is now clear that the effects of vitamin D in the human body extend far beyond bone mineralization. Vitamin D is recognized to play significant roles in processes such as blood glucose regulation, gut homeostasis and inflammatory responses.\textsuperscript{6-12} Perhaps one of the most intriguing topics in contemporary vitamin D research is the compound’s seemingly protective activity against neurological disorders such as multiple sclerosis.\textsuperscript{13}

Vitamin D is also involved in respiratory health and function in humans. From a historical perspective, physicians unknowingly used vitamin D to treat respiratory conditions long before the compound was characterized. Treatment methods involving vitamin D were most important in the years prior to the discovery of antibiotics. For instance in 1849, Chapman
reported that cod liver oil – now recognized to contain vitamin D – was effective in treating tuberculosis, known at that time as consumption.\textsuperscript{14,15} Administration of cod liver oil to children would then become common practice in North America in later years.\textsuperscript{15} Beginning in the 1800s, tuberculosis sanatoriums were also established in Europe and North America where patients were regularly exposed to sunlight as a course of treatment.\textsuperscript{16} Unbeknownst to the physicians at the time, sun exposure was initiating vitamin D synthesis in the skin of their patients.\textsuperscript{16,17}

Modern-day science has granted us with a much greater understanding of vitamin D and its mechanisms of action in the respiratory system. It has now been established that vitamin D is involved in the innate and adaptive immune responses at multiple levels.\textsuperscript{7-11} However, the link between vitamin D and respiratory health is thought to be primarily mediated via the transcriptional regulation of certain protective antimicrobial peptides (AMPs) in human airways.\textsuperscript{18-20} These AMPs have been shown to possess activity against a large number of microbes, including the influenza virus.\textsuperscript{21-24}

Influenza is a respiratory illness that is responsible for 250,000 to 500,000 deaths globally each year, of which roughly 28,000 to 111,000 occur in young children.\textsuperscript{25,26} Children are often among the first to fall ill during influenza outbreaks and are hugely influential in the transmission of the virus within communities.\textsuperscript{27-29} In terms of outpatient visits and hospitalizations, influenza infections are responsible for considerable healthcare utilization costs across North America.\textsuperscript{30-32} It is also important to note that physiological responses to influenza infection are unpredictable and can vary greatly among individuals. For instance in children, outcomes such as hospitalization and development of complications following influenza infection are frequently observed in some individuals and not others. Interestingly, this variability is also observed among otherwise healthy children.\textsuperscript{33,34}
Much of the current knowledge surrounding vitamin D and influenza has been inferred from studies investigating 25(OH)D serum levels and respiratory tract infections (RTIs) in general. Recent observational studies conducted examining RTIs in children indicate that low serum vitamin D levels are associated with increased risk of infection as well as symptom severity. The observed associations between vitamin D status and respiratory health is troubling given that an estimated 24% of Canadian children aged 6 to 11 years have circulating vitamin D levels that fall below the recommended cut-off of 50nmol/L.

1.2 Limitations of Current Data

As mentioned above, a large proportion of the current understanding of vitamin D and influenza has been extrapolated from studies examining vitamin D serum levels and incidence of RTIs in general. RTIs such as pneumonia, respiratory syncytial virus and tuberculosis are of particular interest in relation to vitamin D status. However, there is an undisputable lack of data pertaining to influenza-specific outcomes. In addition, there is currently a shortage of information regarding factors that may influence the severity of influenza-related symptoms. Furthermore, no data are available to assess the role of AMPs during influenza infection in humans. No observational study or randomized controlled trial (RCT) has been conducted to date that examines vitamin D or AMP status and influenza severity in humans.

1.3 Statement of Purpose

This thesis aims to examine and describe the relationship between influenza severity and biochemical status of vitamin D, as well as of several vitamin D-related biomarkers, in children. To accomplish this, two objectives were defined: 1) Examine the relationship between serum
vitamin D levels and influenza severity in a population of influenza-positive, otherwise healthy children and 2) Investigate the associations between serum levels of three vitamin D-regulated antimicrobial peptides and influenza severity in the same study population. Results from this project are hoped to aid in identifying potentially vulnerable populations of children at risk of developing severe influenza infections in Canada, as well as worldwide.

This thesis is presented in chapters. CHAPTER TWO is a review of the current literature and is separated into five main sections: Influenza Infection, Vitamin D, Vitamin D & Respiratory Health, Study Rationale and finally, Study Objectives & Hypotheses. CHAPTER THREE is a data chapter and outlines the study Biochemical status of vitamin D and related biomarkers as predictors of severe influenza infection in children. CHAPTER FOUR is an overall Discussion and CHAPTER FIVE details Conclusions and potential Future Directions. Lastly, CHAPTER SIX and SEVEN contain References and Appendices respectively.
CHAPTER TWO – LITERATURE REVIEW

2.1 Influenza Infection

2.1.1 Etiology

Influenza is a well-characterized, acute, respiratory disease. Classified within the Orthomyxoviridae family, influenza viruses are divided into three distinct types: A, B and C. Influenza A and B viruses are the predominant pathogens involved in influenza infection in humans, while influenza C viruses are less prevalent and tend to cause mild disease. Infection with influenza B viruses appears to be generally less common than influenza A infection. Despite these differences, all influenza viruses replicate in the respiratory mucosa and induce the destruction of infected respiratory cells. The resultant inflammation, via cytokines such as interleukin (IL)-6 and IL-8, is thought to be the primary mechanism governing influenza symptoms.

2.1.2 Clinical Presentation in Children

Physiological responses to influenza infection are unpredictable and vary greatly among individuals. Outcomes such as hospitalization and development of complications following influenza infection are observed in some children but not others. This variability is also observed among otherwise healthy children. Various determinants may be at the root of the observed differences in influenza severity, including clinical, viral, environmental and genetic factors.

Uncomplicated Disease. Infection with the virus frequently results in the sudden onset of common, systemic symptoms such as fever, myalgia and fatigue. Manifestations such as nasal congestion, sore throat, headache and cough are also common. In uncomplicated disease, these
respiratory and systemic symptoms are typically self-limiting and persist for a total of one to five days. 46, 55

Complicated Disease. When complications arise or multiple organ systems become involved, an individual is at risk of developing a more severe form of disease. In addition to the common flu-like symptoms listed above, children may experience complications such as otitis media and dehydration from nausea, vomiting and/or diarrhea over the course of an influenza infection. 44, 56 Pneumonia is another common complication brought about by an influenza infection. 46 Pneumonia of solely viral origin is associated with an extremely high mortality rate; however secondary bacterial pneumonia is more prevalent. 46, 57 Although infrequent, neurological complications such as encephalitis may arise, and have been shown to occur more often in young children than in adults. 44, 58 Influenza-related deaths are uncommon among Canadian children; however severe influenza-related complications such as febrile seizures and respiratory failure arise frequently. 59, 60

2.1.3 Epidemiology

Viral Transmission. While the influenza B virus is largely considered to be specific to humans, influenza A is a pathogen capable of infecting multiple hosts, including non-human animals (animals) such as pigs and birds. 61 Animal hosts play influential roles in the evolution of influenza strains, and thus are highly involved in the emergence of pandemics in human populations. 61 Knowledge surrounding the transmission of influenza from animal hosts to humans is extremely limited, however communication of the disease between humans is better understood. 61
The influenza virus is spread between people via three principal routes: (1) airborne through the inhalation of small virus-containing droplets (aerosols), (2) airborne through the inhalation of large virus-containing droplets and (3) through direct contact with the virus.\textsuperscript{62,63} Airborne droplets may be created when an individual coughs, sneezes or speaks.\textsuperscript{62} Direct contact transmission occurs when an infected surface is touched and the individual’s hands then come in contact with mucous membranes of the nose or eyes, for example.\textsuperscript{62} Aerosol transmission is the predominant pathway and has been found to account for the majority of influenza transmissions within households.\textsuperscript{62-65}

In addition to households, the transmission of influenza occurs in schools, daycares, workplaces and other locales where individuals coexist in close proximity.\textsuperscript{66} Recently, it has been proposed that enforcing strict prophylactic vaccination policies to all school-aged children (specifically those 2-16 years of age) would be cost-effective and significantly decrease influenza transmission within communities.\textsuperscript{67} There is a plethora of evidence to support this notion. For instance, children are among the first to fall ill during influenza outbreaks and are highly influential in the transmission of influenza within communities.\textsuperscript{27-29,66} It is thought that their prominent role in disease transmission may be partially explained by the fact that children (<13 years of age) experience longer viral shedding periods, resulting in a need for longer isolation periods that may not be achieved.\textsuperscript{67,68} Moreover, a study investigating the immune status of German children found that individuals younger than 6 years old had significantly lower serum levels of antibodies against influenza A and B, compared to adults.\textsuperscript{69} These results demonstrate that young children may have less of the acquired immunity needed to protect against the virus.\textsuperscript{67}
**Seasonality.** Populations may be affected by influenza infections in the form of epidemics as well as through sporadic, pandemic outbreaks.\textsuperscript{43,46} While epidemics affect a specific location such as a city or region, pandemics affect larger geographical areas and typically result in more deaths and greater societal disruption. As epidemics, influenza infections occur cyclically within populations with distinct peaks and troughs throughout the year. In the temperate climates of the Northern and Southern Hemispheres, seasonal influenza infection outbreaks are most frequent during winter months.\textsuperscript{70} In tropical and subtropical climates, the influenza season is less clearly defined however it has been shown to coincide with the rainy season (July-September).\textsuperscript{71-73}

There are a number of factors that have been proposed as determinants of influenza infection seasonality in humans. Environmental elements such as decreases in air temperature and humidity levels appear to be strongly related to rates of infection, possibly by affecting the virus’s ability to survive.\textsuperscript{70,74} Societal and cultural changes such as school attendance and indoor crowding in winter months have been proposed to increase transmission rates.\textsuperscript{75} Notably, the decrease in solar ultraviolet (UV) radiation exposure that occurs during winter and rainy season months has also been postulated to be involved in the seasonality of the disease. Firstly, it has been shown that the influenza virus is inactivated by UVB rays – a model for solar radiation – and therefore may not survive in the air as readily during summer months.\textsuperscript{76,77} Furthermore, the host’s ability to fight infection may be compromised during winter months when there is less UV radiation and levels of immunosupportive compounds such as melatonin and vitamin D are subsequently altered in the body.\textsuperscript{75}

**Burden of Disease in Children.** Approximately 3-5 million cases of influenza leading to severe disease arise annually worldwide, wherein roughly 1 million of these severe cases occur
In children under 5 years of age.\textsuperscript{25,26} Influenza accounts for 250,000 to 500,000 deaths globally each year, of which roughly 28,000 to 111,000 are in young children.\textsuperscript{25,26} In Canada it has been estimated that 3,500 deaths can be attributed to influenza each year.\textsuperscript{78} Although occasionally viewed as a relatively innocuous illness and chiefly harmful in the very young, the very old and those with underlying comorbidities, influenza is also a real threat to healthy children.\textsuperscript{33} In a Canadian multicenter study examining the 2004/2005 to 2008/2009 influenza seasons, 50\% of children hospitalized for influenza A had no prior underlying medical condition.\textsuperscript{33} Thus, influenza is a pervasive illness that poses risks to susceptible and healthy populations alike.

In terms of outpatient visits and hospitalizations, influenza infection is responsible for considerable healthcare utilization costs in North America.\textsuperscript{30-32,79} It has been estimated that 1,400 hospitalizations attributable to influenza infection occur annually in Canada among children 0-19 years of age.\textsuperscript{80} An American study found that the total mean cost for influenza-related hospitalization in children was $13,159 USD per child during the 2000-2004 flu seasons.\textsuperscript{81} The total mean cost for pediatric patients cared for in the Intensive Care Unit (ICU) was $39,792 USD per child.\textsuperscript{81} Influenza in children also leads to significant economic productivity losses due to the increased need for caregivers and the resultant loss of parental time spent at work.\textsuperscript{82}

**Control of Influenza Infection.** Influenza infection is predominantly controlled in populations through the implementation of seasonal, prophylactic vaccination policies. In infected children, antiviral therapy is occasionally used in instances of severe illness. However, there are known limitations to the modern prophylactic and treatment strategies used in the management of the disease. The National Advisory Committee on Immunization (NACI) recommends that all children and adolescents ≥6 months of age be vaccinated annually against the influenza virus in Canada.\textsuperscript{83} While data regarding influenza vaccine coverage in children are
limited, it has been estimated that less than 20% of Canadian children <2 years old had been vaccinated against influenza in 2006, 2009 and 2011.\textsuperscript{84} In addition, the efficacy of contemporary vaccines is limited by antigenic drift, which results in the need to reformulate the seasonal influenza vaccine annually.\textsuperscript{85} In cases of severe influenza, antiviral treatment with drugs such as oseltamivir and zanamivir is recommended. Although effective, these drugs are used with caution owing to the virus’ ability to mutate rapidly, leaving populations at risk of antiviral resistance.\textsuperscript{86}

**Variation in Influenza Severity among Children.** As previously stated, the observed symptoms and repercussions of influenza infection fall into a remarkably broad spectrum. While some infected children remain asymptomatic, or present with symptoms similar to the common cold, others may experience pulmonary and extrapulmonary complications leading to organ damage and death.\textsuperscript{87-89} Multiple determinants may account for these observed discrepancies. Clinical, viral, genetic, environmental and nutritional risk factors for severe influenza infection are reviewed below.

**Clinical.** The severity of influenza symptoms has been shown to be dependent upon a number of clinical factors, including age and sex. The rate of hospitalization – an indicator of severe disease – is highest in young children (<5 years of age).\textsuperscript{45,90,91} Male children are at higher risk of developing influenza-related complications than females.\textsuperscript{92-94} In addition, children of certain ethnicities (African-American, Aboriginal and Hispanic) are at higher risk of developing severe influenza-related symptoms than Caucasian children.\textsuperscript{91,95-97} Preexisting conditions such as obesity, lung disorders and developmental delays also predispose children to severe disease outcomes.\textsuperscript{45,91,97,98} Both prior influenza vaccination and antiviral therapy early in the course of infection have been shown to attenuate disease symptoms in infected children.\textsuperscript{99-103}
Viral Elements. Associations between viral genetics and influenza infection outcomes have been heavily studied. Influenza type and sub-type are known to be associated with disease severity in humans.\textsuperscript{43,46} For instance, influenza C viruses do not induce outbreaks and tend to cause symptoms similar to a common cold.\textsuperscript{43,104} Conversely, influenza A viruses have been responsible for the hugely devastating human pandemics of the 18\textsuperscript{th}, 19\textsuperscript{th} and 20\textsuperscript{th} centuries.\textsuperscript{43,46,104}

In seasonal influenza outbreaks, influenza A and B viruses may circulate simultaneously.\textsuperscript{44,45,105} Symptom presentation is generally similar across the two types, however some slight differences in disease outcomes have been observed. Complications such as myalgia and myositis have been reported more commonly in influenza B-positive children.\textsuperscript{106-108} It has also been shown that influenza B may affect children with underlying medical conditions such as oncological disorders and cardiac abnormalities at a higher frequency than influenza A.\textsuperscript{109}

It has been postulated that influenza A strains may result in more severe disease than influenza B infection in children.\textsuperscript{109-111} Increased need for supplemental oxygen therapy as well as increased rates of co-infection with other viruses has been observed following influenza A infection.\textsuperscript{109,112} However, a retrospective study conducted by Peltola et al. covering 20 influenza seasons, found that when compared to influenza B, influenza A infections presented more commonly in slightly younger children (A = 2.0 years, B = 4.2 years, p-value<0.001).\textsuperscript{44} Similar findings have been reported in a number of other studies.\textsuperscript{112-114} Therefore, it is conceivable that influenza A infection may result in more severe disease due to the slightly younger population that it has been shown to affect.

