Dynamics of Influenza Protection and Infection:
Implications for Vaccination Development

by

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Dedication

To my family, who supported my passion for infectious disease epidemiology long before there was a pandemic to validate it, and without whom none absolutely none of this would have been possible. And to Cammi, who will never read a word of this dissertation, but who was present for every single chapter.

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Preface

Chapter 2 (**Antibody responses to influenza A(H1N1)pdm infection**) has been published in Vaccine. The complete author list is: Steph Wraith, Raffael Nachbagauer, Angel Balmaseda, Daniel Stadlbauer, Sergio Ojeda, Arvind Rajabhathor, Roger Lopez, Andrea F. Guglia, Nery Sanchez, Fatima Amanat, Lionel Gresh, Guillermina Kuan, Florian Krammer, Aubree Gordon

Chapter 3 (**Homotypic protection against influenza in a pediatric cohort in Managua, Nicaragua**) has been accepted for publication in Nature Communications. The complete author list is: Steph Wraith, Angel Balmaseda, Fausto Andres Bustos Carrillo, Guillermina Kuan, John Huddleston, John Kubale, Roger Lopez, Sergio Ojeda, Amy Schiller, Brenda Lopez, Nery Sanchez, Richard Webby, Martha I. Nelson, Eva Harris, Aubree Gordon

Chapter 4 (**Impact of heterotypic and heterosubtypic repeat influenza infection patterns in a pediatric cohort in Managua, Nicaragua**) will be submitted for publication. The complete author list will be: Steph Wraith, Angel Balmaseda, Fausto Andres Bustos Carrillo, Guillermina Kuan, John Kubale, Roger Lopez, Sergio Ojeda, Amy Schiller, Brenda Lopez, Nery Sanchez, Eva Harris, Aubree Gordon

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List of Abbreviations

ARI	Acute respiratory infections
ALRI	Acute lower respiratory tract infections
CI	Confidence interval
LMICs	Low- and middle-income countries
RT-PCR	Reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
CDC	U.S. Centers for Disease Control and Prevention
NIAID	National Institute of Allergy and Infectious Disease
WHO	World Health Organization
FDA	U.S. Food and Drug Administration
CNDR	Centro Nacional de Diagnóstico y Referencia
HCSFV	Health Center Sócrates Flores Vivas
NPICS	Nicaraguan Pediatric Influenza Cohort Study
HITS	Household Influenza Transmission Study
A/H1N1pdm	Influenza A H1N1 from 2009 swine flu pandemic
HAI	Hemagglutination inhibition assay
ELISA	Enzyme-Linked Immunosorbent Assay

HA	Hemagglutinin
NA	Neuraminidase
IRB	Institutional review board
SSI	Sustainable Sciences Institute
OR	Odds ratio
aOR	Adjusted odds ratio
OAS	Original antigenic sin

Abstract

Influenza is a major contributor to respiratory morbidity and mortality worldwide and is a particular concern for high-risk populations including children. Although influenza has been studied for over 100 years, major gaps remain in our understanding of influenza transmission and infection. In particular, how individuals respond to natural influenza infection (including those individuals who do not form a traditional response) and the effect of repeated influenza infection on patterns of response across seasons are not well-characterized.

In chapter 2 of this dissertation we investigate humoral immune response to influenza A(H1N1)pdm infection among Nicaraguan households. In chapters 3 and 4 we examine the impacts of repeated influenza infections over a nearly ten-year period among a cohort of Nicaraguan children aged 0-14 years. To examine these questions of immunity to natural infection and patterns of repeated infection we utilize data from the Nicaraguan Pediatric Influenza Cohort Study (NPICS) from 2011-2019, as well as data collected as part of the Household Influenza Transmission Study (HITS) from 2013-2015.

In chapter 2 of we found that a group of the infected individuals identified by PCR failed to produce a ≥4-fold hemagglutinin inhibition assay (HAI) response; a subset of these individuals produced an alternate antibody response (against full-length HA, HA stalk, or neuraminidase). These individuals had lower pre-existing HAI antibody titers and showed a pattern of milder illness. An additional subset did not produce an alternate antibody response, had higher pre-existing antibody titers against full-length & stalk HA, and were less sick. These

findings demonstrate that some individuals mount an alternate antibody response to influenza infection.

In chapters 3 and 4 we investigate the periods of protection from repeat infection following symptomatic influenza. We examine the effects of natural influenza virus infection on subsequent infection with homotypic and heterotypic influenza virus subtypes/lineages across multiple seasons. We observed homotypic protection from repeat infection in children infected with influenza A/H1N1pdm, A/H3N2, and B/Victoria. Overall, protection waned as time or antigenic distance increased. Individuals infected with one subtype or lineage of influenza virus have significantly lower odds of homologous reinfection for the following one to two years; after two years this protection waned. While we found no significant protection from heterotypic/heterosubtypic infection within influenza seasons, we did find that individuals infected with a given type or subtype of influenza in a season where two types/subtypes circulated were at increased risk for the other circulating type/subtype in the subsequent season. This heightened risk was present for both older and younger children and held true even after adjustments were made for healthcare-seeking behavior and pre-exposure antibody titer levels. Better understanding these dynamics of varied immune response and repeated infection and exposure is critical to addressing future risk patterns for individuals and populations and will allow for improvements in influenza vaccine design.

Chapter 1 - Introduction

1.1 Influenza

1.1.1 History and Burden

Influenza is a significant contributor to global annual morbidity and mortality, with epidemics in the winter seasons of the Northern and Southern hemispheres resulting in up to 650,000 deaths annually according to WHO estimates [1-3]. Up to 20% of the population can be infected each year, and there is a significant economic impact associated with influenza, including healthcare costs and time lost to illness. It can be difficult to accurately assess the cumulative burden of influenza due to challenges in both surveillance and testing, but we know that the impact of influenza is particularly high in vulnerable populations including young children who are at significantly higher risk of severe outcomes [4-5].

Influenza virus was first identified and isolated in the 1930s by research groups in the UK and the US [6-7]. Influenza A and B, the dominant influenza types among humans, are both negative sense single-strand RNA viruses with eight segments. These segments encode a range of key viral components, including HA and NA surface glycoproteins. These glycoproteins are the most variable and mutations in them drive the antigenic drift we observe between influenza seasons. The classification and nomenclature of influenza A viruses is determined by these surface proteins, with HA and NA being abbreviated and numbered to result in names like H1N1 and H3N2, the current dominant subtypes of influenza A [8-9]. By comparison, influenza B is classified by lineage, with the Yamagata and Victoria lineages of the virus currently co-

circulating. Those two lineages and two subtypes are generally what is included in most influenza vaccines.

Influenza A has generally exhibited a high level of genetic variation, in part due to the ability of A viruses to circulate in both humans and animals, which leads to a greater diversity of segments available for reassortment following co-infection [6,10]. This high rate of mutation and drift leads to seasonal epidemics and even occasionally, in the event of a major viral shift, a pandemic. There were four influenza pandemics in the 20th century, and one so far in the 21st century – the 2009 influenza pandemic, driven by a novel H1N1 virus. Influenza B, on the other hand, only diverged into two lineages relatively recently, and has no known animal reservoirs so tends to exhibit lower overall rates of antigenic change [10].

1.1.2 Treatment and Prevention

There are a range of options for influenza detection, treatment, and protection. The gold standard tests for influenza are viral culture or RT-PCR. RT-PCR is particularly useful as it is a molecular test with high sensitivity and specificity that is both fast and capable of subtyping viruses. Hemagglutination-inhibition assays (HAIs) are also often used to identify the HA subtype of an unknown viral isolate or two assess the specificity or level of antibody response to an HA subtype [10]. More recently ELISA testing has allowed for comparable assessments of the responses of NA antibodies [11].

The main treatment approach for influenza infection is antivirals. In a typical season of influenza, these antivirals will generally only be used for those who are most severely ill. There are two main classes of antiviral drugs against influenza are currently approved: adamantanes and NA inhibitors [10]. The adamantanes (amantadine and rimantadine) are orally administered and were approved by the FDA in 1966 and 1993, respectively. Both target the M2

transmembrane channel; however, due to concerns regarding resistance in circulating viruses, these drugs are no longer routinely recommended for clinical treatment of influenza. NA inhibitors, on the other hand, target the NA protein and are still regularly in use following the approval of the first NA inhibitor, Oseltamivir, in 1999 [12]. While resistance to NA inhibitors is currently only rarely reported, it has happened in past seasons and remains a cause for concern, particularly as higher levels of resistance have been reported among children [13]. This resistance concern underlines both the importance of continued exploration of alternative antiviral therapies and the value of strong preventative measures, including vaccines.

Seasonal influenza vaccination remains the best tool available for reducing annual disease burden, but the effectiveness of these vaccines has been shown to vary widely from season to season, with vaccine efficacy ranging from 10-60% over the past decade [14-15]. Even though frequent antigenic changes in the circulating influenza viruses are a known factor and the HA head region targeted by the vaccine is one of the most variable regions, the process of developing and manufacturing seasonal influenza vaccines has not greatly altered over the past few decades. The prevalence of circulating strains is determined by the WHO and that surveillance drives the selection of strains for the annual vaccine. While there is ample evidence that these seasonal vaccines still have great value in preventing millions of infections annually, there is clearly room for improvement – in 2018 NIAID released their strategic plan for the development of a universal influenza vaccine, highlighting the global priority of strengthening our ability to prevent influenza epidemics and pandemics [16-17].

1.1.3 Research Gaps

While our knowledge of influenza has expanded greatly since its original isolation in the 1930s, there is still a great deal to explore, particularly with regards to immunologic response to infection and lifetime patterns of exposure. The burden and dynamics of influenza in tropical countries are currently poorly characterized; gaining an enhanced understanding of influenza seasonality and risk characteristics as well as vaccine impact is crucially important in these lower-income settings where respiratory illnesses are a leading cause of child mortality

As we highlighted, one major area of research interest over the past decade has been the development of a universal influenza vaccine, as a replacement for our current model of generating a new seasonal influenza vaccine each year in response to viral changes over the previous season. In pursuit of that, it is critical to enhance our understanding of transmission and natural history of influenza virus infection in populations as well as improving our knowledge of correlates of immune protection, including characterizing responses to natural influenza infection.

1.2 Influenza Repeat Infections

1.2.1 Original Antigenic Sin

One of the key concepts when considering immune system response to infections and the implications of those responses is that of "original antigenic sin". This concept, first proposed by Thomas Francis Jr. in 1960, proposes that our immune systems are bound by the "sin" of their first imprinting on whichever virus is encountered earliest in the life course [18-19]. As proposed by Dr. Francis, this theory suggests that the first antigenic variant encountered early in life conditions lifelong immunity, with the antibody response to influenza strains from childhood dominating the anti-influenza virus antibody response over time; as a person ages and acquires

antibodies to other strains, those original antibodies are still always maintained at the highest levels. As in the biblical passage alluded to in the original hypothesis though, this knowledge comes at a potential cost – the immune response to a related strain later in life may potentially be less effective than a response elicited in the absence of any prior immunological knowledge of the strain. The importance of primary exposure on driving antibody repertoires was not only observed among humans infected with influenza; evidence in support of OAS has also been observed with other infectious diseases as well as in animal populations [20].

Over time, this theory of OAS has been refined, and various parallel or alternative theories have been suggested, including that of "antigenic seniority": the idea that dominant antibody responses are driven by repeated exposures rather than the initial imprinting event central to OAS. Various cohort studies have, over the years, contributed evidence to the impact of repeated influenza infections and the role of early and subsequent infections on driving immune responses over time [21].

1.2.2 Historical Studies

A range of historical cohort and household-based studies laid the groundwork for much of the research outlined in this dissertation. The Tecumseh Study tracked influenza in Tecumseh, Michigan during a nearly sixteen-year period from 1965-1972 and 1976-1981, examining ARI etiology through virus isolation and serology [22-25]. The number of individuals tracked per year was around 1,000 – only in 1978 was the study restricted to families with school-aged children or younger. The researchers examined influenza illness duration and the age-related factors driving this, as well as differences in specimens drawn from the community as compared to hospital-based settings. They were able to determine age-specific influenza infection rates (via serology, not RT-PCR) and identify peak ages for influenza type A and B infections in children.

Because of the reliance on HAI, they struggled to examine children under the age of 5 and were only examining pathogenicity through serology data. The researchers generally focused on the temporality of influenza and other respiratory viruses and did establish that influenza reinfection occurred during the study among the school-aged children. Homotypic protection from influenza was not examined by this study. For the first portion of the study, such examination would not even have been possible as households were not maintained in the study beyond 1 year. Individual longitudinal history of infection was not a focus of this study, and thus repeat infections were difficult to analyze. Additionally, due to the time frame in which the study was conducted, the testing mechanisms utilized were far less sensitive than currently available methods and focused on serodiagnosis, making analyzing the timing of illnesses difficult.