Host Genetic. In contrast to the influence of viral genetic factors on influenza severity, much less is known regarding the effects of human genetic variation. In 2008, Albright et al.
published a study that investigated the influenza-related deaths of 4855 Americans between 1904 and 2004. Both close and distant relatives of individuals who died of influenza infections were found to have a significantly higher relative risk (RR) of also dying from influenza (RR=1.54; 95% CI 1.42-1.67; p-value<0.0001). The RR was calculated based on a standardized mortality rate (i.e. expected death rate) in the population. These findings indicate that there may be heritable traits that predispose certain individuals to severe influenza infection.

More recently, a number of studies have reported on genetic risk factors for severe disease during the influenza A(H1N1) pandemic of 2009. These findings have related specifically to certain single nucleotide polymorphisms (SNPs) in genes encoding cytokine-regulating transcription factors, such as IRF7. However, contradictory results have also been published. For example, a recent Spanish study concluded that no genetic factors predisposed individuals to severe influenza-related complications or death during the H1N1 pandemic. It is the opinion of some specialists that, to date, studied cohorts have been too limited to definitively support the hypothesis that there are genetic risk factors involved in influenza disease outcomes.

Environmental. Numerous environmental determinants of influenza infection outcomes have been investigated. Lower socioeconomic status has consistently been shown to correlate with increased risk of severe disease in children. In the same vein, children living in crowded conditions also develop complications at a higher rate. Air quality is another important determinant of respiratory health. Children living in urban settings have been reported to experience a higher incidence of influenza infection as well as more serious disease outcomes than children living in rural areas. A recent study published in The Journal of Pediatrics reported that children regularly exposed to secondhand tobacco smoke were 4.7 times more
likely to be admitted to the ICU and experienced a 70% longer length of hospital stay (LOS) over the course of the influenza infection.\textsuperscript{129}

**Nutritional.** Nutritional inadequacies and respiratory tract infections (RTIs) such as influenza are tightly linked health concerns in children, particularly in developing countries.\textsuperscript{53} In 2004, the dominant nutritional risk factors leading to RTI-related mortality in young children in Latin America, Asia and Africa were: stunting, low birth weight, suboptimal breast feeding and underweight.\textsuperscript{130} While zinc deficiency was estimated to account for a fraction of these deaths, specific nutrient deficiencies were not considered to be major contributing factors in pediatric RTI-related deaths in these regions.\textsuperscript{130} In addition, it is important to note that in developing countries, individuals are often deficient in numerous nutrients concurrently. Therefore it is difficult to determine the individual effects of specific micronutrients on respiratory health. Nonetheless, investigating particular nutrients independently with regard to respiratory disease has merit.

Certain antioxidants such as selenium and vitamin C have been identified as participatory in regulating influenza severity in murine models.\textsuperscript{131,132} Mice with diets supplemented in selenium experienced significantly lower mortality rates post-influenza A(H1N1) infection than selenium-deficient mice.\textsuperscript{131} Following intranasal inoculation with influenza A(H3N2), vitamin C-insufficient mice had significantly lower levels of an important antiviral cytokine, IFN-α/β.\textsuperscript{132} Furthermore, levels of pro-inflammatory cytokines such as TNF-α and IL-α/β were significantly increased in the murine lung tissue.\textsuperscript{132} Vitamin A-deficiency has also been investigated in relation to influenza infection in mice.\textsuperscript{133} High dietary levels of vitamin A in the form of retinyl palmitate significantly increased titer concentrations of the protective antibody Immunoglobulin A (IgA) in saliva post-influenza A(H3N2) infection.\textsuperscript{133} Lastly, while the studied effects of the
above nutrients appear promising, vitamin D status is of particular interest in relation to influenza severity.\textsuperscript{134}

2.2 Vitamin D

2.2.1 Structure and Function

Vitamin D is a fat soluble vitamin required for numerous metabolic processes in the human body. It is essential for modeling and remodeling of bone tissue, and has more recently been shown to be involved in metabolic processes such as lipid and glucose metabolism.\textsuperscript{17} Vitamin D is naturally present in a small number of foods as either: ergocalciferol (vitamin D\textsubscript{2}) from plant sources such as fungi or cholecalciferol (vitamin D\textsubscript{3}) from animal sources including fatty fish such as salmon, mackerel and snapper.\textsuperscript{17,135} The slight structural differences between D\textsubscript{2} and D\textsubscript{3} lead to a decreased affinity of D\textsubscript{2} for vitamin D binding protein (VBP), an important circulatory protein.\textsuperscript{17,136} As a result, D\textsubscript{2} is cleared more rapidly from the circulation and raises circulating levels of vitamin D less effectively than D\textsubscript{3}.\textsuperscript{17,137,138} Historically D\textsubscript{2} has been more commonly used in food fortification in North America; however it appears that manufacturers may be shifting to include D\textsubscript{3}.\textsuperscript{17,139} In Canada, many food products are fortified with vitamin D including milk, yogurt, margarine and orange juice.\textsuperscript{135,140}

While vitamin D occurs naturally in select dietary sources, a crucial fraction of vitamin D is created endogenously in humans. The endogenous process necessitates direct UVB radiation from sunlight onto exposed skin. During sun exposure, 7-dehydrocholesterol – a cholesterol metabolite – is converted to previtamin D\textsubscript{3} through a non-enzymatic process.\textsuperscript{17,141} Previtamin D\textsubscript{3} then undergoes thermal isomerization to become vitamin D\textsubscript{3}.\textsuperscript{141} Vitamin D\textsubscript{3} is then carried to the liver to undergo further modification into 25-hydroxyvitamin D [25(OH)D].\textsuperscript{7,141} Following the
liver, 25(OH)D is metabolized to 1,25-hydroxyvitamin D [1,25(OH)D] in renal cells, as well as in immune cells such as macrophages and dendritic cells. In the human body, 25(OH)D is the major circulating form of vitamin D. However, it is widely accepted that 1,25(OH)D is the most biologically active form, regulating hundreds of biological processes.

2.2.2 Biochemical Assessment of Vitamin D Status

More than fifty vitamin D metabolites have been identified in humans. However, very few have been quantified in blood and only 25(OH)D and 1,25(OH)D have garnered significant attention. It is generally considered that biochemical assessment of 25(OH)D is the gold standard in determining vitamin D status. The long half-life of 25(OH)D (>3 weeks) makes it a more practical, predictive biomarker than 1,25(OH)D which has a half-life of only four hours. Moreover, circulating 1,25(OH)D levels are tightly regulated and subject to rapid fluctuations depending on physiological inputs such as hormonal activity of parathyroid hormone (PTH). Lastly, it is thought that 1,25(OH)D levels may not decrease significantly until the late stages of a vitamin D deficiency.

Given that 25(OH)D is highly lipophilic and that a large portion is tightly bound to VDP in circulation, measuring 25(OH)D levels in blood can be challenging. Several assays exist to assess 25(OH)D concentrations, and in the majority of commercially available 25(OH)D assays, either serum or plasma may be used. Various methods have been developed over the past fifty years including immunoassays and competitive protein-binding assays. Automated immunoassays such as the DiaSorin LIAISON 25 OH vitamin D total assay have become widely used in clinical and research settings due to their simplicity, efficiency and relatively low cost. However, it has been observed that at lower 25(OH)D serum levels (<12.2 nmol/L) these assays
do not satisfy performance standards such as maximum coefficients of variation.\textsuperscript{145} In addition, it has been repeatedly demonstrated that there is a lack of consistency in the results reported between automated assays.\textsuperscript{136,145,146} Chromatographic assays, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS), offer additional methods of assessing levels of 25(OH)D and 25(OH)D metabolites in serum or plasma.\textsuperscript{136,142,145,147} However, this method requires costly equipment and highly trained staff.\textsuperscript{147}

2.2.3 Recommended Vitamin D Serum Concentrations and Intakes for Children

Organizations such as the Canadian Pediatric Society, the Institute of Medicine (IOM), Health Canada and the American Academy of Pediatrics have put forth recommendations regarding optimal serum 25(OH)D levels for clinical practice (Table 1).\textsuperscript{38,39,148-151} The IOM recommendations state that 25(OH)D serum levels lower than 50 nmol/L are suboptimal in children.\textsuperscript{38,39,148-151} These recommendations have primarily been founded on the basis of bone-health outcomes and many experts believe new guidelines derived from evidence surrounding non-skeletal health outcomes should be created.\textsuperscript{148,152} In terms of recommended intakes, new Dietary Reference Intakes based on the most recent IOM report, were announced by Health Canada in 2010.\textsuperscript{39,148} The Recommended Dietary Allowance (RDA) for all children older than 12 months is 600 IU.\textsuperscript{39,148} No RDA for vitamin D has been established in infants.\textsuperscript{39,148} However, an Adequate Intake (AI) has been determined and is set at 400 IU for children under 12 months of age.\textsuperscript{39,148}
Although controversial, vitamin D deficiency is typically defined as levels where bone disease is likely to occur, while insufficiency is used to describe levels associated with non-skeleton disease outcomes.\textsuperscript{153} 

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<th>Institution</th>
<th>Year Implemented</th>
<th>Serum 25(OH)D levels (nmol/L)</th>
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2.2.4 Prevalence of Pediatric Vitamin D Deficiency

An estimated 24\% of Canadian children aged 6 to 11 years, and 29\% of children aged 12 to 19, have vitamin D levels that fall below the recommended cut-off of 50 nmol/L set by the IOM.\textsuperscript{38,39} Children under the age of 5 years are less likely to be vitamin D deficient, with approximately 11\% of young children falling below the 50 nmol/L cut-off in Canada.\textsuperscript{38} This discrepancy in the prevalence of vitamin D deficiency among age groups may be partially explained by more significant amounts of fortified dairy products consumed by younger children, as well as potential pubertal affects in teenagers.\textsuperscript{154,155} It has been hypothesized that during adolescence, 25(OH)D stores are depleted more rapidly due to the characteristic increase in bone development during this period.\textsuperscript{155} As expected, the vitamin D status of Canadians fluctuates depending on the season.\textsuperscript{38} Although data is not available for children specifically, an estimated 25\% of Canadians have serum 25(OH)D levels below the 50 nmol/L cut-off during summer months, compared to 40\% between November and March.\textsuperscript{38}

Certain populations of Canadian children are at higher risk of being vitamin D insufficient or deficient. In a population of James Bay Cree Canadians, 78.7\% of females and 65.1\% of males age 15 to 19 years were found to have circulating 25(OH)D levels <50
Within the same population of Cree Canadians, 43% of children sampled, age 9 to 13 years, had 25(OH)D levels below the 50 nmol/L cut-off. These percentages are significantly higher than the reported national averages for school-aged children. In addition, a 2014 study examining a large, pediatric population in Toronto found that children who were born, or whose parents were born, outside of a Western country were significantly more likely to have serum 25(OH)D levels <50 nmol/L (OR: 1.9, 95% CI: 1.3-2.9). Mean differences in serum levels were found to be primarily influenced by disparities in vitamin D supplementation between the groups of children.

In developing countries, much higher rates of pediatric vitamin D deficiency and insufficiency have been reported. A study conducted in Mongolia found that approximately 76% of children (ages 9-11 years) had 25(OH)D serum levels below 50 nmol/L. In 2002 it was reported that 80% of newborns in a Turkish cohort had serum 25(OH)D levels below 25 nmol/L. Despite sufficient sunlight year-round, individuals in many Middle-Eastern and African countries have been consistently reported to have low circulating vitamin D levels. It is thought that degrees of skin pigmentation and cultural practices involving traditional clothing styles may play a role.

2.2.5 Factors that Affect Vitamin D Status

Skin pigmentation is a particularly important factor that governs vitamin D status in humans. Darker-skinned individuals experience less effective sunlight-induced vitamin D production than lighter-skinned people. Cultural practices such as veiling and other conservative clothing styles are thought to decrease the amount of skin exposed to sunlight and thereby decrease dermal vitamin D synthesis. Factors such as a socioeconomic status
and sex are also thought to influence vitamin D status in developing countries. Specifically, individuals in low-income families, and females of all ages, are at an increased risk of being vitamin D deficient. Seasonality and the amount of time an individual spends outdoors are also important contributing factors to vitamin D status. However, it has been previously postulated that skin pigmentation, season, and outdoor play time may be less predictive of vitamin D status in young, Canadian children due to contemporary sun avoidance customs (i.e., use of sunscreen and protective clothing).

Geographical region is another significant predictor of vitamin D status. In regions greater than approximately 55° North or South of the equator, no vitamin D₃ is produced in the skin during fall and winter months (≈ six months of the year). It has recently been proposed that the total level of ambient UVB radiation is a more significant determinant of vitamin D status than latitude. Calculations of ambient UVB exposure take into account latitude as well as factors such as cloud cover and condition of the ozone layer in specific geographic regions. It has been proposed that these calculations, used in conjunction with ethnicity data, could be adopted in large epidemiological studies when direct measurements of vitamin D status are not available.

Adiposity has also been proposed to affect vitamin D metabolism and storage in humans. Many observational studies have reported positive associations between vitamin D deficiency and obesity. While the mechanisms remain uncertain, it has been proposed that higher amounts of adipose tissue may lead to increases in stored vitamin D, thereby decreasing bioavailable levels of the vitamin. Along these lines, Drincic et al. found that obese individuals required higher levels of supplemental vitamin D₃ to attain the same serum levels of
25(OH)D, compared to people with healthy body mass indices (BMIs). The authors proposed that new dietary recommendations should be set forth based on weight.

Dietary intake of vitamin D is a highly influential determinant of vitamin D status. Exclusively breast-fed infants are at risk of vitamin D deficiency due to the fact that vitamin D metabolites are present in human milk at very low levels. To meet recommendations established by the various pediatric societies mentioned above, nursing infants generally must obtain vitamin D₃ in supplemental form. However, a recent RCT conducted by Hollis et al. found that lactating mothers supplemented with 6400 IU of vitamin D₃ daily safely increased their breast milk vitamin D levels to amounts that satisfied their infants’ requirements. Similar results were observed in another recent RCT, where only 2000 IU of vitamin D₃ daily beginning in gestation sufficiently increased breast milk levels of vitamin D to meet the infants’ requirements up to at least 8 weeks postpartum.

In older children and adults in North America, dietary consumption of vitamin D is largely in the form of fortified dairy products. Data from the Canadian Health Measures Survey (Cycle 2) show that within the sampled population, children who consumed cow’s milk once or more per day had markedly higher 25(OH)D serum levels than children who consumed milk less than once per day. It is important to note that non-cow’s milk beverages such as rice, almond and soy milk are not required to be fortified with vitamin D in Canada. Results from a recent study conducted in Toronto indicated that the consumption of milk substitutes in lieu of fortified dairy products may leave children at risk of vitamin D deficiency.
2.3 Vitamin D and Respiratory Health

Although long associated with bone health outcomes, vitamin D has more recently been proposed to be involved in respiratory health and function in humans. Many beneficial properties of 25(OH)D have been investigated, including the compound’s ability to lower multiple inflammatory markers, both in vitro and in humans. Importantly, oral administration of 25(OH)D has been reported to decrease inflammation in the airways of asthmatic children. Vitamin D is also involved in the immune response at multiple levels (Figure 1). However, it is thought that the primary avenue by which vitamin D acts in the respiratory system is through the transcriptional regulation of antimicrobial peptides (AMPs).

![Figure 1](Image)

**Figure 1** – Summary of the involvement of 1,25(OH)D in the innate and adaptive immune response.

2.3.1 Vitamin D Regulated AMP Production

AMPs are integral components of the innate immune system and are known to possess antibacterial, antiviral and antifungal properties. Certain AMPs have also been shown to play a role in chemotaxis and cytokine production. According to The Antimicrobial Peptide Database© operated out of the University of Nebraska, 112 AMPs have been identified in humans to date. Upregulated in response to certain pathogens, these peptides are primarily
synthesized in, and subsequently excreted from, epithelial cells and phagocytes such as neutrophils. As a result, AMPs are the second line of defense in the respiratory system, following the protective mucosal layer. In addition to the respiratory system, these compounds play crucial roles in the defense of many organ systems including the gastrointestinal tract, the integumentary system and the urogenital system. AMPs have been investigated in relation to numerous pathogens such as *Helicobacter pylori*, HIV, *Mycobacterium tuberculosis*, the herpes simplex virus and *Candida albicans*.

AMP research is particularly intriguing with regard to influenza. At present, novel anti-influenza regimes are being extensively studied and certain AMPs are under investigation as potential anti-viral therapies. As both the current and novel therapies have a wide range of physiological targets, combination antiviral therapy may become common practice in the treatment of severe influenza in the future. While achievements in areas such as *de novo* AMP synthesis are compelling, much work remains to be done. Research regarding the use of AMPs in treating influenza infection has not passed *in vitro* and animal model stages thus far. Efforts should now be focused on obtaining high-quality, observational data in human populations.

While there are numerous families of AMPs, two particular classes are most commonly studied in relation to influenza: cathelicidins and defensins. Direct inhibition of the virus by several cathelicidins and defensins has now been observed. The endogenous expression of AMPs is modulated in a number of ways, however certain members of both aforementioned AMP families are regulated by 1,25(OH)D – the bioactive form of vitamin D. Specifically, 1,25(OH)D activates gene expression by functioning as a transcription factor. Acting via a Toll-like Receptor (TLR) pathway, 1,25(OH)D binds to Vitamin D
Response Elements (VDREs) in the promoter regions of AMP genes.\textsuperscript{20,202,203} It is important to note that respiratory epithelial cells are known to convert \(25(\text{OH})\text{D}\) to \(1,25(\text{OH})\text{D}\).\textsuperscript{20,205} The AMPs that have been shown to possess anti-influenza activity and that are regulated by \(1,25(\text{OH})\text{D}\) are reviewed below.

**Cathelicidins.** Human cathelicidin LL-37 is one of the main AMPs regulated by \(1,25(\text{OH})\text{D}\). The LL-37 peptide is the only known cathelicidin in humans, and has been shown to be involved in the host defense of the lung through its expression in airway epithelial cells.\textsuperscript{18,206} Four of the most noteworthy experiments regarding LL-37 and influenza were conducted in 2011 by Barlow *et al.*\textsuperscript{23} The research group first observed that, in mice infected with a strain of influenza A, the delivery of nebulized LL-37 molecules significantly increased survival rates compared to mice nebulized with a saline solution (LL-37:~60\%, saline:~15\%, \(p\)-value<0.001).\textsuperscript{23} Infected mice nebulized with LL-37 also had significantly decreased viral concentrations in their bronchoalveolar lavage fluid (BALF) compared to mice who received the saline solution.\textsuperscript{23} A second treatment group of infected mice was nebulized with the anti-influenza drug zanamivir.\textsuperscript{23} The protection offered by the drug was found to be comparable to the protection provided to the mice by LL-37.\textsuperscript{23}

To elucidate LL-37’s mechanisms of action, Barlow *et al.* conducted three subsequent, well-designed experiments.\textsuperscript{23} With the goal of determining if the peptide decreased viral activity by altering the inflammatory response, BALF of infected and uninfected mice was analyzed.\textsuperscript{23} It was reported that the BALF of infected mice, who received either nebulized LL-37 or saline, contained significantly different levels of only two among many cytokines known to be involved in the inflammatory response against influenza.\textsuperscript{23,207} The authors then investigated the hypothesis that LL-37 inhibits the virus directly, at the cellular level.\textsuperscript{23} It was observed that when a strain of
influenza A(H1N1) was incubated in vitro with various physiologically relevant concentrations of LL-37, virus titers were decreased by 90% over one hour (p-value<0.05).23

The authors were next interested in determining if LL-37 was acting via a receptor-mediated pathway on the viral membrane. Barlow et al. used a synthesized version of LL-37 containing only D-amino acids, as opposed to the L-amino acids present in the naturally occurring LL-37 peptide.23 It was determined that the D-peptide demonstrated the same degree of antiviral activity as the L-enantiomer, both in vitro and in nebulized mice.23 Collectively, data from the multiple experiments performed by Barlow et al. suggest that the antiviral activity of LL-37 is likely not governed by: (1) a modified inflammatory response or (2) stereoisomer-specific receptors. It appeared to the authors that LL-37 impaired the influenza virus directly.23 These results were supported by in vitro findings two years later.21 In 2013, it was observed that LL-37 inhibits several influenza A virus strains directly, via degradation of the viral membrane.21

The anti-influenza properties of LL-37 have been firmly demonstrated in murine and in vitro models. Despite this fact, no study has been conducted in humans that relates LL-37 levels to influenza-specific outcomes. However, numerous observational studies have investigated systemic levels of LL-37 in relation to tuberculosis, another respiratory pathogen against which the peptide is active.202 In 2010, it was observed that high serum levels of LL-37 were correlated with clinical markers of infection in a group of North American tuberculosis patients.208 More recently, a Turkish research group determined that circulating LL-37 levels were significantly higher in tuberculosis patients than in healthy controls (p-value = 0.01).209 Similar results were reported in a Chinese cohort.210

Not surprisingly, tuberculosis patients are routinely reported to have lower 25(OH)D serum levels than healthy individuals in observational studies.211-213 It has been previously
demonstrated that circulating 25(OH)D levels correlate with plasma concentrations of LL-37 in humans.\textsuperscript{214,215} This correlation is controversial, as a number of \textit{in vivo} studies have also reported that no relationship was observed between circulating 25(OH)D and LL-37 levels.\textsuperscript{208,216,217} Further research is required to elucidate the relationships between serum 25(OH)D status, both systemic and local LL-37 levels, and clinical outcomes associated with influenza infection.

**Defensins.** 1,25(OH)D is also responsible for the transcriptional regulation of beta-defensin 2 (BD2) and beta-defensin 3 (BD3), two additional endogenous antimicrobial peptides.\textsuperscript{20,218} While there are numerous known defensins involved in the human immune system, BD2 and BD3 are inducible by 1,25(OH)D and are recognized as having anti-influenza activity.\textsuperscript{22,219,220} In addition, BD2 and BD3 are expressed in the airway epithelium in humans.\textsuperscript{221-223} Both BD2 and BD3 have been shown to be up-regulated in the upper and lower airways of mice during influenza A infection.\textsuperscript{22,224} However in contrast to LL-37, much less research has been conducted with regard to the anti-influenza activity of BD2 and BD3 in murine and \textit{in vitro} models.

In 2005, a novel mechanism by which beta-defensins inhibit viral proliferation was identified.\textsuperscript{22} Leikina \textit{et al.} observed that the defensins, including BD3, linked and immobilized glycoproteins found on the influenza virus membrane \textit{in vitro}.\textsuperscript{22} Viral fusion to target cells, as well as viral mobility, was thereby inhibited by the defensins.\textsuperscript{22} It has also been demonstrated \textit{in vitro} that BD2 acts synergistically with immune cells to increase influenza A virus-uptake by neutrophils.\textsuperscript{225} However, as is the case with LL-37, there is no data relating BD2 and BD3 levels to influenza-specific outcomes in humans.

A Turkish research group has performed the only two studies examining BD2 or BD3 levels with respect to acute respiratory infections in humans.\textsuperscript{209,226} Both studies were conducted
in pediatric populations. In 2014, Cakir et al. observed that average BD2 levels in the BALF of tuberculosis patients were higher than in the BALF of healthy controls.\textsuperscript{209} However, the results were not statistically significant (p-value = 0.11).\textsuperscript{209} The following year, the authors examined circulating, systemic levels of BD2 in pediatric patients with post-infectious bronchiolitis obliterans.\textsuperscript{226} Serum levels of BD2 were found to be significantly higher in children with bronchiolitis compared to healthy controls (1.06±0.24 and 0.67±0.72 ng/mL respectively, p-value≤0.001).\textsuperscript{226} Neither study detected a correlation between BD2 concentrations and 25(OH)D levels in the children.\textsuperscript{209,226}

2.3.2 Vitamin D Status and Influenza Infection

A large proportion of the current knowledge surrounding vitamin D and influenza has been extrapolated from studies examining outcome measures indirectly related to influenza infection. For instance multiple observational studies, examining RTIs in general, indicate that low serum 25(OH)D levels are associated with increases in both incidence of disease and symptom severity in children.\textsuperscript{35-37,227-229} RTIs such as pneumonia, respiratory syncytial virus (RSV) and tuberculosis are of particular interest in relation to 25(OH)D status.\textsuperscript{40-42} In addition, findings regarding self-reported “influenza-like illness” are presented frequently in the literature without laboratory assessment of virus type.\textsuperscript{230-235} As mentioned above, the relationship between influenza and vitamin D has also been studied indirectly by exploring the role of 1,25(OH)D in the innate immune system via AMPs. In summary, data regarding vitamin D status and laboratory-confirmed, influenza-specific outcomes are scarce. The current evidence pertaining to vitamin D and influenza is reviewed below.
In vitro. To investigate the cellular response to influenza A(H1N1) infection, Khare et al. undertook a study using a human lung epithelial cell model. Cells were bathed in a 1,25(OH)D solution either pre- or post-infection with the virus. It was determined that treatment with 1,25(OH)D at both time points significantly altered the cellular inflammatory response typically observed during influenza A infection. Specifically, 1,25(OH)D treatment reduced the pro-inflammatory cascade via IL-6 and IL-8. These findings were determined through the analysis of cytokine mRNA expression in the cells. It is important to note that increased in vivo serum levels of IL-6 and IL-8 have been shown to positively correlate with outcome severity in critically ill influenza A(H1N1) patients.

Animal. Two studies, conducted in 1949 and 1956 respectively, investigated vitamin D₂ supplementation and influenza infection susceptibility in murine models. Influenza severity was examined as the primary outcome in both trials. Severity was quantified using a scoring method where scores were calculated based on the extent of lung consolidation observed post-sacrifice. In 1949, Young et al. separated mice (n=593) into eight treatment groups. Diets were supplemented with vitamin D₂ in concentrations ranging from 2.5 IU to 1000 IU per 100 grams of feed. The vitamin D₂ was sourced from Fleischman’s brewer yeast type 9F (9,000 IU per gram). Both treatment and control groups received the diets for a total of 38 days: 28 days prior to viral inoculation and 10 days post-inoculation. Mice were sacrificed and severity scores were derived on the 38th day of feeding. Mice were inoculated intranasally with a virus suspension. The authors refer to the virus as “swine influenza S-15.” Although unclear, the virus was likely a subtype of influenza A. By analyzing severity scores, Young et al. determined that mice fed diets higher in vitamin D₂ experienced less severe influenza symptoms in comparison to mice fed diets low in vitamin D₂ (p-value<0.01).
Young’s group conducted a similar trial in 1956, this time examining diets supplemented with various fat-soluble vitamins. Following weaning, young mice (n=1,832) were separated into nine feeding groups: a control group, a vitamin A group, a vitamin E group, a vitamin D₂ group and fives groups fed various combinations of the three vitamins. Similarly to the 1949 trial, vitamin D₂ was sourced from Fleischman’s yeast. However it is important to note that in 1956, mice in groups fed vitamin D₂ received a fixed amount of only 10 IU per 100 grams of feed. Following the same feeding and inoculation schedule as the earlier trial, mice were sacrificed 10 days post-infection. No significant difference was observed in lung consolidation levels between the control group and the vitamin D₂ group (p-value=0.72). Mice were also stratified into two additional groups for analysis: (1) all mice fed dietary vitamin D₂ (n=814) and (2) all mice that received no dietary vitamin D₂ (n=801). Severity scores were not significantly different between the two groups.

Observational Studies in Humans. As of September 2016, no observational study had been conducted examining the relationship between vitamin D and laboratory-confirmed, influenza-specific outcomes in humans.