The Houston Family Study was a longitudinal family study initiated in Houston in 1975 and followed through 1980 [26-29]. Families were recruited from the Houston area and enrolled at the time of birth of an infant in their family. This was a household-based study with regular sample collection during the respiratory virus season as well as symptom data collection – once again the primary laboratory methodology for determining influenza infection was HAI. Researchers in this study were able to track influenza outbreaks including an outbreak of H3N2 in 1976 and were able to characterize incidence of symptomatic infection – however, once again due to the reliance on antibody rise as a determinant of infection, researchers could not time infection events precisely. They were able to establish some level of support for the Original Antigenic Sin (OAS) first put forth by Thomas Francis in 1960 due to the enrollment of infants at birth and ability to examine titer changes in HAI in response to circulating strains. The researchers did find that higher antibody titers to specific H3N2 strains were associated with protection of family members but were not able to characterize the duration of that protection

and did not always find consistent results regarding protection from reinfection. They did note the presence of statistically significant protection from influenza re-infection in some seasons but not others, but with a small, uncontrolled analysis.

The Cleveland Family Study was an active surveillance study in which illnesses of members of approximately 60 households were monitored from 1947-1957, with a study design focused on young couples with children [30-31]. Symptom diaries and HAI were again the main mechanisms used for data collection and outcome determination in this study. The study was able to establish that children were significantly more likely to experience re-infection with influenza than adults and sought to examine some of the dynamics of infection with influenza in the community examining both children and adults. The researchers established that the occurrence of ILI within a given family during one epidemic was influenced by whether there was influenza virus isolated from that same family during a previous pandemic, a finding that laid the groundwork for much of the subsequent examinations of homotypic protection from influenza; it was critically important for establishing much of the baseline knowledge of influenza dynamics we now have today, but was limited by both its power and the laboratory techniques of the time.

1.2.3 Modern Studies

Present-day studies of influenza have built upon the previously outlined historical work and have also provided insight that led to the research aims outlined in this dissertation. The Flu Watch cohort in the UK began in 2006 with the aim of estimating community burden of influenza and ILI, to measure immune responses, and to use those findings to better inform parameters for modeling influenza [32-34]. This was a household-based study with follow-up conducted for a period of six years after the initial startup of the study. The initial enrollment was

602 households with subsequent response rate in later seasons not available. RT-PCR was used to assess influenza infection status, with blood samples also collected to characterize antibody titer levels. Using this data, the authors published on national estimates of disease burden, demonstrated evidence that T-cell based immunity provided protection from influenza infection in both seasonal and pandemic periods, and the impact of age as a predictor of T-cell responses.

A longitudinal study in Hong Kong was begun in July 2009 and ran through 2015 and involved recruitment from across the population of Hong Kong via telephone survey sampling [35-38]. Serum samples were collected annually from participants, who needed to be 2 years of age or older. HAI was utilized to assess titer levels in response to A/H1N1, A/H3N2, and B strains. This study was able to characterize influenza epidemics in Hong Kong across the study period and examined effects of age and other risk factors [35]. In an analysis of repeated A/H3N2 epidemics, the researchers established that repeat infections were rare and showed that the largest epidemic occurred when there was the lowest level of pre-epidemic immunity for A/H3N2 strains [36]. Waning rates of HAI titers have been shown by this study in the context of A/H3N2 [36]. This research group has also utilized broad surveillance data from Hong Kong to analyze levels of cross-immunity between influenza virus strains; they have additionally conducted observational studies of older adults as well as vaccination randomized controlled trials [37-38].

Influenza virus surveillance has been carried out nationally in Singapore since 1973. In addition, various short-term cohort studies have been conducted to examine various aspects of influenza infection [39-43]. A cohort study from October 2009-September 2010 collected blood samples and demographic data to characterize the epidemiology and severity of influenza subtypes post the 2009 H1N1 pandemic and analyzed the impact of birth cohort on outcomes

[42]. A subset of these individuals was re-sampled to examine antibody titer waning one year after the pandemic; however, the total number of participants was less than 100 and all were 21 years or older in age [39]. A prospective cohort in 2009 collected paired serum samples and symptom diaries from military personnel to characterize seroconversion against influenza and examine the effects of various public health interventions [41]. A cohort study among university students in 2007 utilized RT-PCR and virus isolation to detect viruses and determine the proportion of ILI attributable to influenza A and B on university campuses in Singapore [40]. Retrospective cohort studies to assess influenza vaccine effectiveness have also been conducted [43]. No cohort studies that we were able to locate in Singapore characterized homotypic protection from influenza among children utilizing RT-PCR across multiple seasons.

A household-based cohort study was conducted in Vietnam between December 2007 and April 2010, with all participants 5 years of age or older providing blood samples and swabs during ILI episodes to allow for confirmation of influenza infection using RT-PCR [44]. Approximately 900 participants were included for each of the three seasons examined; this study was one of the first conducted in a tropical setting to quantify influenza incidence in the same households and individuals across multiple consecutive seasons. This study assessed risk of infection, with some examination of co-infection and re-infection. Further analyses characterized chains of transmission to establish probability of transmission between household members. The authors established that high pre-season titers were protective against infection in a given season and determined the amount of infection attributable to household transmission [45-46].

1.2.4 Research Gaps

Many of our most pressing questions around repeat infection implications for influenza require longitudinal data that is hard to come by. While the historical studies discussed here have

helped provide a framework for future influenza research, many of them lack the laboratory data necessary to examine these questions. Present day studies such as Flu Watch have provided valuable insight but are frequently conducted in high income, temperate countries with high levels of vaccination that make analyzing natural infection impacts difficult. There is a major gap in the literature examining the role of repeat influenza infections, particularly among children, and how these infection patterns drive immune responses of individuals and populations. Many of these questions require a large-scale, long-term longitudinal study in order to effectively analyze and respond to these issues.

1.3 Dissertation Aims

1.3.1 Aim 1. Examine the association between HAI non-response and response level to alternate correlates of influenza protection.

In Chapter 2, I will discuss Aim 1 of my dissertation, focused on characterizing HAI non-response and the response level to alternate correlates among a household transmission study in Nicaragua. We hypothesized that individuals infected with influenza who fail to generate a meaningful seroconversion in response to HAI generate a meaningful and detectable response to other antigenic targets on the influenza virus. We sought to assess the demographic characteristics and patterns of immune response to influenza infection among these HAI non-responders through the analysis of data collected as part of a household case-ascertainment study conducted in Managua, Nicaragua. We identified non-responders and analyzed how these non-responders differ from those who undergo a "normal" response to HAI post-influenza infection by demographic characteristics, symptom response patterns, and shedding duration. We additionally examined the response to previously identified correlates of protection (full HA, HA stalk, NA) among both the normal and non-responder individuals by comparing pre- and post-

exposure serology and assessed whether a meaningful number of the non-responders generated a 4-fold or greater response to one or more of these alternate correlates.

1.3.2 Aim 2 - Characterize the strength and level of protection conferred by homotypic influenza infection within and across seasons.

In Chapter 3, I will discuss Aim 2 of my dissertation, examining the effects of homotypic repeat infections on a group of children in a cohort in Managua, Nicaragua. We hypothesized that individuals infected with a given subtype of influenza would be conferred a degree of protection from repeat infections with the same subtype, and that this protection would exist between seasons of influenza in addition to the previously established within season homotypic protection. Further, we hypothesized that this protection would last for at least several years and the duration of protection will vary depending on infection history and the rate of antigenic change in the virus. We characterized these patterns of homotypic protection between specific strains of influenza among participants in a pediatric cohort in Managua, Nicaragua. We expect to see that this protection is strongest in the seasons immediately following infection and then diminishes as the virus strain undergoes antigenic drift. We analyzed the impacts of age and cohort effects upon these associations, using advanced modeling techniques. This analysis provides the first nearly decade-long examination of homotypic protection from repeat infection among children.

1.3.3 Aim 3 - Characterize the strength and level of protection conferred by heterotypic and heterosubtypic influenza infection within and across seasons.

In Chapter 4, I will discuss Aim 3 of my dissertation, exploring the role of heterotypic and heterosubtypic repeat infections among the same pediatric cohort in Nicaragua. We hypothesized

that individuals infected with a given subtype of influenza would be conferred a degree of protection from repeat infections with different types/subtypes of influenza, and that this protection exists and fluctuates only within a single season of influenza before weakening as the time since infection increases. We additionally hypothesized that these same individuals would be at increased risk of heterotypic and heterosubtypic infection in the following seasons. We sought to examine and characterize these patterns of heterosubtypic and heterotypic infection against various influenza strains among participants in a pediatric cohort in Managua, Nicaragua. Establishing the effects of these repeat infections as influenza viruses shift over time will provide valuable insights into both the role of OAS and the potential avenues for improvement of annual influenza vaccines.

The overall goal of this dissertation is to characterize the varied immune responses to influenza virus infection in a tropical LMIC setting, and to establish the effects of repeated infections across nearly a decade among a cohort of children.

Chapter 2 - Antibody Responses to Influenza A(H1N1)pdm Infection

2.1 Author Summary

We found that not all influenza-infected individuals generated HA inhibition antibodies. A subset of individuals responded exclusively to alternate viral targets of infection; these findings suggest that including NA and HA stalk could improve both surveillance efforts and enhance the degree of protection offered by the annual influenza vaccine.

2.2 Abstract

We investigated humoral immune response to influenza A(H1N1)pdm infection and found 32 (22%) of the infected individuals identified by PCR failed to produce a ≥4-fold hemagglutinin inhibition assay (HAI) response; a subset of 18 (56%) produced an alternate antibody response (against full-length HA, HA stalk, or neuraminidase). These individuals had lower pre-existing HAI antibody titers and showed a pattern of milder illness. An additional subset of 14 (44%) did not produce an alternate antibody response, had higher pre-existing antibody titers against full-length & stalk HA, and were less sick. These findings demonstrate that some individuals mount an alternate antibody response to influenza infection. In order to design more broadly protective influenza vaccines it may be useful to target these alternate sites. These findings support that there are influenza cases currently being missed by solely implementing HAI assays, resulting in an underestimation of the global burden of influenza infection.

2.3 Introduction

As part of an ongoing effort to improve influenza vaccines and develop our understanding of the dynamics of the immune response to infection, there is a great deal of interest in investigating alternate correlates of protection against influenza [16, 47].

The influenza virus has two surface glycoproteins; hemagglutinin (HA) and neuraminidase (NA) [48]. Most individuals experience a strong hemagglutination-inhibition (HAI) response to infection with influenza virus, which is currently the only generally accepted correlate of protection for influenza [49-51]. There is variation in response levels, however, and some individuals do not produce a strong HAI antibody response to infection [52].

Importantly, HAI only measures a subset of antibodies that target the HA head. Additional antibody responses can be captured by using enzyme-linked immunosorbent assays (ELISA) against HA stalk region, full-length HA protein, and NA [47,48,53]. These regions are all potential universal influenza vaccine targets, due to their conserved nature and impact on virus fitness and spread [47, 49]. Here we assess whether individuals with a limited HAI response after natural influenza virus infection produce alternate immune responses to the HA stalk, full-length HA, or NA, and examine how these atypical responders differ from those presenting a typical HAI response to infection.

2.4 Methods

2.4.1 Study Design

To investigate the immune response patterns to HAI and potential alternate correlates of protection, a case-ascertainment study of naturally occurring influenza virus transmission was performed in households in Managua, Nicaragua. Study design has been previously described [52, 53]. Subjects provided daily symptom assessment, and respiratory swabs (nasal and oropharyngeal) were taken every 2-3 days over a 10-14 day period. Blood samples were collected at enrollment and 3-5 weeks later. Households eligible for inclusion in the study were those with ≥2 individuals and an index case that experienced acute respiratory infection (ARI) symptom onset within 48 hours and tested positive for influenza. For this analysis, 66 RT-PCR confirmed influenza A(H1N1)pdm index cases from the 2013 and 2015 influenza seasons and their 423 household contacts were considered. 123 participants were excluded due to absence of paired blood samples for testing, resulting in a final analysis group of 366 individuals. This study received ethical approval from the institutional review boards at the Ministry of Health of Nicaragua and the University of Michigan. Informed consent was collected for all participants and verbal assent obtained from children ≥6 years.