Randomized Controlled Trials in Humans. Presently only two randomized controlled trials (RCTs) have been conducted examining vitamin D supplementation and influenza infection specifically. Uras hima et al. championed both projects in pediatric populations between 2008 and 2014. The primary outcome in both trials was incidence of laboratory-confirmed influenza A infection. The first RCT was performed in a population of 324 Japanese children (6-15 years old). The treatment arm (n=167) received 1200 IU of vitamin D₃ daily for a period of four winter months between December and March. In the treatment group, a significantly lower incidence of seasonal influenza A infection was observed over the course of the
supplementation period (10.8% vs. 18.6%, p-value=0.04). However, the incidence of influenza B infection was not significantly different between the supplementation and placebo groups. In addition to the limitation of a relatively small sample size, neither baseline or follow-up 25(OH)D serum levels were analyzed.

In 2009, Urashima et al. began their second RCT in collaboration with Harvard Medical School. A population of 247 Japanese high-school students was randomized to receive 2000 IU of vitamin D₃ per day or a placebo. The study took place over only two months, between October and December. Supplementation adherence was assessed using daily logs. Over the first month, influenza incidence was significantly lower in the treatment arm (1.4% vs. 8.1, p-value=0.009). However, the supplementation group then experienced higher infection rates than the placebo group in the second month, rendering the overall results insignificant (13.5% vs. 12.1%, p-value=0.75). The limitations of a small sample size and a lack of 25(OH)D serum level data are also a limitation of this trial. Furthermore, a supplementation period of only two months may not have been sufficient for blood levels of 25(OH)D to reach steady-state. Lastly, important factors such as vaccination status do not appear to have been assessed in either trial.

### 2.4 Study Rationale

In conclusion, the purported capacities of vitamin D in reducing susceptibility to influenza infection appear promising. However much of the current knowledge surrounding vitamin D and influenza has been inferred from studies investigating 25(OH)D serum levels and RTIs in general. The indiscriminate investigation of RTIs caused by numerous, distinct microbiologic agents has been beneficial in acquiring a broad understanding of 25(OH)D and respiratory
illnesses. Nevertheless, there is a lack of data pertaining to influenza-specific outcomes. Respiratory pathogens behave uniquely and outcomes related to RTIs such as pneumonia and tuberculosis may not be applicable to influenza infections. In addition, there is currently a shortage of information regarding factors that may influence the severity of influenza-related symptoms. This is a significant limitation of the current literature as influenza infection is known to have an extremely broad spectrum of illness.

Furthermore, no data are available to assess the role of AMPs during influenza infection in humans. It is important to note that while serum 25(OH)D is believed to be the best indicator of vitamin D status, it is 1,25-hydroxyvitamin D that governs the production of AMPs. Consequently, analysis of AMPs such as BD2, BD3 and LL-37 may strengthen research investigating the role of vitamin D in respiratory diseases such as influenza. Circulating 25(OH)D levels have been shown to correlate with plasma concentrations of LL-37 in humans. Hence LL-37 levels may not only be indicative of degree of immune response, they may be indicative of vitamin D status. Examination of BD2, BD3 and LL-37 levels may provide mechanistic insight into the relationship between vitamin D status and influenza severity. In addition, data regarding the AMP status of influenza-positive individuals may prove influential as the investigation of novel influenza treatment strategies continues.

Further investigation of vitamin D and AMP status as they relate to influenza severity is intended to contribute to the creation of targeted means to combat severe influenza infection in children. As a modifiable risk factor, vitamin D status may be easily and safely rectified in children through prophylactic supplementation regimes. AMPs may ultimately prove to be protective and be incorporated into clinical care techniques. It is anticipated that results from this
study may also aid in identifying potentially vulnerable populations of children across Canada, as well as worldwide.

2.5 Study Objectives and Hypotheses

**Objective 1** - To examine the relationship between serum 25(OH)D levels and influenza severity in influenza-positive, otherwise healthy children.

**Hypothesis.** Serum 25(OH)D levels will be inversely associated with influenza severity.

**Objective 2** - To investigate associations between serum levels of three vitamin D-regulated antimicrobial peptides: (1) LL-37, (2) BD2, (3) BD3 and influenza severity in this study population.

**Hypothesis.** Biochemical serum levels of LL-37, BD2 and BD3 will be associated with influenza severity in the population of children.
CHAPTER THREE

BIOCHEMICAL STATUS OF VITAMIN D AND RELATED BIOMARKERS AS PREDICTORS OF SEVERE INFLUENZA INFECTION IN CHILDREN

3.1 Abstract

**Background.** Influenza is a well-characterized respiratory infection with the potential to give rise to life-threatening complications. Previous observational studies that have examined respiratory tract infections in general have found that low serum 25(OH)D levels are associated with increased risk of both infection and symptom severity in children. It is thought that the primary avenue by which vitamin D acts in the respiratory system is through transcriptional regulation of certain antimicrobial peptides (AMPs). Presently however, few data exist concerning the impact of 25(OH)D or AMP status on influenza severity specifically.

**Objectives.**
1) To examine the relationship between serum 25(OH)D levels and influenza severity in a population of influenza-positive, otherwise healthy children.
2) To investigate the associations between serum levels of three vitamin D-regulated antimicrobial peptides: 1) human cathelicidin-derived antimicrobial peptide (LL-37); 2) beta-defensin 2 (BD2); 3) beta-defensin 3 (BD3) and influenza severity in the same population of children.

**Methods.** Eighty-two children (<18 years) with laboratory-confirmed influenza were recruited prospectively from the inpatient units of four Canadian pediatric referral centers. Serum levels of LL-37, BD2 and BD3 were measured using commercially available immunoassays, while 25(OH)D levels were assessed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Influenza severity was quantified using the maximum Comprehensive Severity Index® score (maxCSI). Comparative statistics were used to examine differences in maxCSI scores within measured variables of interest. Multivariable models were then created by controlling for age, sex, BMI, maternal education, vaccination status and influenza type.

**Results.** Serum 25(OH)D levels were inversely associated with influenza severity within the study population (B = -0.43, p-value = 0.03). Participants whose serum 25(OH)D levels fell below 50 nmol/L had significantly higher maxCSI scores than those with adequate serum levels (medians of 56 vs. 37, p-value = 0.01). Serum LL-37 levels were also found to be inversely associated with maxCSI scores among participants (B = -0.78, p-value = 0.03). Analyses were not performed for BD3 as 96% of patient samples contained BD3 levels under the lower limit of detection (12.5 pg/mL). Only serum 25(OH)D levels remained a significant predictor of influenza severity when incorporated into a multivariable model (B = -0.50, SE = 0.20, p-value = 0.04).

**Conclusions.** Serum 25(OH)D levels were found to be inversely associated with influenza severity in this population of children. Results from this study indicate that serum 25(OH)D levels <50 nmol/L may be associated with the development of influenza-related complications in children, possibly via the transcriptional down-regulation of LL-37.
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3.2 Background

Influenza is a respiratory infection that accounts for 250,000 to 500,000 deaths globally each year, of which roughly 28,000 to 111,000 are in young children.\textsuperscript{25,26} Children are among the first to fall ill during influenza outbreaks and are highly influential in the transmission of influenza within communities.\textsuperscript{27-29} In terms of outpatient visits and hospitalizations, influenza infection is responsible for considerable healthcare utilization costs in North America.\textsuperscript{30-32} Influenza in children also leads to significant economic productivity losses due to the increased need for caregivers and the resultant loss of parental time spent at work.\textsuperscript{82}

Physiological responses to influenza infection vary greatly among individuals. In children, outcomes such as hospitalization and development of complications following influenza infection are frequently observed in some children and not others. Interestingly, this variability is also observed among otherwise healthy children.\textsuperscript{33,34} Various determinants may be at the root of the observed differences in outcomes, including clinical, viral and genetic elements.\textsuperscript{49-52} Environmental factors such as socioeconomic status, tobacco smoke exposure and nutritional status may also be involved.\textsuperscript{53,54,129}

In the context of nutrition, nutrient status of vitamin D is of particular interest.\textsuperscript{134} Although long associated with bone health outcomes, recent observational studies have found associations between vitamin D deficiency and increased risk of respiratory traction infection (RTI) as well as severity of RTI symptoms in children.\textsuperscript{35-37,228} The observed associations between vitamin D status and respiratory health is troubling given that an estimated 24\% of Canadian children aged 6 to 11 years have circulating vitamin D levels that fall below the recommended cut-off of 50 nmol/L set by the Institute of Medicine (IOM).\textsuperscript{38,39} The link between vitamin D and respiratory health is thought to be largely mediated via the transcriptional regulation of certain
protective antimicrobial peptides (AMPs) in human airways. A number of these peptides are currently being investigated as novel anti-influenza treatment regimes, in lieu of the antiviral therapies currently available.

A large proportion of the current understanding of vitamin D and influenza has been extrapolated from studies examining 25(OH)D serum levels and risk of RTIs in general. There is currently a lack of information regarding nutritional factors, such as vitamin D status, that may influence degree of severity observed in influenza-related symptoms. Furthermore, no data are available to assess the role of AMPs during influenza infection in humans. To our knowledge, no observational study or RCT has been conducted to date that examines vitamin D or AMP status and influenza severity in humans. The primary objective of this study was to first examine the relationship between serum levels of 25(OH)D and influenza severity in a population of otherwise healthy children. The secondary objective was to then investigate the associations between serum levels of three AMPs (LL-37, BD2, BD3) and influenza severity in the same population of children.

3.3 Methods

The project described here builds upon an observational, prospective, multicenter project (dubbed “FluGene”) where patient recruitment has been ongoing since the 2007/2008 influenza season. FluGene aims to investigate a large number of clinical, viral, environmental and, in future, host genetic factors associated with influenza severity in children.

3.3.1 Patient Population

Eighty-two children with laboratory-confirmed influenza A or B infection were recruited from the inpatient units of four Canadian, pediatric referral centers: The Hospital for Sick
Children (Toronto, ON), Children’s Hospital of Eastern Ontario (Ottawa, ON), Centre hospitalier de l’Université Laval (Québec, QC) and Montreal Children’s Hospital (Montréal, QC). Recruitment occurred for a total of nine influenza seasons, beginning in the fall of 2007 and ending in the spring of 2016.

Children we required to be under the age of 18 at presentation in order to participate. Exclusion criteria included: (1) Any underlying condition leading to higher risk of complications from influenza according to the National Advisory Committee on Immunization (NACI) such as chronic pulmonary, cardiovascular, gastrointestinal, neurologic, neuromuscular, metabolic, endocrine or renal disease, immunosuppressive condition, underlying hemoglobinopathy, malignancy, upper airway abnormality, or pregnancy (except for morbid obesity and “past” asthma, defined as having no symptoms or need for medications in the preceding 12 months); (2) Insufficient command of English/French and absence of a translator; (3) Circumstances preventing follow-up assessments (e.g. no telephone).

Ethics approval was obtained from the Research Ethics Board at SickKids, as well as at the local ethics committees of each additional pediatric referral center from which patients were recruited (n=3). Written, informed consent was obtained from the parent(s)/legal guardian(s) of each subject prior to enrollment. Parent(s)/legal guardian(s) also consented to the storage of biological samples for future research.

3.3.2 Study Design

Laboratory virology reports of hospitalized patients were reviewed daily to identify those confirmed as influenza A or B positive. Influenza infection was detected by an immunofluorescence assay, viral culture or polymerase chain reaction (PCR). Patient health
records were examined to assess eligibility criteria before parents were approached and consent was obtained.

Following enrollment, patients were followed for 28 days to obtain data on symptoms and influenza-related complications that may have arisen. Follow-up visits and collection of biological samples were arranged by research personnel. Follow-up assessments took place at 48 hours, 1 week, 2 weeks and 4 weeks post-enrollment. These assessments were performed in-person for children still admitted to hospital and via telephone for discharged patients.

Blood samples (1 mL/kg to a maximum of 10 mL) were collected during phlebotomy for clinical care, when feasible. Unused fractions of clinical chemistry samples were also retrieved and banked. For the purpose of this study, all subjects for whom serum samples were available were included in the analysis (n=82).

3.3.3 Data Collection

In addition to biological samples, extensive clinical and demographic data were collected from the parent(s)/guardian(s) of participants using a standardized data collection form. Data was gathered in interview form based on parental recall and facilitated by trained clinical research personnel over a number of study visits. Information regarding prior influenza infection and vaccination history was compiled. Ethnicity of the child’s four biological grandparents was also documented and was used to evaluate the ethnicity of the patient. In the case that all four grandparents belonged to the same ethnic group, the child was classified as that ethnicity. However, if the ethnicities differed, the patient was designated as “mixed ethnicity.” Information regarding years of formal maternal education and household smoking exposure was also retrieved from parents. Body mass index z-scores (zBMI) were calculated using WHO Anthro ® v3.2.2 and WHO AnthroPlus ® v1.0.4 software.
3.3.4 Laboratory Methods

The analysis of 25(OH)D serum levels was performed at Queen’s University in Kingston, ON. Liquid chromatography-tandem mass spectrometry (LC-MS/MS), a highly sensitive, selective and clinically acceptable assay for the measurement of serum 25(OH)D levels was used.\textsuperscript{147,246,247} The LC-MS/MS method at Queen’s University is accredited by the Vitamin D External Quality Assessment Scheme (DEQAS). The assessment of serum levels of LL-37, BD2 and BD3 was performed at Mount Sinai Laboratory Services in Toronto, ON. The following ELISA kits were used: LL-37 (human) ELISA kit by Hycult Biotech, Beta-defensin 2 (human) ELISA kit by Pheonix Pharmaceuticals Inc and Beta-defensin 3 (human) ELISA kit by Pheonix Pharmaceuticals Inc. Samples were run in duplicate to ensure within-batch precision.

3.3.5 Quantification of Influenza Severity

The maximum Comprehensive Severity Index \textsuperscript{®} score (maxCSI) was used as the primary outcome measure of influenza severity. The CSI is a composite, continuous measure of a patient’s clinical state that has been validated extensively in predicting severity-dependent outcomes (e.g. mortality, cost, length of stay) in inpatient and ambulatory settings for children and adults.\textsuperscript{248-253} It has also been validated in a subpopulation comprised of the first 317 influenza-positive children recruited to the FluGene study (unpublished data). It is important to note that the CSI contains criteria matrices specific to influenza and related complications that are age-specific.\textsuperscript{251} The CSI does not include inputs that are related to clinical care received in-hospital, and is therefore not influenced by treatments provided to a patient.\textsuperscript{251}

Trained study staff abstracted data (e.g. symptoms, imaging results) from patient medical charts in order to determine CSI scores for each participant. Reliability testing was conducted by personnel at the University of Utah (Salt Lake City, UT) to establish sufficient inter-scorer
reliability (≥95%). A maxCSI score was determined for every medical visit (e.g. emergency department visit, hospital admission). If a child had >1 visit over the course of their illness, the highest maxCSI score was used in analyses.