2.4.2 Laboratory Methods

Respiratory samples were tested at the Nicaraguan National Virology Laboratory via real-time RT-PCR following U.S. Centers for Disease Controls and Prevention protocols. Samples were tested for influenza A virus; positive samples were then subtyped as H1N1 or H3N2, with RT-PCR for both universal A and subtype repeated for initially unsubtypable samples to reduce probability of a false positive. Hemagglutinin inhibition assays were conducted to measure HAI titers; ELISAs were performed to measure anti-HA stalk, full-length

HA, and NA antibodies as previously described [7]. Full-length recombinant HA constructs corresponded to vaccine strains from the respective seasons (2013: H1 A/California/4/09, 2015: H1 A/Michigan/45/15) were used. To measure HA stalk antibodies, a recombinant chimeric HA with the head domain from an H6 HA (A/mallard/Sweden/81/02) and a stalk domain from A/California/4/09 was used (cH6/1); to measure NA antibodies, a recombinant NA of A/California/4/09 was used [52].

2.4.3 Statistical Analysis

The main outcomes of this study were PCR-confirmed influenza virus infection, seroconversion by HAI (defined as a ≥4-fold rise in antibody titer), and the ratio of antibody response comparing the post- and pre-infection measurements for HA stalk, full-length HA, and NA antibodies. "HAI responders" were defined as individuals with PCR-confirmed influenza virus infection and a ≥4-fold rise in HAI titer. "HAI non-responders" were defined as individuals with PCR-confirmed influenza infection who did not exhibit a ≥4-fold rise in HAI titer. "Alternate responders" were defined as "HAI non-responders" who had a ≥4-fold response to full-length HA, HA stalk and/or NA. All definitions were established prior to the statistical analysis of the data. While there is some debate in the literature as to whether a 2-fold response to HAI can truly be considered non-response, we elected to define our seroconversions by the currently accepted standard of a 4-fold rise [54]. Chi-squared analyses, t-tests, and ANOVA modeling were used to compare groups. Statistical analyses were conducted using SAS. Graphics were constructed in R using the packages ggplot, plotly, and reshape2.

2.5 Results

2.5.1 Study Population

Among the total study population (n=366), 149 (41%) individuals experienced PCR-confirmed influenza virus infection and 147 (40%) individuals had serological evidence of infection using traditional criteria of a ≥4-fold rise in HAI titer (Table 2.3). Antibody levels (both pre- and post-exposure) were more strongly correlated between HAI, full-length HA, HA stalk, and NA among children than among adults (Figure 2.2). HAI, full-length HA, and HA stalk antibodies were most strongly correlated irrespective of age category or exposure timing. Among both children and adults, NA titer correlation to HAI and full-length HA increased post-exposure; among children the correlation between NA titer and HA stalk also increased while among adults correlation slightly decrease. Children displayed a higher proportion of HAI responders as well as increased correlation of HA stalk and NA compared to adults.

2.5.2 Characteristics of HAI responders

A total of 117 (79%) PCR-positive participants experienced a ≥4-fold HAI antibody titer rise. Among these "HAI responders", 52 (44%) were male, 79 (68%) were children (defined as ≤14 years of age), and 95 (81%) exhibited a symptom profile for influenza-like illness (ILI) (Table 2.1). 39% exhibited ≥4-fold rise in anti-HA stalk antibodies, 51% exhibited ≥4-fold rise in anti-full-length HA antibodies, and 32% exhibited ≥4-fold rise in anti-NA antibodies (Table 2.4).

2.5.3 Characteristics of HAI non-responders

The "HAI non-responders" group consisted of 18 (56%) individuals who had a ≥4-fold response against HA stalk, full-length HA, and/or NA, hereafter termed "alternate responder", and 14 (44%) individuals who had ≤4 fold response, hereafter termed "no response". Among the

"alternate responder" group, 6 (33%) were male, 12 (67%) were children, and 13 (72%) exhibited a symptom profile for ILI (Table 2.2). Among this "alternate responder" population, 11 (61%) exhibited ≥4-fold antibody response to HA stalk, 14 (78%) exhibited ≥4-fold antibody response to full-length HA, and 4 (22%) exhibited ≥4-fold response to NA (Table 2.4). Among the "no response" group, 8 (57%) were male, 7 (50%) were children, and 7 (50%) exhibited a symptom profile for ILI (Table 2.2).There was no evidence of differences by age (p=0.44), sex (p=0.96), or symptoms (p=0.14) between those who developed an alternate antibody response and those who did not, although these comparisons are limited in power by the sample size of n=32 (Table 2.5).

2.5.4 HAI non-responders compared to HAI responders

Among PCR-positive individuals, 32 (22%) did not exhibit a ≥4-fold HAI antibody response. "HAI non-responders" did not differ significantly from "HAI responders" by age (p=0.3894), sex (p=0.94), or index patient status (p=0.11) (Table 2.1). "HAI non-responders" were significantly less sick then "HAI responders", as they were less likely to report fever ($p<0.05$), be classified as an ILI ($p<0.01$) or ARI episode ($p<0.05$; Table 2.1). There was a significant difference in coughing duration (Table 2.1). "HAI non-responders" also had lower fold-change antibody titers to full-length HA and NA than "HAI responders" across all age groups (Figure 2.3a, c). The HA stalk response was similar for "HAI responders" and "HAI non-responders" under 40 years of age (Figure 2.3b).

2.5.5 No response individuals versus HAI and alternate responders

When this analysis was further subset into three groups ("HAI responders", "alternate responders", "no response"), significant patterns emerged. There was a difference in both symptoms and pre-exposure antibody levels between the "no response" population and the HAI

and alternate responders. “No response” individuals were less ill than HAI responders and alternate responders, with only 64% exhibiting fever (p-value = 0.1070), 50% exhibiting ILI (p-value 0.0274), and 57% exhibiting ARI (p-value = 0.0006). “No response” individuals also had significantly higher levels of pre-existing full-length HA (mean titer 104.78, p-value 0.0173) and stalk antibodies (mean titer 116.75, p-value 0.0001) than HAI responders or alternate responders. There was no significant difference in age or sex amongst the three groups.

2.5.6 Alternate responders compared to HAI responders

The “alternate responders” were less symptomatic and had shorter duration of symptoms than “HAI responders”, but otherwise did not differ (Table 2.6). There was no significant difference in pre-exposure antibody levels between the “alternate responders” and the “HAI responders” except for the HAI pre-titer (Table 2.6). Among the “alternate responders”, only 1 (6%) had a detectable HAI pre-titer; among the “HAI responders”, 30 (26%) had a detectable HAI pre-titer. Antibody titers (both pre- and post-exposure) had lower correlation on average among “alternate responders” than among “HAI responders” (Figure 2.1). In general, these “alternate responders” had a pattern of increased correlation in their post-exposure titers, similar to the magnitude of change in correlation pre- and post-exposure among the “HAI responders”. To visually examine potential patterns in antibody responses, individuals were mapped onto a 3D plot reflecting their relative responses to each of the antibodies (Figure 2.4). “Alternate responders” varied in their antibody response and did not exhibit a consistent pattern outside of their low HAI response compared to “HAI responders”. To display the distribution of how “alternate responders” overlap each other, a pie chart was created (See Appendix A); the greatest percentage responded to both HA stalk and full HA (34%), followed by those who responded only to full HA (19%) or only NA (19%). We assessed whether alternate response patterns

among the “HAI responders” made any additional benefits (Table 2.7) and found that HAI responders who also produced an “alternate response” experienced more severe symptoms (p-value 0.0193) and were more likely to be under 15 years of age (p-value 0.0035) and male (p-value 0.0272). A tree diagram depicting each subpopulation analysis presented across tables in the study has been included in the Supplemental Materials (See Appendix A).

2.6 Discussion

Here we found that within the overall population of influenza A(H1N1)pdm PCR-positive individuals, there are those who produce a ≥4-fold HAI response (“HAI responders”) but also a sub-population that fails to produce the ≥4-fold rise typically associated with infection (“HAI non-responders”). These “HAI non-responders” were less sick and had shorter symptom duration than the “HAI responders”. Within this “HAI non-responder” population, there were individuals who produced a ≥4-fold response to one of the alternate antibody targets (HA, HA stalk, NA), who we designated as “alternate responders”. These “alternate responders” did not differ significantly from the “HAI responders” by any individual variables but did display an overall pattern of less severe symptoms. These findings broadly provide evidence that some individuals with limited HAI response after influenza virus infection produce alternate immune responses.

We also identified individuals who were confirmed positive for influenza by PCR but exhibited no serologic response to infection. Notably, this population had higher pre-existing HAI titers and higher pre-existing full-length and stalk HA antibody titers. The fact that these antibody markers have been associated with protection [52, 53] might explain why this population tends to exhibit less symptoms that the “HAI responders” or the “alternate responders”. Alternatively, the level of influenza exposure among those individuals may have

been too weak to induce any immune response. Prior exposure to influenza could have an impact on whether individuals exhibit an alternate response to subsequent infection as it has been well-established in the literature that memory B cells produced in response to prior infection have an advantage generating antibodies over naive B cells [55].

These findings are consistent with prior research which identified alternative responses to influenza infection [52, 56]. Additionally, the level of HAI non-response in this study is similar to other studies [51, 57-58]. While there have been studies identifying non-responders to HAI [52], and studies highlighting alternate correlates of protection that may be used in assessing immune response to influenza [7, 59-60], there has not been an extensive examination of the overlap between these two areas. What these findings demonstrate is that among the previously identified population of those with limited response to HAI, there is a sub-population responding meaningfully to one of the alternate markers of infection previously identified in other studies. With regards to the differential results between adults and children, while it is known that HA stalk and NA antibody levels increase with age, children exhibiting higher correlation in this analysis doesn't indicate that their actual antibody levels are higher, so this finding does not contradict the established literature [57-58]. Strengths of this study include the intensive nature of follow-up. Limitations include the fairly small number of non-responders identified, decreasing the statistical power of the study; we also did not assess any potential contribution of cellular immune responses.

Prior studies have identified HA stalk and NA as alternative and independent correlates of protection for influenza and suggested that inclusion of these correlates could result in development of a more robustly protective influenza vaccine [59-62]. In particular, Huang et al. found in a cohort study in New Zealand that among those infected with influenza, nearly a third

only seroconverted in response to NA, not hemagglutinin [62]. Their work highlights the importance of considering other antigen responses in determining the overall burden of influenza, as they noted distinct patterns of NA-only response based on age and virus type; within their study, children under 5 and those infected with influenza B were significantly more likely to experience only NAI seroconversion. Our work expands on these findings by including assays for HA stalk and full HA (in addition to HAI and NA), and examining these associations in response to influenza A(H1N1)pdm, which the Huang study was unable to do as they were limited to a single influenza season with low H1N1 circulation. We find that in addition to individuals responding to NA, there are also influenza-infected individuals responding to the HA stalk or other regions of the HA not detected by traditional HAI.

Our findings support the conclusions of prior studies in this area and suggest that including these alternative correlates as serological markers of infection would allow us to capture influenza cases currently being missed by the serology standard of HAI, which results in both an underestimation of influenza infections and a bias in cases identified. Additionally, these findings indicate that designing influenza vaccines to elicit immune responses such as those that occur in the “alternate responders”, may result in broader protection for the general population and that HAI antibodies are not required to clear influenza infection in all individuals.