3.3.6 Sample Size and Power

It was originally projected that serum samples would be available for 324 patients. For the continuous primary outcome measure maxCSI, a sample size of 324 would have provided 80% power to detect effect sizes ($f^2 \geq 0.025$) attributed to the tested predictor (i.e. the independent variable of interest) after having adjusted for an additional 10 independent variables (i.e. covariates) in multiple linear regression analysis, assuming $\alpha=0.05$. However, it was determined that less than a third of the anticipated number of patients had serum available for analysis. The number of covariates planned to be included in the analysis was therefore decreased from ten to six. A sample size of 82 provided 80% power to detect effect sizes ($f^2$ $\geq 0.15$) following the adjustment for six covariates in multiple linear regression, assuming $\alpha=0.05$.

3.3.7 Statistical Analysis

Descriptive statistics were determined for all variables of interest. Frequency and percent were calculated for categorical variables whereas mean, standard deviation, median and interquartile range (IQR) were determined for continuous variables. Comparative statistics were used to examine differences in maxCSI scores within measured variables of interest. Non-normal, continuous data were analyzed using Wilcoxon or Kruskal-Wallis tests (as appropriate). Preliminary, univariate, linear regression analyses were conducted to determine the unadjusted relationship between maxCSI scores and serum levels of 25(OH)D, LL-37, BD2 respectively.
Analyses were not performed for BD3 as 96% of patient samples contained BD3 levels under the lower limit of detection (12.5 pg/mL). Serum levels of 25(OH)D, LL-37, BD2 were analyzed as continuous variables. However, clinically relevant cut-offs of <50 nmol/L and <75 nmol/L for serum 25(OH)D levels were also explored.

Separate multiple linear regression models were subsequently created for the three predictors of interest – serum levels of 25(OH)D, LL-37 and BD2. Six covariates were included in the models: age, sex, zBMI, maternal education, vaccination status and influenza type. Covariates were chosen based on: 1) biological plausibility; 2) completeness of data; 3) adequate variability within the data. Goodness of fit was evaluated using the $R^2$ coefficient of determination for all models. Covariates were assessed for collinearity using the variance inflation factor (VIF), where collinearity was considered absent for VIF values ≤ 5. All hypothesis tests were two-sided and p-values < 0.05 were considered to be statistically significant. Analyses were performed using SAS 9.4 (SAS Institute, Cary, NC).

3.4 Results

3.4.1 Patient Characteristics

Data collected from 82 children were included in the final analyses. Participant characteristics are shown in Table 1. The mean age of patients was 5.0 years with the majority of the children falling between 2 and 10 years of age (64.7%). The study sample was 67.1% male (n=55) and 73.7% (n=59) of the population was of non-European ethnicity. The vast majority of the study population did not receive the seasonal influenza vaccination (91.4%). Of the 47 patients for whom tobacco smoke exposure data was available, 23.4% (n=11) of the children were regularly exposed to smoke in their homes. Over the course of their hospital stay, 15.9% of patients received early antiviral therapy. Seasonal influenza A infection accounted for 56.1%
(n=46) of the hospitalizations in the study population, while pandemic influenza A [H1N1] and influenza B accounted for 15.9% (n=13) and 28.0% (n=23) respectively. Within the study population, 50.6% (n=40) of participants had serum 25(OH)D levels that fell below 50 nmol/L.

3.4.2 Influenza Severity

Patients between the ages of 6 months and 2 years, as well as patients over the age of 10, had the highest median maxCSI scores within the study population (Table 2). Median maxCSI scores were also found to be significantly higher among children who were regularly exposed to tobacco smoke in their homes (61 vs. 33, p-value = 0.04). Median maxCSI scores were significantly higher in children infected with the pandemic H1N1 strain of influenza A compared to seasonal influenza A and influenza B, respectively (105 vs. 39 vs. 41, p-value < 0.0001). Patients treated with early antiviral therapy did not have significantly higher maxCSI scores than those who were not (50 vs. 42, p-value < 0.41). Serum 25(OH)D levels were also significantly associated with influenza severity within the study population (B = -0.43, p-value = 0.03). Participants whose serum 25(OH)D levels were below 50 nmol/L had significantly higher median maxCSI scores than those with serum levels greater than 50 nmol/L (56 vs. 37, p-value = 0.01). Serum LL-37 levels were also found to be inversely associated with maxCSI scores among participants (B = -0.78, p-value = 0.03). Lastly, biochemical levels of BD2 were not found to be associated with influenza severity in univariate analyses (B = 4.46, p-value = 0.62).

The three multivariable models used to examine the relationships between maxCSI scores and serum levels of 25(OH)D, LL-37 and BD2 are shown in Tables 3, 4 and 5.

**Primary analysis.** After controlling for patient sex, age, zBMI, maternal education, vaccination status and influenza type, serum 25(OH)D levels remained a significant predictor of patient maxCSI scores (B = -0.50, SE = 0.20, p-value = 0.04, R²=0.21).
Secondary analysis. Conversely, serum LL-37 levels were no longer significantly associated with maxCSI scores following the adjustment for the same aforementioned covariates (B = -0.80, SE = 0.40, p-value = 0.06, $R^2=0.21$). Serum levels of BD2 were not found to be associated with maxCSI scores in the multivariable model (B = 2.0, SE = 9.6, p-value = 0.84, $R^2=0.15$). Multicollinearity was not detected among predictors (VIF > 5).
TABLE 1 – Patient demographics of 82 influenza-positive children recruited at four Canadian pediatric referral centers between the 2007/2008 and 2015/2016 influenza seasons

<table>
<thead>
<tr>
<th>Patient Demographics</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>55 (67.1)</td>
</tr>
<tr>
<td>Female</td>
<td>27 (32.9)</td>
</tr>
<tr>
<td><strong>Age, years</strong></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>5.0 (4.4)</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>3.8 (2.1, 6.2)</td>
</tr>
<tr>
<td>Distribution, n (%)</td>
<td></td>
</tr>
<tr>
<td>&lt; 0.5 years</td>
<td>9 (11.0)</td>
</tr>
<tr>
<td>0.5 to &lt; 2 years</td>
<td>11 (13.4)</td>
</tr>
<tr>
<td>2 to &lt; 5 years</td>
<td>28 (34.2)</td>
</tr>
<tr>
<td>5 – 10 years</td>
<td>25 (30.5)</td>
</tr>
<tr>
<td>≥ 10 years</td>
<td>9 (11.0)</td>
</tr>
<tr>
<td><strong>BMI-for-Age, z-score, (n=64)</strong></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>0.05 (1.8)</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>0.16 (-1.0, 1.0)</td>
</tr>
<tr>
<td><strong>Ethnicity, n (%), (n = 80)</strong></td>
<td></td>
</tr>
<tr>
<td>European</td>
<td>21 (26.2)</td>
</tr>
<tr>
<td>Non-European</td>
<td>51 (63.8)</td>
</tr>
<tr>
<td>Mixed</td>
<td>8 (10.0)</td>
</tr>
<tr>
<td><strong>Maternal Education, n (%), (n = 81)</strong></td>
<td></td>
</tr>
<tr>
<td>≤ High school</td>
<td>21 (25.9)</td>
</tr>
<tr>
<td>≥ College or university</td>
<td>60 (74.1)</td>
</tr>
<tr>
<td><strong>Household tobacco smoke exposure, n (%), (n = 47)</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11 (23.4)</td>
</tr>
<tr>
<td>No</td>
<td>36 (76.6)</td>
</tr>
<tr>
<td><strong>Received current seasonal vaccination, n (%), (n = 81)</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7 (8.6)</td>
</tr>
<tr>
<td>No</td>
<td>65 (80.3)</td>
</tr>
<tr>
<td>N/A (child &lt; 6 months of age)</td>
<td>9 (11.1)</td>
</tr>
<tr>
<td><strong>Received early antiviral therapy, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>13 (15.9)</td>
</tr>
<tr>
<td>No</td>
<td>69 (84.1)</td>
</tr>
<tr>
<td><strong>Influenza type, n (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Influenza A (seasonal)</td>
<td>46 (56.1)</td>
</tr>
<tr>
<td>Influenza A (pandemic, H1N1)</td>
<td>13 (15.9)</td>
</tr>
<tr>
<td>Influenza B</td>
<td>23 (28.0)</td>
</tr>
<tr>
<td><strong>Serum 25(OH)D levels (nmol/L), (n = 78)</strong></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>52.2 (20.5)</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>50.2 (39.0, 66.4)</td>
</tr>
<tr>
<td>Distribution, n (%)</td>
<td></td>
</tr>
<tr>
<td>&lt; 50 nmol/L</td>
<td>40 (50.6)</td>
</tr>
<tr>
<td>&lt; 75 nmol/L</td>
<td>28 (35.4)</td>
</tr>
<tr>
<td><strong>Serum LL-37 levels (ng/mL), (n = 49)</strong></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>10.83 (12.8)</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>7.94 (2.7-14.0)</td>
</tr>
<tr>
<td><strong>Serum BD2 levels (ng/mL), (n = 81)</strong></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>0.40 (0.5)</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>0.24 (0.1-0.4)</td>
</tr>
</tbody>
</table>

Total N=82 unless otherwise specified


TABLE 2 – Associations between maxCSI scores and various patient demographics of 82 influenza-positive children recruited at four Canadian pediatric referral centers between the 2007/2008 and 2015/2016 influenza seasons

<table>
<thead>
<tr>
<th>Patient Demographics</th>
<th>Median maxCSI score (IQR)</th>
<th>Regression coefficient (95% CI)</th>
<th>Unadjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>46 (25-75)</td>
<td></td>
<td>0.34</td>
</tr>
<tr>
<td>Female</td>
<td>42 (17-61)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age, years</strong></td>
<td></td>
<td>1.80 (0.1, 3.5)</td>
<td>0.04</td>
</tr>
<tr>
<td>Distribution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 0.5 years</td>
<td>18 (16-41)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 to &lt; 2 years</td>
<td>53 (48-66)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 to &lt; 5 years</td>
<td>47 (25-70)</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>5 – 10 years</td>
<td>33 (17-70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 10 years</td>
<td>79 (29-100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BMI-for-Age, z-score, (n=64)</strong></td>
<td></td>
<td>0.10 (-4.6, 4.8)</td>
<td>0.97</td>
</tr>
<tr>
<td><strong>Ethnicity, (n = 80)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>European</td>
<td>32 (25-68)</td>
<td></td>
<td>0.82</td>
</tr>
<tr>
<td>Non-European</td>
<td>48 (25-70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td>43 (25-66)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Maternal Education, (n = 81)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ Highschool</td>
<td>54 (34-79)</td>
<td></td>
<td>0.12</td>
</tr>
<tr>
<td>≥ College or university</td>
<td>42 (21-66)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Household tobacco smoke exposure, (n = 47)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>61 (25-79)</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>No</td>
<td>33 (17-54)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Received seasonal vaccination, (n = 81)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>75 (43-100)</td>
<td></td>
<td>0.17</td>
</tr>
<tr>
<td>No</td>
<td>45 (25-68)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Received early antiviral therapy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>50 (32-81)</td>
<td></td>
<td>0.41</td>
</tr>
<tr>
<td>No</td>
<td>42 (25-70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Influenza type</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Influenza A (seasonal)</td>
<td>39 (25-54)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Influenza A (pandemic, H1N1)</td>
<td>105 (96-119)</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Influenza B</td>
<td>41 (16-54)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Serum 25(OH)D levels (nmol/L), (n = 78)</strong></td>
<td>-0.43 (-0.8, -0.1)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Distribution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 50 nmol/L</td>
<td>37 (16-54)</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>&lt; 50 nmol/L</td>
<td>56 (29-94)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Serum LL-37 levels (ng/mL), (n = 49)</strong></td>
<td>-0.78 (-1.5, -0.1)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td><strong>Serum BD2 levels (ng/mL), (n = 81)</strong></td>
<td>4.46 (-13.2, 22.1)</td>
<td>0.62</td>
<td></td>
</tr>
</tbody>
</table>

Total N=82 unless otherwise specified.
Interval data: Wilcoxon or Kruskal-Wallis tests were used as appropriate.
Continuous data: univariate linear regression was used.
TABLE 3 – Multiple linear regression model data examining the association between 25(OH)D serum levels and maxCSI scores in the study population

<table>
<thead>
<tr>
<th>Predictor Variables</th>
<th>Regression Coefficient (B)</th>
<th>Standard Error (SE)</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex [Male:Female], (n=82)</td>
<td>-2.1</td>
<td>8.9</td>
<td>(-20.03, 15.79)</td>
<td>0.81</td>
</tr>
<tr>
<td>Age years , (n=82)</td>
<td>0.9</td>
<td>1.0</td>
<td>(-1.21, 3.00)</td>
<td>0.40</td>
</tr>
<tr>
<td>BMI-for-Age z-score, (n=64)</td>
<td>-1.8</td>
<td>2.3</td>
<td>(-6.52, 2.82)</td>
<td>0.43</td>
</tr>
<tr>
<td>Maternal Education [≤Highschool: ≥College/University], (n=81)</td>
<td>-5.5</td>
<td>10.9</td>
<td>(-27.39, 16.43)</td>
<td>0.62</td>
</tr>
<tr>
<td>Received seasonal vaccination [Yes:No], (n=81)</td>
<td>-23.6</td>
<td>11.0</td>
<td>(-45.70, -1.58)</td>
<td>0.04</td>
</tr>
<tr>
<td>Influenza type [A(seasonal):A(pandemic):B], (n=82)</td>
<td>-0.3</td>
<td>4.9</td>
<td>(-10.00, 9.49)</td>
<td>0.90</td>
</tr>
<tr>
<td>Serum 25(OH)D levels [nmol/L], (n=78)</td>
<td>-0.5</td>
<td>0.2</td>
<td>(-0.89, -0.03)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Abbreviations – CI: confidence interval, BMI: Body Mass Index, 25(OH)D: 25-hydroxyvitamin D

$R^2 = 0.21$
TABLE 4 – Multiple linear regression model data examining the association between serum levels of LL-37 and maxCSI scores in the study population