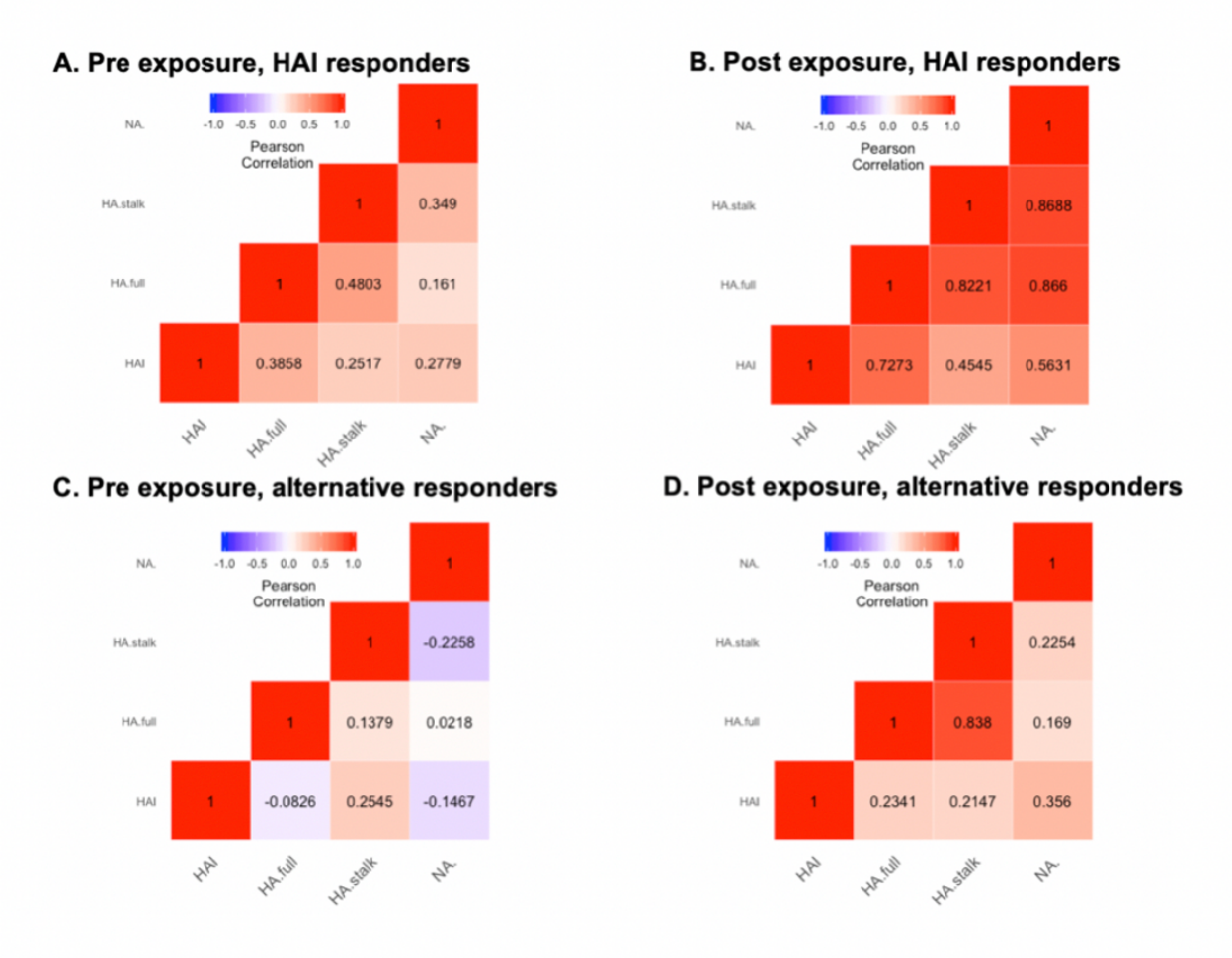

Figure 2.1: Pre-exposure and post-exposure correlation plots among HAI responders & alternative responders

Correlation of antibody levels as measured by Pearson correlation coefficient, comparing pre (A, C) and post (B, D) exposure levels for both HAI responders and non-responders. Brighter colors indicate greater correlation. Red colors indicate positive correlation, purple colors indicate negative correlation.

Table 2.1 HAI Responder versus Non-Responder Characteristics

	PCR-positive HAI responder[a] (n = 117)	PCR-positive HAI non-responder[b] (n=32)	P-value
Male	52 (44%)	14 (44%)	0.94
Age			0.39
0-14 years	79 (68%)	19 (59%)	
15+ years	38 (32%)	13 (41%)	
Symptoms			
Fever[c]	99 (85%)	22 (69%)	<0.05
ILI[d]	95 (81%)	20 (65%)	<0.01
ARI[e]	108 (92%)	23 (72%)	<0.05
Index case	55 (44%)	10 (31%)	0.11
Symptom duration (mean days, SD)			
Cough	7.39 (3.54)	5.83 (4.17)	<0.05
Shedding	4.23 (3.07)	3.28 (2.50)	0.11

Data are no. individuals with characteristic/no. of individuals.
Abbreviations: ILI, influenza-like illness; ARI, acute respiratory infection; SD, standard deviation.
[a]HAI responder is an individual who generated a 4-fold or greater HAI response.
[b]HAI non-responder is an individual who failed to generate a 4-fold or greater HAI response.
[c]Fever is reported fever or measured temperature ≥ 37.5 °C.
[d]ILI is fever as described above plus either cough or sore throat.
[e]ARI is defined as any of the following symptoms: fever, cough, sore throat, runny nose.

Table 2.2: HAI responder versus Alternate responder characteristics

	Alternate responders[a] (n=18)	HAI responders[b] (n=117)	P-value
Male	6 (33%)	52 (44%)	0.38
Age			0.94
0-14 years	12 (67%)	79 (68%)	
15+ years	6 (33%)	38 (32%)	
Symptoms			
Fever[c]	13 (72%)	99 (85%)	0.19
ILI[d]	13 (72%)	95 (81%)	0.38
ARI[e]	15 (83%)	108 (92%)	0.21
Illness duration (mean days, SD)			
Cough	6.14 (4.00)	7.39 (3.54)	0.06
Shedding	3.72 (2.87)	4.23 (3.07)	0.50
Pre-exposure antibody levels (mean level, SD)			
HAI	0.56 (2.36)	7.469 (17.83)	<0.01
HA full	34.84 (102.4)	39.13 (72.99)	0.87
HA stalk	24.15 (49.12)	34.38 (56.28)	0.43
NA	6.57 (10.49)	9.83 (20.91)	0.31

Data are no. individuals with characteristic/no. of individuals.
Abbreviations: ILI, influenza-like illness; ARI, acute respiratory infection; SD, standard deviation; HAI, hemagglutinin inhibition assay; HA, hemagglutinin; NA, neuraminidase.

[a]Alternate responder is an individual who failed to generate a 4-fold or greater HAI response but did generate a 4-fold or greater response to HA full-length, HA stalk, or NA.
[b]HAI responder is an individual who generated a 4-fold or greater HAI response.
[c]Fever is reported fever or measured temperature ≥ 37.5 °C.
[d]ILI is fever as described above plus either cough or sore throat.
[e]ARI is defined as any of the following symptoms: fever, cough, sore throat, runny nose.

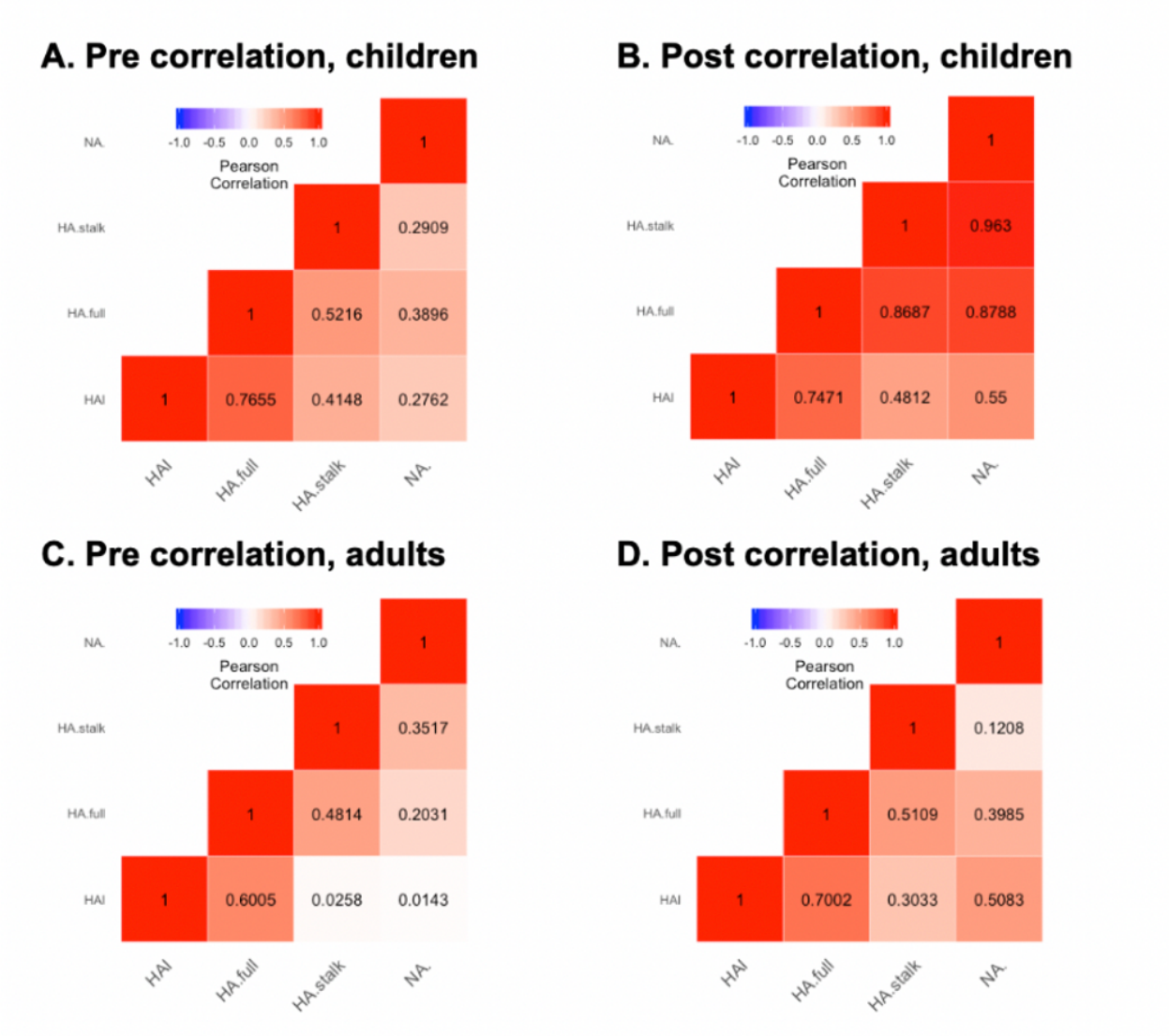

Figure 2.2: Pre-exposure and post-exposure correlation plots among children and adults

Correlation of antibody levels as measured by Pearson correlation coefficient, comparing pre (A, C) and post (B, D) exposure levels for children (14 years of age or younger) and adults. Brighter colors indicate greater correlation. Red colors indicate positive correlation, purple colors indicate negative correlation.

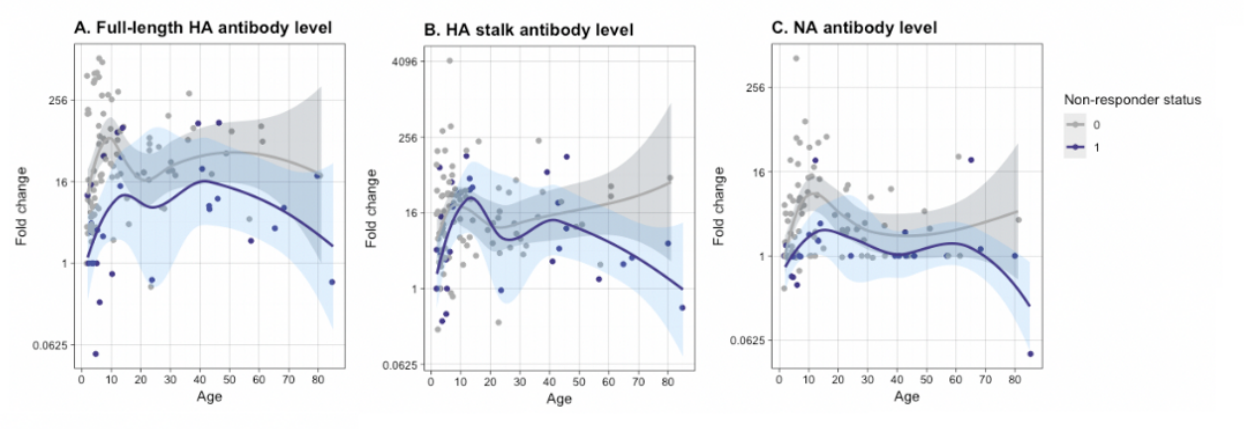

Figure 2.3: Fold-change responses by age comparing HAI responders and non-responders

Distribution of fold-change responses across ages to (a) full-length HA, (B) HA stalk, and (C) NA antibody; normal responders to HAI are represented in grey, with HAI non-responders represented in blue.

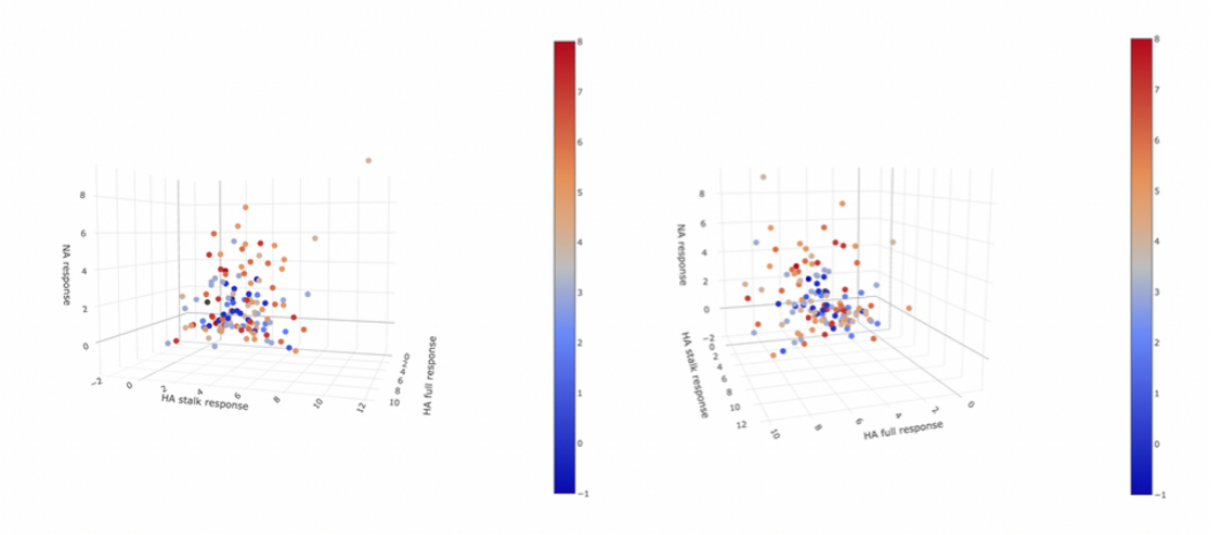

Figure 2.4: Three-dimensional distribution of log-change responses to antibodies, HAI responder versus alternate responder

Each individual's antibody response is plotted on a 3D system corresponding to their log-change response to each of the alternative correlates of protection; each data point is color-coded by the individual's level of response to HAI with highest HAI responders in red and alternate (non-HAI responders) in blue. Two views of the same 3D plot are presented above.

Table 2.3: Overall Study Population Characteristics

	≥4-fold HAI titer rise (n = 147)	<4-fold HAI titer rise (n=219)	P-value
PCR positive	117 (80%)	32 (15%)	<0.0001
Male	61 (42%)	76 (35%)	0.19
Age			<0.0001
0-14 years	86 (59%)	61 (28%)	
15+ years	78 (41%)	141 (72%)	
Symptoms			
Fever[a]	107 (73%)	45 (21%)	<0.0001
ILI[b]	103 (70%)	38 (17%)	<0.0001
ARI[c]	122 (83%)	87 (40%)	<0.0001
Index case	55 (37%)	11 (5%)	<0.0001

Data are no. individuals with characteristic/no. of individuals.

Abbreviations: ILI, influenza-like illness; ARI, acute respiratory infection.

[a]Fever = reported fever or measured temperature ≥ 37.5 °C.

[b]ILI is fever as described above plus either cough or sore throat.

[c]ARI is defined as any of the following symptoms: fever, cough, sore throat, runny nose.

Table 2.4: Distribution of ≥4-fold antibody responses to HA stalk, full-length HA or NA among Alternate responders and HAI responders

	Alternate responders[a] (n=18)	HAI responder 4-fold or greater response[b] (n=117)	P-value
HA stalk	11 (61%)	51 (44%)	0.16
HA	14 (78%)	67 (57%)	0.10
NA	4 (22%)	42 (36%)	0.25

Data are no. individuals with characteristic/no. of individuals.

Abbreviations: HA, hemagglutinin; NA, neuraminidase.

[a]Alternate responder is an individual who failed to generate a 4-fold or greater HAI response. but did generate a 4-fold or greater response to HA full, HA stalk, or NA.

[b]HAI responder is an individual generated a 4-fold or greater HAI response.

Table 2.5: HAI Non-responder Population Characteristics

	HAI non-responders[a] with alternate response[b] (n=18)	HAI non-responders[a] with no alternate response[b] (n=14)	P-value
Male	6 (33%)	8 (57%)	0.96
Age			0.44
0-14 years	12 (67%)	7 (50%)	
15+ years	6 (33%)	7 (50%)	
Symptoms			
Fever[c]	13 (72%)	9 (64%)	0.14
ILI[d]	13 (72%)	7 (50%)	0.08
ARI[e]	15 (83%)	8 (57%)	0.11
Illness duration (mean days, SD)			
Cough	6.14 (4.00)	5.41 (4.49)	0.64
Shedding	3.72 (2.87)	2.71 (1.90)	0.27

Data are no. individuals with characteristic/no. of individuals.
Abbreviations: ILI, influenza-like illness; ARI, acute respiratory infection; SD, standard deviation.

[a]HAI non-responder is an individual who failed to generate a 4-fold or greater HAI response.

[b]Alternate response is a 4-fold or greater response to HA full, HA stalk, or NA.

[c]Fever is reported fever or measured temperature ≥ 37.5 °C.

[d]ILI is fever as described above plus either cough or sore throat.

[e]ARI is defined as any of the following symptoms: fever, cough, sore throat, runny nose.

Table 2.6: HAI responders with alternate responses versus HAI responders without alternate responses characteristics

	PCR-positive HAI responders with alternate responses (n=90)	PCR-positive HAI responders without alternate responses (n=27)	P-value
Male	45 (50%)	7 (26%)	0.0272
Age			0.0035
0-14 years	67 (74%)	12 (44%)	
15+ years	23 (26%)	15 (56%)	
Symptoms			
Fever[c]	80 (89%)	19 (70%)	0.0193
ILI[d]	86 (96%)	22 (81%)	0.0161
ARI[e]	76 (84%)	19 (70%)	0.1007
Illness duration (mean days, SD)			
Cough	7.61 (3.30)	6.67 (4.21)	0.2250
Shedding	4.52 (3.15)	3.29 (2.62)	0.0683
Pre-exposure antibody levels (mean level, SD)			
HAI	8.94 (11.65)	19.63 (25.04)	0.0024
HA full	32.04 (68.84)	74.65 (72.85)	0.0070
HA stalk	23.07 (37.16)	83.33 (77.79)	0.0007
NA	10.18 (16.19)	23.23 (25.84)	0.0022

Data are no. individuals with characteristic/no. of individuals.

Abbreviations: ILI, influenza-like illness; ARI, acute respiratory infection; SD, standard deviation; HAI, hemagglutinin inhibition assay; HA, hemagglutinin; NA, neuraminidase.

[a]Alternate responder is an individual who failed to generate a 4-fold or greater HAI response but did generate a 4-fold or greater response to HA full-length, HA stalk, or NA.

[b]HAI responder is an individual who generated a 4-fold or greater HAI response.

[c]Fever is reported fever or measured temperature ≥ 37.5 °C.

[d]ILI is fever as described above plus either cough or sore throat.

[e]ARI is defined as any of the following symptoms: fever, cough, sore throat, runny nose.

Chapter 3 - Homotypic Protection Against Influenza in a Pediatric Cohort in Managua, Nicaragua

3.1 Author Summary

We observed homotypic protection from repeated infection with influenza using 9 years of data from a prospective pediatric cohort. This protection was shown across multiple seasons, subtypes, and lineages and was consistent for older and younger children. We found strong protection from repeated homotypic infection for a period of three years, depending on the degree of antigenic change across a given season. The strength of this protection suggests that incorporating infection histories may be useful in understanding population-level risk in a given year and may also inform vaccine development decisions.

3.2 Abstract

The period of protection from repeat infection following symptomatic influenza is not well established due to limited availability of longitudinal data. Using data from a pediatric cohort in Managua, Nicaragua, we examine the effects of natural influenza virus infection on subsequent infection with the same influenza virus subtype/lineage across multiple seasons, totaling 2,170 RT-PCR-confirmed symptomatic influenza infections. Logistic regression models assessed whether infection in the prior influenza season protected against homologous

reinfection. We sequenced viruses from 2011-2019 identifying dominant clades and measuring antigenic distances between hemagglutinin clades. We observe homotypic protection from repeat infection in children infected with influenza A/H1N1pdm (OR 0.12, CI 0.02-0.88), A/H3N2 (OR 0.41, CI 0.24-0.73), and B/Victoria (OR 0.00, CI 0.00-0.14), but not with B/Yamagata viruses (OR 0.60, CI 0.09-2.10). Overall, protection wanes as time or antigenic distance increases. Individuals infected with one subtype or lineage of influenza virus have significantly lower odds of homologous reinfection for the following one to two years; after two years this protection wanes. This protection is demonstrated across multiple seasons, subtypes, and lineages among children.

3.3 Introduction

Influenza poses a significant public health threat, with the effects of repeated infections still not well understood [1, 63]. Children experience particular influenza risk and high attack rates during outbreaks [64]. The effects of homotypic influenza protection have been explored previously but rarely in longitudinal studies [65-72]. Several important studies have previously examined influenza infection patterns in a range of contexts, and with this study we seek to build upon this historical work. The Tecumseh Study examined illness duration and the age-related factors driving this from 1965-1972 and 1976-1981, identifying age-specific infection rates and peak ages for influenza A and B infection [22]. The Cleveland and Houston family cohorts both conducted household-based studies to track outbreaks and characterize incidence and noted the presence of protection from re-infection in some seasons but not others [26-27, 31]. While these historical studies laid important groundwork for the examination of homotypic protection, they were limited in both power and by the laboratory techniques of the time, which restricted ability

to time infection events precisely. More recently, a range of cohort studies have been conducted in both temperate and tropical contexts to explore influenza burden and measure immune responses. The Flu Watch cohort in the UK demonstrated evidence that T-cell based immunity provided protection from influenza infection in both seasonal and pandemic periods [33-34]. In children in Hong Kong, protection from reinfection for both A/H1N1pdm and H3N2 in one subsequent homologous season has been observed using serological samples and model simulation [35-36]. Other studies in Singapore and Vietnam have examined influenza dynamics and incidence but have not characterized the level of homotypic protection from symptomatic infection [39, 44]. Additionally, no data exist showing protection from clinical infection or for multiple seasons which is crucial for influenza given varying antigenic change between seasons. Here we examine homotypic influenza protection in children utilizing natural symptomatic infections covering a much broader nine-year time span.

Immunity to influenza is driven by antibody responses to surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). Antibody responses to infection drive evolutionary pressures and antigenic drift, necessitating the creation of yearly influenza vaccines. For the development of next-generation influenza vaccines, it is critical to determine the extent of immunity conferred by natural infection [6, 73-75]. In this analysis, we explore interactions between patterns of virus drift and duration of time between infections, showing how these dynamics drive protection from symptomatic influenza infection.

3.4 Methods

3.4.1 Study Procedures

The Nicaraguan Pediatric Influenza Cohort Study (NPICS) protocol has been described previously [76]. Briefly, NPICS includes children aged 0-14 years and is conducted in Managua, Nicaragua, at the Health Center Sócrates Flores Vivas (HCSFV). Clinical history, sociodemographic information, and household characteristics are collected at enrollment. Annual visits are performed to collect blood, height and weight data, and to re-administer surveys. All data is initially collected and managed via OpenClinica (OpenClinica LLC, Waltham, MA, https://openclinica.com/), which has been adapted and validated for the NPICS study. This study received ethical approval from the institutional review boards at the Ministry of Health of Nicaragua and the University of Michigan. Written informed parental consent is obtained for all participants, and verbal assent is obtained for all children ≥six years. Parents agree to bring participants to the HCSFV at the first indication of fever; respiratory samples are collected for children meeting the testing definition of feverishness or fever of ≥37.8°C with a cough, runny nose and/or sore throat or lower respiratory symptoms.

Between January 2011 and December 2019, participants were followed from enrollment to their exit date or through 2019; additional children were enrolled each year to account for those aging out of the study and occasional loss to follow-up [Figure S1]. Confirmed, acute infections were categorized based on their influenza infection type (A or B) and subtype (H1N1pdm or H3N2) or lineage (Victoria or Yamagata).

Participants were included in the analysis if they were followed during the entirety of an influenza season. For the primary analysis, individuals with homotypic infections (same subtype/lineage) during intervening years between the exposure year and outcome year under

consideration in a given model were excluded from the analysis. Sensitivity analyses included such individuals.

Influenza vaccination levels are historically quite low in the study population; while in certain seasons children aged 6 months to 2 years of age were prioritized for influenza vaccination by the government, influenza vaccinations at the health center are dependent upon donations and annual vaccination percentages within the cohort were never above 6%, with an overall yearly vaccination rate among all children in the cohort of 2.7% [Table 3.3]. There were no significant differences in vaccination rates between the two age groups under comparison in the study.

3.4.2 Laboratory Methods

Sample collection and storage details have been described [76]. For RT-PCR testing, RNA was extracted from nasal/oropharyngeal swabs using the QIAamp® Viral RNA Mini Kit (Qiagen Corporation, Valencia, CA). Influenza A and B viruses were amplified and typed/subtyped/lineaged according to CDC protocols. In total there were 6 influenza A samples that could not be subtyped, and 50 influenza B samples without an identifiable lineage – these samples were subsequently excluded from all subtype/lineage specific analyses.