<table>
<thead>
<tr>
<th>Predictor Variables</th>
<th>Regression Coefficient (B)</th>
<th>Standard Error (SE)</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex [Male:Female], (n=82)</td>
<td>1.3</td>
<td>10.6</td>
<td>(-20.41, 23.03)</td>
<td>0.90</td>
</tr>
<tr>
<td>Age years, (n=82)</td>
<td>1.3</td>
<td>1.2</td>
<td>(-1.21, 3.72)</td>
<td>0.31</td>
</tr>
<tr>
<td>BMI-for-Age z-score, (n=64)</td>
<td>-3.4</td>
<td>2.6</td>
<td>(-8.74, 2.00)</td>
<td>0.21</td>
</tr>
<tr>
<td>Maternal Education [≤Highschool: ≥College/University], (n=81)</td>
<td>-8.4</td>
<td>14.0</td>
<td>(-36.91, 20.09)</td>
<td>0.55</td>
</tr>
<tr>
<td>Received seasonal vaccination [Yes:No], (n=81)</td>
<td>-5.5</td>
<td>13.1</td>
<td>(-32.29, 21.37)</td>
<td>0.68</td>
</tr>
<tr>
<td>Influenza type [A(seasonal):A(pandemic):B], (n=82)</td>
<td>5.1</td>
<td>6.0</td>
<td>(-7.17, 17.28)</td>
<td>0.40</td>
</tr>
<tr>
<td>Serum LL-37 levels [ng/mL], (n=49)</td>
<td>-0.8</td>
<td>0.4</td>
<td>(-1.63, 0.02)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Abbreviations – CI: confidence interval, BMI: Body Mass Index, LL-37: Cathelicidin LL-37; \( R^2 = 0.21 \)
**TABLE 5 – Multiple linear regression model data examining the association between serum levels of BD2 and maxCSI scores in the study population**

<table>
<thead>
<tr>
<th>Predictor Variables</th>
<th>Regression Coefficient (B)</th>
<th>Standard Error (SE)</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong> [Male:Female], (n=82)</td>
<td>-0.9</td>
<td>8.8</td>
<td>(-18.52, 16.68)</td>
<td>0.92</td>
</tr>
<tr>
<td><strong>Age</strong> years, (n=82)</td>
<td>1.7</td>
<td>1.1</td>
<td>(-0.47, 3.87)</td>
<td>0.12</td>
</tr>
<tr>
<td><strong>BMI-for-Age</strong> z-score, (n=64)</td>
<td>-1.6</td>
<td>2.4</td>
<td>(-6.37, 3.08)</td>
<td>0.49</td>
</tr>
<tr>
<td><strong>Maternal Education</strong> [≤Highschool: ≥College/University], (n=81)</td>
<td>-5.3</td>
<td>10.6</td>
<td>(-26.53, 15.97)</td>
<td>0.62</td>
</tr>
<tr>
<td><strong>Received seasonal vaccination</strong> [Yes:No], (n=81)</td>
<td>-15.4</td>
<td>10.8</td>
<td>(-37.03, 6.16)</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Influenza type</strong> [A(seasonal):A(pandemic):B], (n=82)</td>
<td>-0.6</td>
<td>4.8</td>
<td>(-10.12, 8.98)</td>
<td>0.90</td>
</tr>
<tr>
<td><strong>Serum BD2 levels</strong> [ng/mL], (n=81)</td>
<td>2.0</td>
<td>9.6</td>
<td>(-17.24, 21.18)</td>
<td>0.84</td>
</tr>
</tbody>
</table>

Abbreviations – CI: confidence interval, BMI: Body Mass Index, BD2: beta-defensin 2

$R^2 = 0.15$
3.5 Discussion

Biochemical levels of 25(OH)D were found to be inversely associated with maxCSI scores in univariate analyses, as well as after controlling for patient sex, age, zBMI, maternal education, vaccination status and influenza type. It was also observed that patients whose serum 25(OH)D levels were below the current IOM-recommended cut-off of 50 nmol/L experienced more severe influenza-related symptoms than children whose 25(OH)D serum levels exceeded 50 nmol/L (median maxCSI scores: 56 vs. 37, p-value = 0.01).\textsuperscript{39}

Among our study population, the proportions of patients whose circulating 25(OH)D levels were below the recommended cut-off of 50 nmol/L were higher than the reported national data for school-aged children (Supplementary Table 2). An estimated 24\% of Canadian children aged 6 to 11 years, and 29\% of children aged 12 to 19, have circulating vitamin D levels that fall below 50 nmol/L.\textsuperscript{38} However, it is important to note that Sarafin et al. recently conducted a study standardizing the Canadian Health Measures Survey (CHMS) data and observed that the proportion of vitamin D deficient Canadians may be higher than previously reported.\textsuperscript{255} In our study population, 53.3\% of children 6 to 11 years old and 75.0\% of children 12 to 19 years old had serum 25(OH)D levels below 50 nmol/L. Children under the age of 5 years are less likely to be vitamin D deficient, with approximately 11\% of children three to five years old falling below the 50 nmol/L cut-off in Canada.\textsuperscript{38} In our study population, 61.5\% of patients between the ages of 3 and 5 years had serum 25(OH)D levels below 50 nmol/L. When examined as a whole, 50.6\% (n=40) of study participants had serum 25(OH)D levels lower than 50 nmol/L.

It is thought that the primary avenue by which vitamin D acts in the respiratory immune system is through the transcriptional regulation of AMPs such as LL-37.\textsuperscript{20,177} Specifically,
1,25(OH)D activates gene expression by functioning as a transcription factor. Acting via a Toll-like Receptor (TLR) pathway, 1,25(OH)D binds to Vitamin D Response Elements (VDREs) in the promoter regions of AMP genes. It is important to note that respiratory epithelial cells are known to convert 25(OH)D to 1,25(OH)D.

It has been previously demonstrated that circulating 25(OH)D levels correlate with plasma concentrations of LL-37 in humans. This correlation is controversial as a number of studies have also reported that no relationship was observed between circulating 25(OH)D and LL-37 levels. In a post-hoc analysis using a subset of our study population, serum levels of 25(OH)D and LL-37 were not found to be significantly correlated (N=49, p-value=0.26). However, univariate analyses revealed that circulating levels of the AMP LL-37 were inversely associated with influenza severity in this population. Due to the fact that 25(OH)D in converted to 1,25(OH)D locally in respiratory epithelial cells, it is possible that local or systemic levels of 1,25(OH)D may be more strongly correlated with LL-37 levels than circulating 25(OH)D levels. Future studies should examine this association.

Results from this study are consistent with previous research that has reported an increase in influenza severity among children regularly exposed to secondhand smoke. Similarly, past studies have also reported that pandemic H1N1-infected individuals experienced more severe symptoms than people infected with seasonal strains of influenza. Disease severity was observed to be significantly higher among children infected with the pandemic H1N1 strain of influenza A when compared to seasonal influenza A or B in our study population. However, the proportions of recruited children who were exposed to tobacco smoke in their homes or who were pandemic H1N1-positive were small (n=11 and n=13 respectively, Table 1).
The investigation of sex, age, maternal education and patient ethnicity in relation to influenza severity yielded results inconsistent with the current literature. While it has frequently been observed that young males experience influenza complications more frequently than young females, sex was not found to be associated with influenza severity in the study population (Table 2).\textsuperscript{92-94} In addition, younger age was not associated with higher maxCSI scores. In fact, children over the age of ten experienced the most severe symptoms within our study population. Maternal education, a marker of socioeconomic status, was not found to be associated with symptom severity. Lastly, ethnicity was not a significant predictor of influenza severity in this population. However, due to the small sample size, patient ethnicity was dichotomized to European and non-European ancestry. These two categories were likely inadequate to identify certain ethnic groups within the study population that have previously reported to be at risk of more severe disease, such as aboriginal populations and people of Asian or African descent.\textsuperscript{95-97,259}

There are three significant limitations to the study design. Firstly, our study is limited by its small sample size (n=82) which may have restricted our ability to detect relevant variation within our outcome measure (maxCSI). Secondly, by including only patients with available serum samples in the analyses, selection bias may have been introduced. More severely ill children typically have blood drawn for clinical care more often than less ill children and the consent rate for blood collection in the absence of clinical blood work is relatively low. This selection bias would have lead to an overestimation of any observed relationships between our study predictors and our outcome measure.\textsuperscript{260} Lastly, the fact that serum samples are collected from patients at one time point during the study period is a limitation of the study as it has been
postulated that serum 25(OH)D levels may vary over the course of an acute inflammatory response, as seen in cases of viral infection.\textsuperscript{261-264}

Strengths of our study include the multicenter strategy, the focus on healthy children, the extensive approach to prospective data collection and the primary outcome measure (maxCSI). By studying this population of otherwise healthy children, we were able to examine the risk factors of interest without the influence of other health conditions that could have potentially confounded results. The use of the maxCSI strengthened this project as it is an objectively measured and validated outcome. The maxCSI, a continuous outcome measure, was also an asset as it allowed for the detection of relatively small effect sizes ($f^2 \geq 0.15$) regardless of the study’s small sample size ($n=82$). Utilizing a continuous outcome variable that did not necessitate classification also reduced the risk of patient misclassification when compared to categorical severity measures.

3.6 Conclusions

Serum 25(OH)D levels were found to be inversely associated with influenza severity in this population of children after controlling for sex, age, zBMI, maternal education, vaccination status and influenza type. Although significant in univariate analyses, serum LL-37 levels were not significantly associated with maxCSI scores following the adjustment for the aforementioned covariates. No association between circulating levels of BD2 and influenza severity was detectable in this population of children. Analyses were not performed for BD3 as 96\% of patient samples contained BD3 serum levels under the lower limit of detection (12.5 pg/mL). Results from this study indicate that serum 25(OH)D levels lower than 50 nmol/L may be associated with the development of influenza-related complications in children, possibly via the transcriptional down-regulation of LL-37. Further research using larger sample sizes, as well as
multiple blood sampling time points, will be important as the protective effects of 25(OH)D and AMPs continue to be investigated in relation to influenza severity.

3.7 Notes

Conflicts of interest. None disclosed.

Funding. Funding for this study was received from three sources. CIHR provided funds through two Operating Grants: 1) Severe influenza in infants, children and youths: Role of clinical and epidemiologic risk factors (21109MOP-259534-PH1-CEAB-164032) and 2) Vitamin D and related biomarkers as predictors of severe influenza infection in children (201503MOP-343630-PH2-CEAB-164032). Funding was also received from the Canadian Foundation for Dietetic Research.

Acknowledgements. Many thanks to Suganya Lee, Monalisa Pedlar, Jalpa Patel, Parag Patel, Lakeshia Daley, Mason Snook, Aunshu Goyal, Julie Coste and Mao Motohashi for their contributions to study management, participant recruitment, data collection, data abstraction and various other crucial tasks. We would also like to thank the Clinical Nurse Coordinators at all study sites for their commitment and hard work.
### SUPPLEMENTARY TABLE 1 – Multiple linear regression model data examining the association between serum levels of 25(OH)D dichotomized to above and below 50 nmol/L and maxCSI scores in the study population

<table>
<thead>
<tr>
<th>Predictor Variables</th>
<th>Regression Coefficient (B)</th>
<th>Standard Error (SE)</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex [Male:Female], (n=82)</td>
<td>-5.2</td>
<td>9.8</td>
<td>(-24.82, 14.50)</td>
<td>0.60</td>
</tr>
<tr>
<td>Age years, (n=82)</td>
<td>0.9</td>
<td>1.1</td>
<td>(-1.21, 3.08)</td>
<td>0.38</td>
</tr>
<tr>
<td>BMI-for-Age z-score, (n=64)</td>
<td>-2.0</td>
<td>2.4</td>
<td>(-6.74, 2.78)</td>
<td>0.41</td>
</tr>
<tr>
<td>Maternal Education [≤Highschool: ≥College/University], (n=81)</td>
<td>-2.2</td>
<td>11.7</td>
<td>(-25.83, 21.34)</td>
<td>0.85</td>
</tr>
<tr>
<td>Received seasonal vaccination [Yes:No], (n=81)</td>
<td>-26.6</td>
<td>14.8</td>
<td>(-56.48, 3.26)</td>
<td>0.08</td>
</tr>
<tr>
<td>Influenza type [A(seasonal):A(pandemic):B], (n=82)</td>
<td>1.4</td>
<td>5.0</td>
<td>(-8.79, 11.50)</td>
<td>0.79</td>
</tr>
<tr>
<td>Serum 25(OH)D levels [&lt;50 nmol/L: ≥50 nmol/L], (n=78)</td>
<td>-24.9</td>
<td>9.5</td>
<td>(-44.14, -5.75)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Abbreviations – CI: confidence interval, BMI: Body Mass Index, 25(OH)D: 25-hydroxyvitamin D

\[ R^2 = 0.22 \]
### SUPPLEMENTARY TABLE 2 – Comparison of the percentage of patients whose circulating 25(OH)D levels fall below 50 nmol/L in the study population vs. reported national data from the CHMS

<table>
<thead>
<tr>
<th>Age Group (years)</th>
<th>Reported national data&lt;sup&gt;38&lt;/sup&gt; (%)</th>
<th>Study population data (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 3 (n=29)</td>
<td>No data available</td>
<td>41.4</td>
</tr>
<tr>
<td>3 to &lt; 6 (n=26)</td>
<td>11.0</td>
<td>61.5</td>
</tr>
<tr>
<td>6 to &lt; 12 (n=15)</td>
<td>24.0</td>
<td>53.3</td>
</tr>
<tr>
<td>12 to 19 (n=8)</td>
<td>29.0</td>
<td>75.0</td>
</tr>
</tbody>
</table>

Abbreviations – 25(OH)D: 25-hydroxyvitamin D, CHMS: Canadian Health Measures Survey
SUPPLEMENTARY TABLE 3 – Multiple linear regression model data examining the association between 25(OH)D serum levels and maxCSI scores in the study population with zBMI removed

<table>
<thead>
<tr>
<th>Predictor Variables</th>
<th>Regression Coefficient (B)</th>
<th>Standard Error (SE)</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>-5.3</td>
<td>8.5</td>
<td>(-22.21, 11.70)</td>
<td>0.54</td>
</tr>
<tr>
<td>[Male:Female], (n=82)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.4</td>
<td>1.0</td>
<td>(-1.49, 2.40)</td>
<td>0.65</td>
</tr>
<tr>
<td>Maternal Education</td>
<td>-11.0</td>
<td>9.7</td>
<td>(-30.30, 8.29)</td>
<td>0.26</td>
</tr>
<tr>
<td>[≤Highschool: ≥College/University], (n=81)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Received seasonal vaccination</td>
<td>-25.1</td>
<td>10.1</td>
<td>(-45.34, -4.91)</td>
<td>0.02</td>
</tr>
<tr>
<td>[Yes:No], (n=81)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Influenza type</td>
<td>1.1</td>
<td>4.6</td>
<td>(-8.12, 10.25)</td>
<td>0.82</td>
</tr>
<tr>
<td>[A(seasonal):A(pandemic):B], (n=82)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum 25(OH)D levels</td>
<td>-0.4</td>
<td>0.2</td>
<td>(-0.80, -0.02)</td>
<td>0.04</td>
</tr>
<tr>
<td>[nmol/L], (n=78)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations – CI: confidence interval, BMI: Body Mass Index, 25(OH)D: 25-hydroxyvitamin D

$R^2 = 0.18$
**SUPPLEMENTARY TABLE 4** – Multiple linear regression model data examining the association between serum levels of LL-37 and maxCSI scores in the study population with zBMI removed

<table>
<thead>
<tr>
<th>Predictor Variables</th>
<th>Regression Coefficient (B)</th>
<th>Standard Error (SE)</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Male:Female], (n=82)</td>
<td>-8.1</td>
<td>10.4</td>
<td>(-29.05, 12.92)</td>
<td>0.44</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>years, (n=82)</td>
<td>0.1</td>
<td>1.1</td>
<td>(-2.23, 2.37)</td>
<td>0.95</td>
</tr>
<tr>
<td><strong>Maternal Education</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[≤Highschool: ≥College/University], (n=81)</td>
<td>-18.1</td>
<td>11.8</td>
<td>(-42.05, 5.81)</td>
<td>0.13</td>
</tr>
<tr>
<td><strong>Received seasonal vaccination</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Yes:No], (n=81)</td>
<td>-13.8</td>
<td>12.0</td>
<td>(-38.07, 10.47)</td>
<td>0.26</td>
</tr>
<tr>
<td><strong>Influenza type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[A(seasonal):A(pandemic):B], (n=82)</td>
<td>6.8</td>
<td>6.8</td>
<td>(-4.62, 18.31)</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>Serum LL-37 levels</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[ng/mL], (n=49)</td>
<td>-1.1</td>
<td>0.4</td>
<td>(-1.90, -0.29)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Abbreviations – CI: confidence interval, BMI: Body Mass Index, LL-37: Cathelicidin LL-37

$R^2 = 0.20$
SUPPLEMENTARY TABLE 5 – Multiple linear regression model data examining the association between serum levels of BD2 and maxCSI scores in the study population with zBMI removed

<table>
<thead>
<tr>
<th>Predictor Variables</th>
<th>Regression Coefficient (B)</th>
<th>Standard Error (SE)</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex [Male:Female], (n=82)</td>
<td>-4.8</td>
<td>8.3</td>
<td>(-21.38, 11.80)</td>
<td>0.57</td>
</tr>
<tr>
<td>Age years, (n=82)</td>
<td>1.1</td>
<td>1.0</td>
<td>(-0.95, 3.13)</td>
<td>0.29</td>
</tr>
<tr>
<td>Maternal Education [≤Highschool: ≥College/University], (n=81)</td>
<td>-11.8</td>
<td>9.6</td>
<td>(-30.91, 7.36)</td>
<td>0.22</td>
</tr>
<tr>
<td>Received seasonal vaccination [Yes:No], (n=81)</td>
<td>-20.1</td>
<td>10.2</td>
<td>(-40.31, 0.18)</td>
<td>0.05</td>
</tr>
<tr>
<td>Influenza type [A(seasonal):A(pandemic):B], (n=82)</td>
<td>0.26</td>
<td>4.5</td>
<td>(-8.76, 9.27)</td>
<td>0.95</td>
</tr>
<tr>
<td>Serum BD2 levels [ng/mL], (n=81)</td>
<td>-0.98</td>
<td>9.6</td>
<td>(-18.93, 16.96)</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Abbreviations – CI: confidence interval, BMI: Body Mass Index, BD2: beta-defensin 2

$R^2 = 0.13$
CHAPTER FOUR – OVERALL DISCUSSION

This thesis aimed to examine the relationship between serum 25(OH)D levels and disease severity in a population of influenza-positive, otherwise healthy children. It was hypothesized that circulating 25(OH)D levels would be inversely associated with influenza severity. This theory was based upon data from previous observational studies that indicated that low circulating 25(OH)D levels were associated with increases in both risk of infection and of more severe presentation in pediatric RTIs.\textsuperscript{35-37,227-229} Findings from our study supported our hypothesis and were consistent with the current literature. Serum 25(OH)D levels were found to be inversely associated with influenza severity in this population of children after controlling for sex, age, zBMI, maternal education, vaccination status and influenza type ($B = -0.50$, SE = 0.20, p-value = 0.04, $R^2 = 0.21$). These results have contributed to our understanding of vitamin D status and pediatric RTI severity by examining influenza specifically – a respiratory infection that has been considerably less studied than TB or pneumonia in relation to vitamin D status.\textsuperscript{134}

The second objective of this thesis was to investigate the associations between serum levels of LL-37, BD2, BD3 and influenza severity in the study population. It was hypothesized that serum levels of the three AMPs would be associated with influenza severity among the children; however the direction of the associations was not specified. Although significant in univariate analyses, serum LL-37 levels were not significantly associated with maxCSI scores following the adjustment for patient sex, age, zBMI, maternal education, vaccination status and influenza type. No association between circulating levels of BD2 and influenza severity was detectable in this population of children. Analyses were not performed for BD3 as 96% of patient samples contained BD3 levels under the lower limit of detection for the Pheonix Pharmaceuticals Inc. ELISA kit (12.5 pg/mL) – an interesting finding in itself.
There is no data in the literature regarding biochemical AMP status in influenza-positive humans. Therefore it remains unclear whether circulating AMP levels are raised or lowered over the course of influenza infection. However, numerous studies investigating TB-related outcomes have observed that LL-37 levels are significantly higher among infected individuals than in healthy controls. It is conceivable that TB infection may result in an increase in AMP production as a compensatory, protective response. Univariate results from our study demonstrate that the data surrounding TB and LL-37 status may be applicable to influenza infection. As the investigation of AMPs as potential therapeutic agents continues, future research will need to elucidate the relationship between disease severity and AMP status in humans. Observational studies investigating AMP status at multiple time points over the course of infection may prove useful.

The bioactive form of vitamin D – 1,25(OH)D – is known to upregulate the production of LL-37, BD2 and BD3 by functioning as a transcription factor in vitro. As such, many researchers have been interested in determining if a relationship exists between vitamin D status and AMP status in humans. The vast majority of these studies have been conducted using circulating levels of AMPs as biomarkers of AMP status, and circulating 25(OH)D levels as biomarkers of vitamin D status. Two observational studies have reported a correlation between circulating AMP and 25(OH)D levels, however many others have noted that no association was observed. A post-hoc analysis in a subset of our study population revealed that serum 25(OH)D levels were not significantly correlated with serum levels of LL-37 (N=49, p-value=0.26) or BD2 (N=81, p-value=0.36). It may be beneficial for future research to examine the association between circulating or local levels of 1,25(OH)D, in lieu of circulating 25(OH)D levels, and AMP status in humans.
It is also possible that circulating levels of AMPs may not be representative of local AMP levels in the respiratory system. No study has been conducted to date that investigates the relationship between local and systemic AMP levels in humans. However, two previous studies have examined the association between vitamin D status and AMP status, quantified using measured AMP levels in human bronchoalveolar lavage fluid (BALF). In a group of pediatric TB patients, Cakir et al. found no correlation between serum 25(OH)D levels and LL-37 or BD2 levels measured in patient BALF. A second research group assessed levels of LL-37, as well as of 1,25(OH)D and 25(OH)D, in the BALF of patients following an allergen challenge in allergic participants. Although an allergic response may not be equivalent to viral or bacterial infection, it may translate as an inflammatory response in the respiratory system. The authors observed positive and statistically significant correlations between BALF concentrations of LL-37 and both 1,25(OH)D (Spearman’s \( \rho = 0.91, \) p-value<0.0001) and 25(OH)D (Spearman’s \( \rho = 0.86, \) p-value<0.0001) of participants. These results call into question the use of circulating, systemic levels of vitamin D and AMPs as biomarkers in studies investigating respiratory disease. Further research is required to determine the validity of circulating AMP and vitamin D levels, as opposed to local AMP and vitamin D levels, as biomarkers in respiratory health research.

As previously mentioned, there are three significant limitations to the study design. Firstly, our study is limited by its sample size (n=82). The small sample size governed the complexity of the variables included in the multivariable models. For instance, patient ethnicity was dichotomized to European or non-European ancestry. As a result, 73.7% of the study population was classified as non-European. Ethnicity was therefore not included in the multivariable models due to insufficient variability within the data. This is a significant
limitation of the study as it has been previously reported that certain ethnicities (e.g. African-American, Aboriginal and Hispanic) are at higher risk of developing influenza-related complications or requiring hospitalization than others.\textsuperscript{95-97,267,268} A Toronto-based study that examined ethnic disparities among children infected with pandemic influenza A H1N1 found that infected children were significantly more likely to be Black (OR: 16.02, 95% CI: 2.85-89.92) compared to other ethnicities after controlling for confounders such as various socioeconomic determinants and clinical risk factors.\textsuperscript{259}

Secondly, by including only patients with available serum samples in the analyses, a selection bias may have been introduced. More severely ill children typically have blood drawn for clinical care more often than less ill children and the consent rate for blood collection in the absence of clinical blood work is relatively low. Therefore, our study population is potentially enriched for severe disease. This selection bias, termed “severity of illness bias” in the literature, would lead to an overestimation of any observed relationships between our study predictors and our outcome measure.\textsuperscript{260} Future studies should implement control groups and use larger sample sizes with a broad spectrum of illness, when feasible.

Lastly, there are potential limitations surrounding the fact that serum samples are collected from patients on one single occasion during the study period. In our cohort, serum collection typically occurs at the approximate time the participants’ symptoms are the most severe. However, it has been postulated that serum 25(OH)D levels may vary over the course of an acute inflammatory response, as seen in cases of viral infection.\textsuperscript{261-264} A recent systematic review examined the results of eight studies that measured 25(OH)D serum levels before and after acute inflammatory responses following surgical interventions and one following malarial infection.\textsuperscript{269} Six of the studies observed decreases in serum 25(OH)D levels over the trial
periods, while two studies found that 25(OH)D levels remained constant.\textsuperscript{269} However, the authors of the systematic review identified major heterogeneity among the articles they reviewed, as well as some important limitations.\textsuperscript{269} Firstly, the observed decreases in serum 25(OH)D levels may be due to hemodilution following the administration of intravenous (IV) fluids during surgery in four of the six studies.\textsuperscript{269} It is also important to note that parathyroid hormone, an indirect indicator of 25(OH)D status, was not found to fluctuate over the trial in one of the reviewed studies.\textsuperscript{261} All but one of the studies investigated populations undergoing surgery, which may not be representative of infection-induced inflammatory responses.\textsuperscript{269} In addition, none of the studies included children as participants. In terms of heterogeneity, 25(OH)D levels were measured by various different assays, blood was drawn at disparate time-points and the inflammatory responses in the studies had different origins.\textsuperscript{269} In a more recent study not included in the systematic review, serum 25(OH)D levels in a population of children with acute bacterial infections were examined.\textsuperscript{270} It was observed that 25(OH)D levels analyzed at the time of infection and again following infection were not significantly different.\textsuperscript{270} Therefore, although 25(OH)D levels analyzed at the time of an inflammatory response should be interpreted with caution, it is our belief that there is not sufficient evidence to discourage its use as a biomarker of vitamin D status in influenza-infected children.

Strengths of our study include the multicenter strategy, the focus on healthy children, the extensive approach to prospective data collection and the primary outcome measure (maxCSI). By studying this population of otherwise healthy children, we were able to examine the risk factors of interest without the influence of other health conditions that could have potentially confounded results. The use of the maxCSI strengthened this project as it is an objectively measured and validated outcome. The maxCSI, a continuous outcome measure, was also an asset.
as it allowed for the detection of relatively small effect sizes ($f^2 \geq 0.15$) regardless of the study’s small sample size ($n=82$). Utilizing a continuous outcome variable that did not necessitate classification also reduced the risk of patient misclassification when compared to categorical severity measures.
CHAPTER FIVE – CONCLUSION AND FUTURE DIRECTIONS

Results from this study indicate that poor vitamin D status may be predictive of severe disease in children over the course of an influenza infection. Within the study population, biochemical levels of 25(OH)D were found to be inversely associated with influenza severity after controlling for patient sex, age, zBMI, maternal education, vaccination status and influenza type. Influenza severity was quantified using the Composite Severity Index® (CSI), a validated measure of influenza severity.\textsuperscript{248,250,251,253} In univariate analyses, patients whose serum 25(OH)D levels fell below the IOM-recommended cutoff of 50 nmol/L had significantly higher maxCSI scores than children whose 25(OH)D levels exceeded 50 nmol/L. Univariate analyses also revealed that circulating levels of the AMP LL-37 were inversely associated with influenza severity in this population. Thus, serum 25(OH)D levels lower than 50 nmol/L may be associated with the development of influenza-related complications in children, possibly via the transcriptional down-regulation of LL-37.

Results garnered from this project have strengthened our understanding of epidemiological risk factors for severe respiratory disease, and may hold considerable implications for clinicians and health policy-makers. However, future research regarding vitamin D and AMP status in relation to influenza severity should focus on the attainment of high-quality data with larger sample sizes. This would allow researchers to control for variables at a more profound level than what this project was able to accomplish. The small sample size in this study (n=82) governed the complexity of the variables included in the multivariable models. For instance, patient ethnicity was dichotomized to European or non-European ancestry. Larger sample sizes would permit researchers to control for the effect of variables, such as ethnicity, on influenza severity more precisely.
Further research is also required to elucidate the relationships between serum 25(OH)D status, both systemic and local AMP levels, and clinical outcomes associated with influenza infection. As previously mentioned, there is currently no consensus in the literature as to whether or not a correlation exists between systemic 25(OH)D and AMPs levels. Moving forward, RCTs investigating biochemical AMP status at multiple time points over the course of vitamin D supplementation would be advantageous. In addition, the investigation of circulating, systemic levels of AMPs such as LL-37, in comparison to local respiratory levels, would be extremely valuable. Obtaining biological samples from the respiratory system (e.g. lung biopsies, BALF) is highly invasive. As such, data regarding the relationship between systemic and local levels of AMPs would be useful in ascertaining the validity of AMP serum levels as biomarkers in respiratory health research.