3.4.3 Outcomes

The primary outcome was laboratory-confirmed symptomatic influenza A or B infection, defined by a positive RT-PCR test. Symptomatic was defined as meeting the testing criteria of feverishness or fever of $\geq$37.8°C with a cough, runny nose and/or sore throat or lower respiratory symptoms.

3.4.4 Statistical analyses

The effect of prior influenza infection on protection against subsequent, homotypic infection was assessed using logistic regression models to estimate adjusted odds ratios (aORs) and 95% credible intervals (CIs). The exposure was influenza infection in a prior season, and the outcome was infection of the same subtype or lineage in a subsequent season, examining individual-level associations between participants enrolled in the study during the relevant comparison years. Individuals with influenza infections in the intervening seasons were dropped from consideration for models comparing seasons separated by more than a year in order to accurately account for the waning effects of immunity. All models were adjusted for both age and sex and were run in R. Other potential confounders such as household crowding and underlying illness were considered and modeled in a DAG [Figure S4]. Ultimately, we decided not to incorporate these variables into the modeling as their overall biasing effect would have been to attenuate the results towards the null, meaning that the findings presented here represent a likely underestimate of the effect of homotypic protection. In models grouped by age (>4 years vs. ≤4 years), the subject's age group was determined by age at the time of the prior infection – this stratification by age was based on patterns of immune system development in children and commonly-accepted breakpoints in the literature, as well as the age structure of the cohort. The number of repeat infections (NRI) was determined for each set of comparisons between influenza seasons. To address the absence of repeat infections for certain comparison years (that caused convergence issues with frequentist models), we used Bayesian logistic models with non-informative priors of N(mean = 0, variance = 1000) on the beta coefficients. All statistical analyses were conducted in R (version 4.0.2).

3.4.5 Phylogenetic analyses and antigenic distances

The final sequence dataset consisted of HA sequences for 189 A/H1N1pdm viruses, 381 A/H3N2 viruses, 151 B/Victoria viruses, and 74 B/Victoria viruses collected from our studies in Nicaragua from January 1, 2011, to December 31, 2019 using clinical samples collected during that time. Sequences were aligned using mafft v7.475 [77] and assigned to clades based on defining amino acid positions using the align_clades.py script available from the seasonal influenza build of Nextstrain, with visual confirmation that viruses from the same clade clustered monophyletically using IQ-TREE 2.0.3 [78]. Distributions of clade assignments by subtype/lineage and year are available in the supplement [Figure S2, S3].

To measure the antigenic distance between influenza HA clades within each lineage, we constructed time-resolved phylogenetic trees (mafft 7.475 Katoh et al. 2013, IQ-TREE 2.0.3 Nguyen et al. 2014, and TreeTime 0.8.1 Sagulenko et al. 2018) that evenly sampled strains across major geographic regions and each month between January 1, 2010 and January 1, 2020, with priority given to strains with available hemagglutination inhibition (HI) measurements. We assigned strains to major historical clades with Nextstrain (Augur 12.0.0 Huddleston et al. 2021, and Auspice 2.25.1 Hadfield et al. 2018). We selected corresponding HI measurements for these strains from data provided by the CDC, the Victorian Infectious Diseases Reference Laboratory, and previously described in [79]. With this approach, we selected 3,015 strains and 6,870 HI measurements for A/H3N2, 2,894 strains and 4,911 HI measurements for A/H1N1pdm, 2,989 strains and 5,087 HI measurements for B/Victoria, and 2,964 strains and 4,427 HI measurements for B/Yamagata.

We calculated $\log_2$ normalized antigenic distances between pairs of strains with HI measurements and estimated serum potencies with a titer substitution model [79-80]. We

calculated mean $\log_2$ antigenic distance between each reference serum and all test strains in each clade, adjusted by serum potency of the corresponding reference strain, and then calculated mean $\log_2$ distance between reference and test clades.

3.5 Results

Between January 2011 and December 2019, we followed 2,764 participants aged 0-14 years who experienced 2,170 episodes of symptomatic, RT-PCR-confirmed influenza. We identified 542 A/H1N1pdm infections, 867 A/H3N2 infections, and 798 influenza B infections, with 37 infections being co-infections.

The dominant influenza A subtype fluctuated from year to year. A/H1N1pdm dominated in 2011, 2015, and 2018, and there were few if any cases in 2012, 2014, 2016 and 2017 [Table 3.3]. A/H3N2 was the dominant influenza A subtype in 2012-2014, 2016-2017, and 2019. Influenza B lineages fluctuated between B/Yamagata and B/Victoria, with B/Victoria being more dominant in recent years when lineage typing was routinely available. The subtypes and lineages underwent highly varied levels of antigenic drift over the study period [Figures 3.1-3.3]. A/H1N1pdm viruses remained fairly stable except for a shift from genetic clade 6 to 6b in 2013 and a subsequent shift to 6b.1a in 2017. A/H3N2 viruses underwent more evolution, with a different genetic clade dominating circulation for each season after 2012. For influenza B, the B/Victoria lineage clade was very stable throughout 2012-2017, while more genetic change occurred in B/Yamagata during 2014-2017.

Overall, A/H1N1pdm exhibited very strongly protective effects against homotypic infection, with an overall subsequent season protection aOR of 0.12 (CI 0.02-0.88) [Table 3.1]. With the exception of being infected in 2011 and experiencing a symptomatic infection in 2013,

all seasons ≤4 years apart exhibited strong protection from repeated symptomatic infection. The strongest protection occurred for A/H1N1pdm cases in 2013, whose aOR for repeated symptomatic infection in 2015 compared to those who were not infected in 2013 was 0.01 (CI: 0.00-0.07) [Table 3.1, Figure 3.1]. A/H1N1pdm cases from 2011 had a similar level of protection in 2015 (aOR: 0.14, CI: 0.01-0.75), as did individuals infected in 2015 against illness in 2018 (aOR: 0.18, CI: 0.04-0.50). Prior symptomatic A/H1N1pdm infection protected from repeated symptomatic infection equivalent to a vaccine effectiveness of 80-99% over two years and 86% over four years. For seasons five or more years apart, no significant protection was observed. The antigenic distances between all seasons were <0.5 on the $\log_2$ scale, indicating relative stability over the study period. There was a shift from clade 6 to 6a from 2011 to 2013 that may explain some of the diminished protection during that time period [See Appendix B].

Upon stratifying A/H1N1pdm infections by age, patterns of protection persisted. There were some variations by age group: children aged ≥5 who were infected in 2011 were protected from repeat illness in 2013 (aOR: 0.00, CI: 0.00-0.27) as compared to children aged ≤4 who were not significantly protected (aOR: 0.23, CI: 0.01-1.32) [Table 3.4, Figure 3.1]. Children aged ≥5 infected in 2011 were also significantly protected in 2018 (aOR: 0.00, CI: 0.00-0.22). Thus, while both age groups were well-protected from repeat symptomatic infections with A/H1N1pdm, older children (aged ≥5) at the time of infection were more strongly protected from subsequent homotypic A/H1N1pdm infection than younger children.

The H3N2 subtype of influenza A tended not to exhibit as strong a protective effect as H1N1pdm, particularly across two or more seasons. Overall, A/H3N2 had a one-year protection aOR of 0.41 (CI 0.24-0.73) and a near-identical two-year protection aOR [Table 3.1]. However, unlike for A/H1N1pdm, we only observed A/H3N2-associated protection lasting for 4

years for a single season-to-season comparison; all other protective durations lasted one or two years. There was significant protection from repeated illness for 2012-2014, 2013-2014, and 2014-2016 [Table 3.1, Figure 3.2]. For each of these associations apart from 2012-2014, antigenic distances between dominant clades in the given seasons were <0.4 on the $\log_2$ scale, indicating that circulating viruses at that time were similar. We were particularly interested in examining associations around the 2014 season, where a major clade shift took place in the circulating A/H3N2 virus, as we wanted to explore whether natural infection would still provide strong protection in the aftermath of a significant viral shift. Among children who were protected in 2014 as a result of infection in 2013, we observed increased risk of repeat infection in 2016 (aOR: 1.67, CI: 0.83-3.18) demonstrating the effect of the clade shift on homotypic protection, though this risk was not statistically significant. Additionally, when we examined the three-year protection from only clade-shifted A/H3N2 3c2.a viruses post-2014, we found a borderline significant protection (aOR: 0.55, CI: 0.30-1.03), indicating that while protection from A/H3N2 among children generally lasts for a period of two years this protection may last up to three years if there is less antigenic drift in the virus, and reinforcing the joint impact of time and antigenic distance on protection from influenza.

When stratified by age, older children were better protected from repeated homotypic infection with A/H3N2 over mostly one- and two-year periods. Children aged ≥5 were significantly protected from 2012 to 2013, 2013 to 2014, 2012 to 2014, 2014 to 2016, and 2012 to 2016 (aOR: 0.00, CI: 0.00-0.26) [Table 3.5, Figure 3.2]. However, children aged ≤4 infected in 2012 appeared to experience longer duration of protection. Younger children infected in 2012 were not significantly protected from illness in 2013 or 2014 but were significantly protected from illness in 2016 (aOR: 0.00, CI: 0.00-0.35), 2017 (aOR: 0.00, CI: 0.00-0.33), and

2019 (aOR: 0.00, CI: 0.00-0.42); this discrepancy may be due to age-driven patterns of exposure and imprinting, or could be due to small number considerations. All results for Influenza A were similar in the sensitivity analyses conducted.

The B/Victoria lineage circulated more frequently in the study area during 2011-2019 and was more prevalent in recent years, coinciding with greater availability of lineage typing and permitting more comparisons. Overall, there was strong homotypic protection against repeated symptomatic infection with the B/Victoria lineage for all seasons one or two years apart (one-year aOR 0.00 (CI 0.00-0.28)) [Table 3.2, Figure 3.3]. This pattern of short-term protection was consistent for both younger and older children [Table 3.6].

We observed limited seasons of B/Yamagata lineage circulation during the study period, so comparisons were only available between the 2014, 2017, and 2019 seasons. Of these, significant protection was only observed between the 2014 and 2019 seasons (aOR: 0.11, CI: 0.00-0.65) [Table 3.2, Figure 3.3]. When stratified by age, this protection remained for children ≤4 but was not significant for older children [Table 3.7]. There were minimal clade changes during the study time period, with no antigenic distances >0.6 on the $\log_2$ scale [Figure 3.3 & Appendix B]. Overall, we did not find the same pattern of protection from repeat infections among children from B/Yamagata as we observed for circulating subtypes of influenza A or B/Victoria. All results for Influenza B were similar in the sensitivity analyses conducted.

3.6 Discussion

Using RT-PCR-confirmed influenza infections and sequencing data from 2011-2019, we found that children infected with influenza A/H1N1pdm, A/H3N2, or B/Victoria were strongly protected from infection in subsequent seasons. Our findings regarding patterns of clade drift and

sequence change in Nicaragua generally align with prior observations of influenza's global circulation, where influenza A/H1N1pdm and B viruses have been observed to have less seasonal drift with slower rates of antigenic evolution and fewer epidemics as compared to influenza A/H3N2 viruses [81-82]. Additionally, these results support prior findings that repeat influenza infections can differentially boost antibody and immune responses depending upon those strains encountered early in life [83-86]. Overall, we found that protection waned with time and greater antigenic distance.

When we examined age-stratified results for A/H1N1pdm infection, we found that children aged ≥5 at infection experienced stronger protection than younger children. This accords with prior analyses showing that due to the slower rate of antigenic evolution in A/H1N1pdm, older children and adults are less susceptible to reinfection than young children [87-88]. This lower susceptibility possibly results from a broader level of immunity due to multiple exposures to A/H1N1pdm across multiple seasons; alternatively, this heightened protection may simply be the result of immune maturation or may be attributable to a broader spectrum of prior exposure to both H1 and H3 among older children who typically have a wider and more varied immune repertoire.

While we observed similar patterns in A/H3N2 repeat infections, these results were more heavily dependent on the level of antigenic change between seasons. Such findings align with antigenic cartography studies demonstrating that A/H3N2 generally evolves faster and with more punctuated evolution than A/H1N1pdm and B lineages [79]. The A/H3N2 3c2.A clade that emerged in 2014 is unique, as it possesses a glycosylation site that protects an important target of neutralizing antibodies. This clade has continued to circulate and has dominated influenza seasons despite individuals being repeatedly infected and acquiring some level of immunity [89].

Despite this, we observed protection from 2013 to 2014 when seasonal influenza vaccine effectiveness was low. Protection against circulating strains wanes faster against A/H3N2 than A/H1N1pdm [79], which may explain why the period of homotypic protection observed here tended to be short, even after accounting for antigenic distances between dominant clades. Children aged ≥5 were generally better protected from repeat infection with A/H3N2, although children ≤4 infected in 2012 displayed a long duration of protection. Early life exposure to A/H3N2 significantly affects anti-3c2.A antibodies, so under-5 children infected in 2012 may have had different exposure patterns than older children, thereby driving an age disparity across season comparisons [89].