Lastly, future studies should continue to explore the use of the Composite Severity Index® (CSI) in clinical research. It would be highly beneficial to standardize the methods used in reporting severity of illness during influenza infection. In our opinion, the CSI has proven itself incredibly valuable as an objectively measured, validated, continuous outcome. Although infrequently used at present, it is conceivable that the CSI could become an important outcome measure of assessing illness severity in many disease states in various clinical research settings in the future.
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CHAPTER SEVEN – APPENDICES

APPENDIX A  Sample Consent Form……………………………………………..81

APPENDIX B  List of Recruiting Sites…………………………………………...88
# APPENDIX A
Sample Consent Form for *Biochemical Status of Vitamin D and Related Biomarkers as Predictors of Severe Influenza Infection in Children*

SickKids®
THE HOSPITAL FOR SICK CHILDREN
Research Ethics Board

**Research consent form for parents to consent for their child (ER patients)**

**Title of Research Project:** Risk factors for severe influenza in children ("FluGene Study")

**Investigator(s):**
- Dr. Dat Tran, Division of Infectious Diseases  
  Tel: 416-813-7654  
  Ext 204649
- Dr. Andrew Paterson, Program in Genetics and Genome Biology, Research Institute  
  Tel: 416-813-6383
- Dr. Deborah O’Connor, Department of Clinical Dietetics  
  Tel: 416-813-5901
- Dr. Jonathan Gubbay, Division of Infectious Diseases  
  Tel: 419-813-6268
- Dr. Jonathon Maguire, Division of Paediatric Medicine  
  Tel: 416-919-3462
- Dr. Moshe Ipp, Division of Paediatric Medicine  
  Tel: 416-913-6933
- Dr. Suzanne Schuh, Division of Paediatric Emergency Medicine  
  Tel: 416-813-7257
- Dr. Susan Richardson, Department of Paediatric Laboratory Medicine  
  Tel: 416-813-5990
- 24-hr pager (Dr. Tran or one of the Infectious Diseases Research Fellows)  
  Pg: 416-530-3155

**Study Staff:**
- Suganya Lee, Clinical Research Nurse Coordinator  
  Tel: 416-813-6286
- Cheryl Arneson, Clinical Research Nurse Coordinator  
  Tel: 416-813-7654  
  Ext 207021
- Kim Simpson, Clinical Research Nurse Coordinator  
  Tel: 416-813-7654  
  Ext 203980
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- Paola Brazal, Clinical Research Nurse Coordinator  
  Tel: 416-813-5625
- Aunshu Goyal, Research Project Coordinator  
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- Kayla Furlong, Clinical Research Project Assistant  
  Tel: 416-813-5625
- Nadine Lombardo-Han, Clinical Research Project Assistant  
  Tel: 416-813-5625
- Sanarya Rashad, Research Project Assistant  
  Tel: 416-813-5625
- Eleanor Reid, Graduate Student  
  Tel: 416-813-5625
- Lakeshia Daley, Phlebotomist  
  Tel: 416-813-5625
- Jalpa Patel, Phlebotomist  
  Tel: 416-813-5625
- Paragkumar Patel, Phlebotomist  
  Tel: 416-813-5625
- Mason Snook, Phlebotomist  
  Tel: 416-813-5625
- Mai Dung Ha, Research Technologist I  
  Tel: 416-813-5625
**Purpose of the Research:**
Influenza is a major cause of illness and death worldwide, and inflicts a heavy economic burden. Yearly influenza epidemics are estimated to affect 5–15% of the world’s population. Although most cases are mild, these epidemics still cause severe illness in 3–5 million people and 250,000–500,000 deaths worldwide. While relatively mild, the 2009 influenza pandemic showed that we were limited in our ability to predict influenza pandemics and to identify healthy individuals at highest risk. Many people are exposed to influenza viruses and get infected by them, yet very few develop serious complications from the infection. A number of factors likely contribute to varying severity of illness from influenza infection. These include factors such as age, sex, and socioeconomic status. The genetic make-up of an individual’s immune system, environmental exposures such as tobacco smoke, and imbalances in micronutrient levels (such as vitamin D and selenium) may also explain why some get sicker than others with influenza infection. This research is being done to increase understanding of who gets severe influenza by looking at all of the potential risk factors so that it may help doctors and public health agencies to better use resources in preventing severe illness in the most vulnerable children.

Over a 4-year period, we will recruit approximately 1,693 children from doctors’ offices, a large emergency department, community hospitals and children’s hospitals across Canada.

Your child is being asked to participate in the study because he/she has signs and symptoms suggestive of influenza infection.

**Description of the Research:**
If you agree for your child to be in this study, we will ask the following of your child, you and your family over the study period (approximately 4 weeks).

**At the start of the study:**
- The research staff will take 2 nasal swabs from your child. One swab will be sent to the SickKids Virology Laboratory to confirm influenza infection and look for other viruses. The other swab will be used to perform a rapid bedside test for influenza (results available in 15 minutes) and stored for future measurement of the substances produced by your child in fighting the virus.
- You will be asked to complete two brief 20-minute questionnaires. One of these questionnaires is about your child’s eating habits.
- A urine sample will also be collected when your child urinates. This will help us to learn more about severe influenza and any secondary bacterial infection(s) your child may have.
- A small sample of blood (½ to 1 tablespoon, depending on your child’s size) or saliva will be collected. We prefer a blood sample since it will allow us to learn much more about risk factors for severe influenza than possible with a saliva sample (see Table below). If there is routine blood ordered by the doctor, this sample of blood will be taken at the same time so that your child will not receive an extra needle prick. The blood sample will be tested for environmental, immune system and genetic factors that may result in more severe influenza.
<table>
<thead>
<tr>
<th>Type of information provided by:</th>
<th>Blood</th>
<th>Saliva</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pattern of immune responses that leads to more severe influenza</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Environmental factors (eg, vitamin D level) that may contribute to more severe influenza</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Quality of genetic information</td>
<td>Higher quality, more information,</td>
<td>Lower quality, less information</td>
</tr>
</tbody>
</table>

If your child provides a blood sample, we will provide you with your child’s vitamin D level when the sample is tested.

- If other types of specimens are taken as part of your child’s doctor’s orders, additional or leftover quantities will be taken and stored for future measures of your child’s immune responses to the virus.
- We would also like a DNA sample from your child’s biological parents.

**At Visit 2, 3, 4 and 5:**

- The research staff will follow up on your child’s condition by phone and ask a 5-minute questionnaire.
- If your child provided blood at the beginning of the study (Day 1), we will ask for additional blood samples (½ to 1 tablespoon depending on your child’s size) if and when blood is already being drawn for clinical care. These samples are **optional**.

The following table summarizes the procedures that will be done:

<table>
<thead>
<tr>
<th>Type of Evaluation</th>
<th>Visit 1 Day 1 Start of Study (In Person)</th>
<th>Visit 2 Day 3 (By Phone)</th>
<th>Visit 3 Day 7 (By Phone)</th>
<th>Visit 4 Day 14 (By Phone)</th>
<th>Day 28* (By Phone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal swabs</td>
<td>✓ 2 swabs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Questionnaire</td>
<td>✓ 20 min</td>
<td>✓ 5 min</td>
<td>✓ 5 min</td>
<td>✓ 5 min</td>
<td>✓ 5 min</td>
</tr>
<tr>
<td>Urine sample†</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood or saliva collection§</td>
<td>✓ (✓)</td>
<td>(✓)</td>
<td>(✓)</td>
<td>(✓)</td>
<td>(✓)</td>
</tr>
<tr>
<td>Food and supplement consumption pattern questionnaire</td>
<td>✓ 20 min</td>
<td>(✓)</td>
<td>(✓)</td>
<td>(✓)</td>
<td>(✓)</td>
</tr>
</tbody>
</table>

*If your child were to be admitted to hospital for longer than two weeks, we would complete the
4th visit 14 days after your child’s discharge from hospital.
§If the blood sample provided is too small than a saliva sample will also be collected.

(✔)If your child provided blood at the beginning of the study (Day 1), we will ask for additional blood samples (½ to 1 tablespoon depending on your child’s size) if and when blood is already being drawn for clinical care. These samples are optional.

†A second urine sample will be collected if your child develops pneumonia or signs/symptoms of a secondary bacterial infection.

Your child will be cared for by the Emergency Department nurses and doctors as your child would have been if he/she were not in the study. For example, the study will not decide whether your child gets admitted to hospital or gets to go home.

At some point after your child has completed the study, the research staff will review your child’s health record to collect information relevant to the study. The research staff will create a study code to identify the research information and samples for your child. Dr. Tran will keep a key telling him the name and medical record number belonging to each study code. However, only the study code will be used to label the questionnaires, nasal swabs and blood specimens. To determine the role environment risk factors (i.e., air pollution), your child’s address will be collected and kept separately. This information will be encrypted and sent to Environment Canada where it will be converted to pollution information. Once your child’s address is converted to pollution information, it will be destroyed.

The other identifiers such as your or your child’s name, phone number, or medical record number will not be included as part of the research information. The recorded information on the questionnaires will be entered into a secure database. All samples will be sent to the Infectious Diseases Research Laboratory at SickKids, where they will be processed for storage and subsequent analysis. The saliva sample or a portion of the blood sample will be sent for genetic studies up to and including sequencing of the whole genome. The genetic information learned cannot yet be understood and therefore will not be used for your or your child’s medical care. Therefore, it will not be part of your or your child’s health records. The urine sample will be sent for testing to determine the chemicals produced by the body’s metabolic processes to develop a profile for secondary bacterial infections and severe influenza. Scientists, laboratory personnel, data entry clerks and any other personnel involved in the above steps will not be able to identify you or your child since only Dr. Tran will have access to the code that identifies the samples.

The samples will be stored indefinitely in a specimen bank under the direction of Dr. Tran at the research laboratory in the Division of Infectious Diseases at SickKids. The reason for storing samples is that new information could be discovered and there is a possibility that information may need to be gathered through additional testing. These samples will be made available only to researchers whose research studies are approved by an authorized review board or ethics committee (committee that reviews and approves research studies to make sure they are safe and ethical).
If changes are made to the study or new information (from this study or other studies) that might affect your willingness to continue to participate in the research becomes available, you will be informed.

**Potential Harms:**
There may be a small amount of bleeding when blood is taken from a vein and there may be slight discomfort and bruising or redness at the site that will usually disappear in a few days.

**Potential Discomforts or Inconvenience:**
The extra tests taken in the study (nasal swab and blood sampling) may be associated with some local discomfort.

**Potential Benefits:**

**To individual subjects:**
If your child is enrolled into the study, he/she may benefit indirectly in that the availability of the result of the rapid bedside test for influenza may help your child’s doctor make decisions about the necessity of other tests or in treating your child while in the Emergency Department. Your child may also benefit from closer follow-up than usual (follow-up at Day 3, Day 7, Day 14 and Day 28 by phone) as a result of participation in the study.
If your child provides a blood sample, we will provide you with your child’s vitamin D level when the sample is tested.

**To society:**
We hope the information learned from this study will benefit other children with or at risk for severe influenza infection in the future.

**Dissemination of study progress and findings:**
We plan to share the progress and results of this study with others in the following ways:
- Involving public health agencies throughout the study
- Presentations at scholarly meetings
- Publications in medical journals

**Confidentiality:**
We will respect your privacy. No information about who your child is will be given to anyone or be published without your permission, unless required by law. For example, the law could make us give information about your child if a child has been abused, if your child has an illness that could spread to others, if your child or someone else talks about suicide (killing themselves), or if the court orders us to give them the study papers.
SickKids clinical research monitors may see your child’s health record to check on the study. By signing this consent form, you agree to let these people look at your child’s records. We will put a copy of this research consent form in your child’s patient health record and give you a copy as well.
The data produced from this study will be stored in a secure, locked location. Only members of the research team (and maybe those individuals described above) will have access to the data. This could include external research team members. Following completion of the research study the data will be kept as long as required then destroyed as required by SickKids policy. Published study results will not reveal your child’s identity.
Reimbursement:
You, your child and/or your family will not be paid to take part in this study. We will reimburse you for all reasonable out of pocket expenses for being in this study, e.g., meals, babysitters, parking and getting you to and from SickKids. If you stop taking part in the study, we will pay you for your expenses for taking part in the study up until that point.

Participation:
It is your choice to take part in this study. You can stop at any time. The care your child gets at SickKids will not be affected in any way by whether he/she takes part in this study. New information that we get while we are doing this study may affect your decision to take part in this study. If this happens, we will tell you about this new information. And we will ask you again if you still want your child to be in the study.
During this study we may create new tests, new medicines, or other things that may be worth some money. Although we may make money from these findings, we cannot give you/your child any of this money now or in the future because your child took part in this study.
If your child becomes ill or are harmed because of study participation, we will treat your child for free. Your signing this consent form does not interfere with your child’s legal rights in any way. The staff of the study, any people who gave money for the study, or the hospital are still responsible, legally and professionally, for what they do.

Sponsorship:
This research is being funded by the Canadian Institutes of Health Research (CIHR).

Conflict of Interest:
There are no conflicts of interest to declare by members of this research team.

Preferences for Additional Consent

Please initial beside EACH of the options below to indicate your preferences about storage of samples, their use for future studies, and about being re-contacted.

_____  _____  I agree to allow my child’s samples to be kept and used for future research on infections other than influenza.
Yes   No

_____  _____  I may be contacted for future research studies related to influenza.
Yes   No

_____  _____  I may be contacted for future research studies not related to influenza.
Yes   No

_____  _____  I agree to provide consent for disclosure of personal health information related only to the “Flugene Study” if my child is seen by health care professionals outside of SickKids.
Yes   No
Consent:

“By signing this form, I agree that:

1) You have explained this study to me. You have answered all my questions.
2) You have explained the possible harms and benefits (if any) of this study.
3) I know what I could do instead of having my child take part in this study. I understand that I have the right to refuse to let my child take part in the study. I also have the right to take my child out of the study at any time. My decision about my child taking part in the study will not affect my child’s health care at SickKids.
4) I am free now, and in the future, to ask questions about the study.
5) I have been told that my child’s medical records will be kept private except as described to me.
6) I understand that no information about my child will be given to anyone or be published without first asking my permission.
7) I agree, or consent, that my child________________________ may take part in this study.”

____________________________________
Printed name of parent/legal guardian

Parent/legal guardian’s signature & date

____________________________________
Printed name of person who explained consent

Signature of person who explained consent & date

____________________________________
Printed witness’ name
(if parent/legal guardian does not read English)

Witness’ signature & date

If you have any questions about this study, please call Dr. Dat Tran at 416-813-7654 Ext 204649

If you have questions about your rights as a subject in a study or injuries during a study, please call the Research Ethics Manager at 416-813-5718.
APPENDIX B  List of Recruiting Sites for Biochemical Status of Vitamin D and Related Biomarkers as Predictors of Severe Influenza Infection in Children

Dr. Dat Tran  The Hospital for Sick Children
555 University Avenue
Toronto, ON
M5G 1X8

Dr. Nicole Le Saux  Children’s Hospital of Eastern Ontario (CHEO)
401 Smyth Road
Ottawa, ON
K1H 8L1

Dr. François Boucher  Centre hospitalier de l’Université Laval
2705, boulevard Laurier
Québec, QC
G1V2L9

Dr. Dorothy Moore  Montreal Children’s Hospital
2300 Rue Tupper
Montréal, QC
H3H 1P3