B/Yamagata tends to circulate more frequently in adults and exhibit greater genetic diversity than B/Victoria [90-91]. B/Victoria tends to infect younger individuals than B/Yamagata, which has been hypothesized to result from differences in the age-varying prevalence of receptor-binding structures [92]. Alternatively, the declining prevalence of B/Victoria infection with age has been attributed to strengthened immunity over time due to accumulated immunity and lower levels of genetic change [90-91]. In general, we observed protection from repeated infections with B/Victoria among children, aligning with previously observed patterns of protection, as children appear to develop immunity over time resulting in strong protection by adulthood. By comparison, we saw very little protection from repeat infection with B/Yamagata, indicating that children may not develop durable protection in response to B/Yamagata infection.

This study is strengthened by our ability to study naturally occurring influenza infections in a population with high numbers of RT-PCR-confirmed positives. Influenza vaccination in Nicaragua is not common, with ~5% of our study population ever being vaccinated against

influenza. Within this study, only symptomatic individuals who met testing criteria were RT-PCR tested, meaning that this analysis examined the effects of repeated symptomatic infection and excluded asymptomatic individuals. As we were primarily interested in the individual health effects of infection, we viewed this as an appropriate population. Because of the testing criteria for inclusion, we may have missed milder infections, which could potentially have biased our findings as the impacts of asymptomatic and mild infections could not be assessed by this study. While our main interest was in the role of symptomatic infections, we acknowledge that asymptomatic infections not captured by this study may have distorted the degree of homotypic protection found here by boosting immunity in between comparison seasons. Additionally, because the cohort used only includes children aged 14 or younger, our findings may not generalize to older populations. However, children are a highly vulnerable population for influenza infection and are major drivers of transmission; thus we believe these findings have significant public health implications and are broadly generalizable to other pediatric populations, particularly those with low levels of annual influenza vaccination.

The presence of homotypic protection against symptomatic infection between seasons of naturally occurring influenza has been explored prior to this study, but not in a setting with many RT-PCR-confirmed infections. Overall, we found that individuals infected with one influenza subtype or lineage had significantly lower odds of being infected with that same subtype or lineage in a subsequent season. These findings show that establishing individuals' prior patterns of influenza infections can predict their subsequent risk and contribute to better, more specific vaccine development.

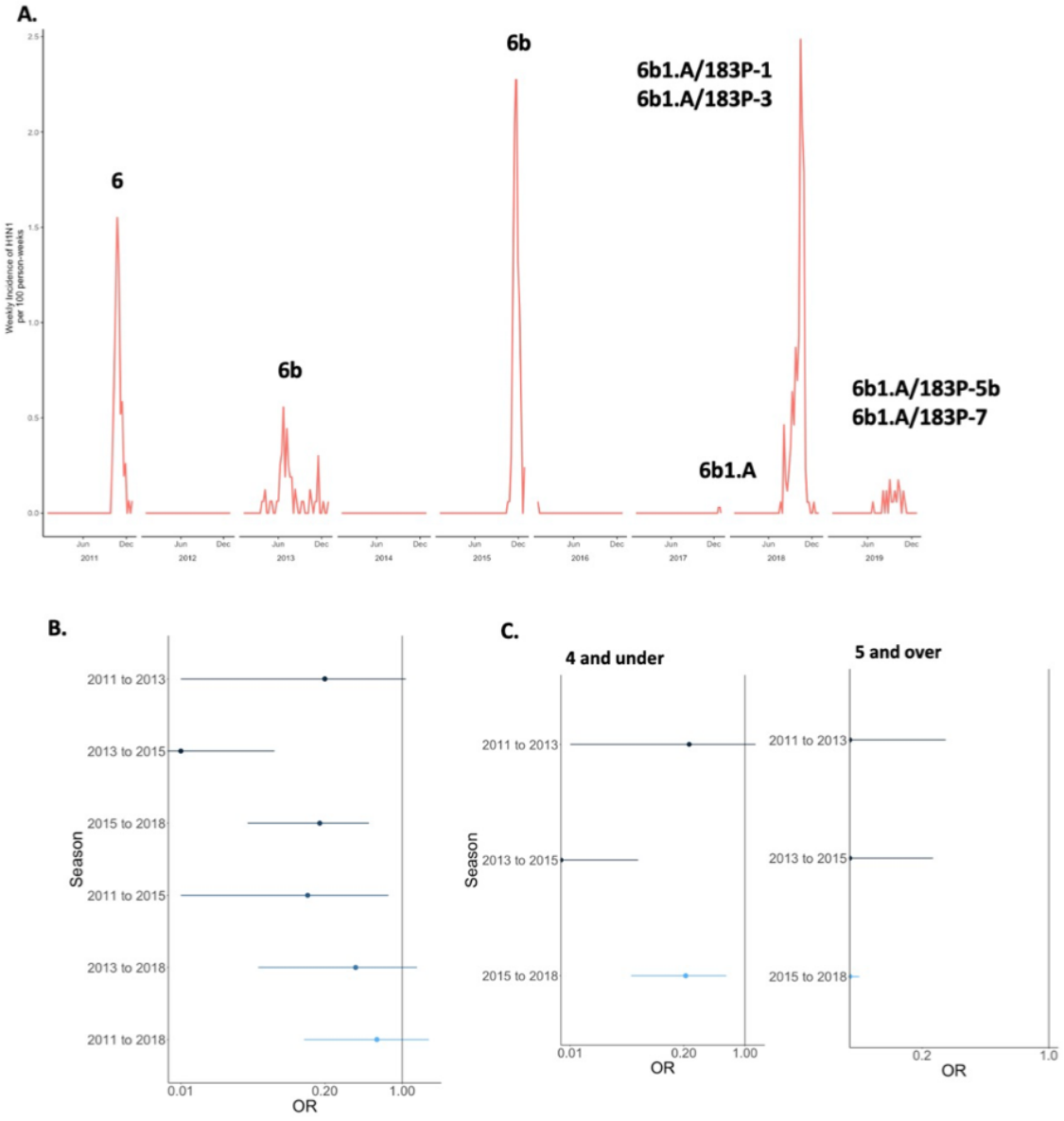

Figure 3.1: Influenza A H1N1pdm Epidemics and Duration of Protection Across Seasons

A. Seasonality and clades of influenza A H1N1pdm cases among children in the study from 2011 to 2019. B. Log-adjusted odds ratios for a given exposure and outcome year looking at protection from repeat infection with error bars for the confidence intervals. C. Log-adjusted odds ratios for a given exposure and outcome year looking at protection from repeat infection, stratified on age and restricted to season distances less than 4 years, with error bars for the

confidence intervals. For B & C, n=2,764 children followed over 9 study years. Darker colors in the plots represent seasons that are closer together in time; lighter colors represent seasons that are further apart.

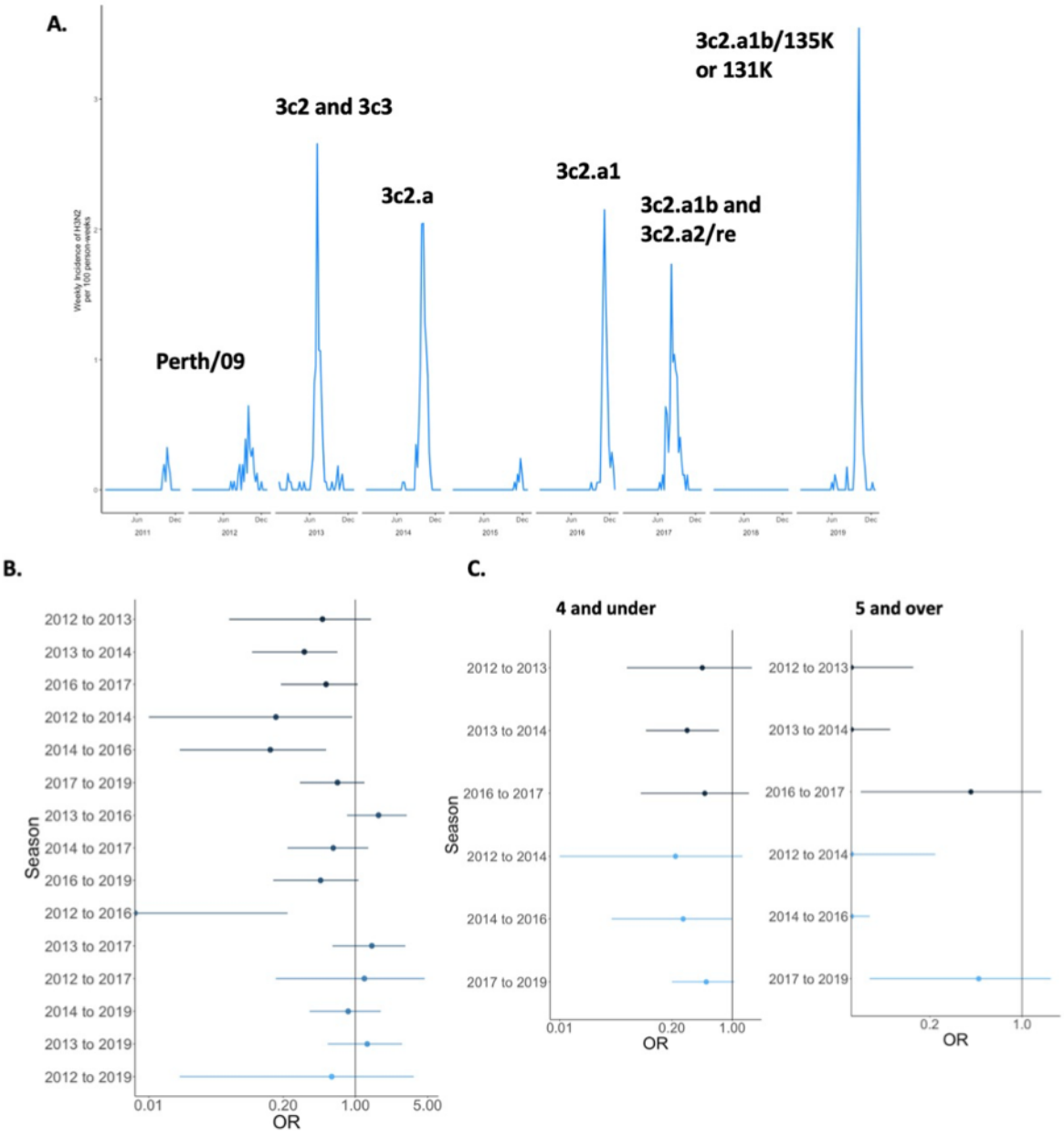

Figure 3.2: Influenza A H3N2 Epidemics and Duration of Protection Across Seasons

A. Seasonality and clades of influenza A H3N2 cases among children in the study from 2011 to 2019. B. Log-adjusted odds ratios for a given exposure and outcome year looking at protection from repeat infection with error bars for the confidence intervals. C. Log-adjusted odds ratios for a given exposure and outcome year looking at protection from repeat infection,

stratified on age and restricted to season distances less than 4 years, with error bars for the confidence intervals. For B & C, n=2,764 children followed over 9 study years. Darker colors in the plots represent seasons that are closer together in time; lighter colors represent seasons that are further apart.

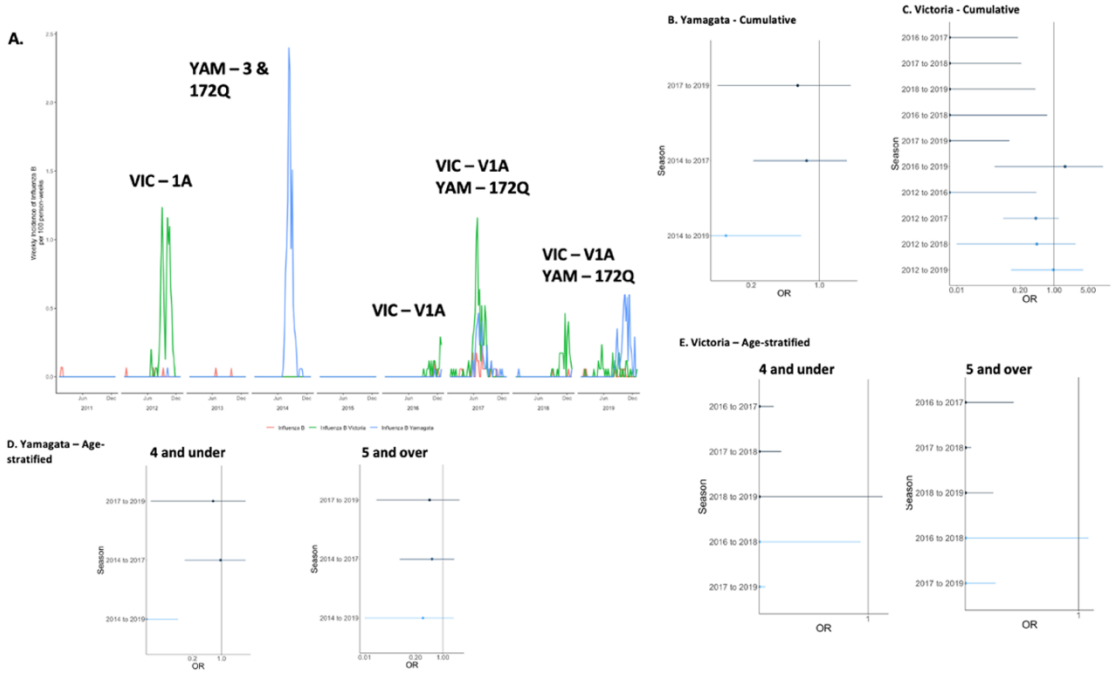

Figure 3.3: Influenza B Epidemics and Duration of Protection Across Seasons

A. Seasonality and clades of influenza B cases among children in the study from 2011 to 2019. B. Log-adjusted odds ratios for a given exposure and outcome year looking at protection from repeat infection with Yamagata lineage, with error bars for the confidence intervals. C. Log-adjusted odds ratios for a given exposure and outcome year looking at protection from repeat infection with Victoria lineage, with error bars for the confidence intervals. D. Log-adjusted odds ratios for a given exposure and outcome year looking at protection from repeat infection with Yamagata lineage, stratified on age and restricted to season distances less than 4 years, with error bars for the confidence intervals. E. Log-adjusted odds ratios for a given exposure and outcome year looking at protection from repeat infection with Victoria lineage, stratified on age and restricted to season distances less than 4 years, with error bars for the confidence intervals. For B-E, n=2,764 children followed over 9 study years. Darker colors in

the plots represent seasons that are closer together in time; lighter colors represent seasons that are further apart.

Table 3.1: Influenza A Repeat Infection Odds

Seasons	N	NRI	OR	95% CI	Antigenic distance
A/H1N1pdm					
Two years apart					
Summary		1	0.12	(0.02-0.88)	
2011 to 2013	1468	1	0.20	(0.01-1.08)	*
2013 to 2015	1456	0	0.01	(0.00-0.07)	0.47
Three years apart					
2015 to 2018	1363	3	0.18	(0.04-0.50)	0.07
Four years apart					
2011 to 2015	1138	1	0.14	(0.01-0.75)	*
Five years apart					
2013 to 2018	885	2	0.38	(0.05-1.36)	0.07
Seven years apart					
2011 to 2018	659	3	0.59	(0.13-1.74)	*
A/H3N2					
One year apart					
Summary		13	0.41	(0.24-0.73)	
2012 to 2013	1566	2	0.48	(0.06-1.41)	0.3
2013 to 2014	1675	5	0.32	(0.10-0.67)	-0.6
2016 to 2017	1725	6	0.52	(0.19-1.05)	0.7
Two years apart					
Summary		12	0.41	(0.23-0.73)	
2012 to 2014	1335	1	0.17	(0.01-0.93)	1.3
2014 to 2016	1464	2	0.15	(0.02-0.52)	0.1
2017 to 2019	1586	9	0.67	(0.29-1.22)	2.1
Three years apart					
Summary		23	0.85	(0.55-1.33)	
Summary for post-2014		11	0.55	(0.30, 1.03)	
2013 to 2016	1105	12	1.67	(0.83-3.13)	0.78
2014 to 2017	1227	6	0.61	(0.22-1.33)	0.06
2016 to 2019	1309	5	0.46	(0.16, 1.07)	0.46
Four years apart					
2012 to 2016	864	0	0.00	(0.00-0.22)	0.5
2013 to 2017	907	8	1.44	(0.60-3.03)	0.9

Five years apart					
2012 to 2017	698	2	1.22	(0.17-4.65)	0.9
2014 to 2019	901	8	0.85	(0.36-1.75)	0.39
Six years apart					
2013 to 2019	632	8	1.30	(0.54-2.82)	0.78
Seven years apart					
2012 to 2019	462	1	0.59	(0.02-3.66)	*

*No antigenic distance available between these seasons' dominant clades
**All models were adjusted for age and sex of participants

Table 3.2: Influenza B Repeat Infection Odds

Seasons	N	NRI	OR	95% CI	Antigenic Distance
B/Victoria					
One year apart					
Summary		0	0.00	(0.00-0.14)	
2016 to 2017	1725	0	0.00	(0.00-0.18)	1.6
2017 to 2018	1727	0	0.00	(0.00-0.21)	1.6
2018 to 2019	1730	0	0.00	(0.00-0.41)	1.6
Two years apart					
Summary		0	0.00	(0.00-0.32)	
2016 to 2018	1457	0	0.00	(0.00-0.72)	1.6
2017 to 2019	1550	0	0.00	(0.00-0.12)	1.6
Three years apart					
2016 to 2019	1295	1	1.68	(0.06-10.05)	1.6
Four years apart					
2012 to 2016	1076	0	0.00	(0.00-0.43)	0.8
Five years apart					
2012 to 2017	935	3	0.42	(0.09-1.23)	0.8
Six years apart					
2012 to 2018	759	1	0.44	(0.01-2.75)	0.8
Seven years apart					
2012 to 2019	637	2	0.96	(0.13-3.91)	0.8
B/Yamagata					
Two years apart					
2017 to 2019	1585	2	0.60	(0.09-2.10)	0.6
Three years apart					
2014 to 2017	1326	4	0.74	(0.21-1.92)	0.6
Five years apart					
2014 to 2019	1018	1	0.11	(0.00-0.65)	0.6

*All models were adjusted for age and sex of participants

Table 3.3: Influenza infections and vaccinations by season and type/subtype

		H1N1		H3N2		IB		Vaccinated	
Influenza Season	N	n	%	n	%	n	%	n	
2011	1,578	102	6.5	16	1.0	2	0.1	10	0.6
2012	1,653	0	0.0	49	3.0	154	9.3	69	4.2
2013	1,790	66	3.7	159	8.9	2	0.1	38	2.1
2014	1,938	0	0.0	168	8.7	205	10.6	48	2.5
2015	1,894	153	8.1	10	0.5	0	0.0	93	4.9
2016	1,874	1	0.1	134	7.2	31	1.7	20	1.1
2017	1,880	1	0.1	150	8.0	220	11.7	25	1.3
2018	1,872	198	10.6	0	0.0	47	2.5	27	1.4
2019	1,873	21	1.2	181	9.7	137	7.3	111	5.9

*In most years, the influenza season in Nicaragua is entirely contained within a single calendar year, however occasionally cases will continue into the next year

Table 3.4: H1N1 Repeat Infection Odds, Age-Stratified

Seasons	N	NRI	OR	95% CI
4 and under				
Two years apart				
Summary		1	0.12	(0.02-0.85)
2011 to 2013	503	1	0.23	(0.01-1.32)
2013 to 2015	501	0	0.00	(0.00-0.06)
Three years apart				
2015 to 2018	550	3	0.21	(0.05-0.61)
Four years apart				
2011 to 2015	408	1	0.12	(0.00-0.69)
Five years apart				
2013 to 2018	379	1	0.16	(0.01, 0.92)
Seven years apart				
2011 to 2018	330	3	0.71	(0.15-2.24)
5 and over				
Two years apart				
Summary		0	0.00	(0.00-0.05)
2011 to 2013	965	0	0.00	(0.00-0.27)
2013 to 2015	955	0	0.00	(0.00-0.23)
Three years apart				
2015 to 2018	814	0	0.00	(0.00-0.09)
Four years apart				
2011 to 2015	730	0	0.00	(0.00-0.22)
Five years apart				
2013 to 2018	506	1	0.74	(0.03-4.50)
Seven years apart				
2011 to 2018	329	0	0.00	(0.00-0.22)

Table 3.5: H3N2 Repeat Infection Odds, Age-Stratified

Seasons	N	NRI	OR	95% CI
4 and under				
One year apart				
Summary		11	0.37	(0.20-0.70)
2012 to 2013	489	2	0.45	(0.06-1.69)
2013 to 2014	560	5	0.30	(0.10-0.70)
2016 to 2017	583	4	0.48	(0.09-1.56)
Two years apart				
Summary		10	0.41	(0.21-0.80)
2012 to 2014	377	1	0.22	(0.01-1.31)
2014 to 2016	507	2	0.27	(0.04-0.99)
2017 to 2019	596	7	0.50	(0.20-1.06)
Three years apart				
Summary		16	0.77	(0.45-1.34)
Summary for post-2014		8	0.54	(0.25-1.14)
2013 to 2016	385	8	1.70	(0.67-3.96)
2014 to 2017	454	5	0.65	(0.21-1.62)
2016 to 2019	479	3	0.36	(0.08-1.05)
Four years apart				
2012 to 2016	267	0	0.00	(0.00-0.35)
2013 to 2017	347	7	1.81	(0.66-4.43)
Five years apart				
2012 to 2017	246	0	0.00	(0.00-0.33)
2014 to 2019	385	5	0.83	(0.27-2.10)
Six years apart				
2013 to 2019	302	5	1.06	(0.33-2.76)
Seven years apart				
2012 to 2019	217	0	0.00	(0.00-0.42)
5 and over				
One year apart				
Summary		2	0.20	(0.05-0.81)
2012 to 2013	1077	0	0.00	(0.00-0.15)
2013 to 2014	1115	0	0.00	(0.00-0.10)
2016 to 2017	1142	2	0.41	(0.06-1.56)
Two years apart				
Summary		2	0.19	(0.05-0.77)
2012 to 2014	958	0	0.00	(0.00-0.22)

2014 to 2016	957	0	0.00	(0.00-0.07)
2017 to 2019	990	2	0.47	(0.07-1.66)
Three years apart				
Summary		7	0.71	(0.32-1.54)
Summary for post-2014		3	0.42	(0.13-1.36)
2013 to 2016	720	4	1.34	(0.38-3.58)
2014 to 2017	773	1	0.21	(0.01-1.20)
2016 to 2019	830	2	0.46	(0.06-1.63)
Four years apart				
2012 to 2016	597	0	0.00	(0.00-0.26)
2013 to 2017	560	1	0.36	(0.01-2.08)
Five years apart				
2012 to 2017	452	2	3.05	(0.40-12.85)
2014 to 2019	516	3	0.73	(0.16-2.23)
Six years apart				
2013 to 2019	330	3	1.51	(0.32-4.97)
Seven years apart				
2012 to 2019	245	1	1.39	(0.05-9.88)

Table 3.6 Influenza B Victoria Repeat Infection Odds, Age-Stratified

Seasons	N	NRI	OR	95% CI
4 and under				
One year apart				
Summary		0	0.00	(0.00-0.28)
2016 to 2017	583	0	0.00	(0.00-0.26)
2017 to 2018	609	0	0.00	(0.00-0.29)
2018 to 2019	625	0	0.00	(0.00-1.23)
Two years apart				
Summary		0	0.00	(0.00-0.56)
2016 to 2018	510	0	0.00	(0.00-0.90)
2017 to 2019	585	0	0.00	(0.00-0.23)
Three years apart				
2016 to 2019	483	0	0.00	(0.00-1.38)
Four years apart				
2012 to 2016	387	0	0.00	(0.00-0.32)
Five years apart				
2012 to 2017	373	1	0.17	(0.01-0.95)
Six years apart				
2012 to 2018	330	1	0.58	(0.02-3.96)
Seven years apart				
2012 to 2019	312	1	0.65	(0.02-4.54)
5 and over				
One year apart				
Summary		0	0.00	(0.00-0.25)
2016 to 2017	1142	0	0.00	(0.00-0.40)
2017 to 2018	1118	0	0.00	(0.00-0.22)
2018 to 2019	1105	0	0.00	(0.00-0.30)
Two years apart				
Summary		0	0.00	(0.00-0.68)
2016 to 2018	947	0	0.00	(0.00-1.15)
2017 to 2019	965	0	0.00	(0.00-0.31)
Three years apart				
2016 to 2019	812	1	3.50	(0.13-23.37)
Four years apart				
2012 to 2016	689	0	0.00	(0.00-2.68)
Five years apart				

2012 to 2017	562	2	0.72	(0.10-2.74)
Six years apart				
2012 to 2018	429	0	0.00	(0.00-0.79)
Seven years apart				
2012 to 2019	325	1	1.16	(0.04-9.13)

Table 3.7: Influenza B Yamagata Repeat Infection Odds, Age-Stratified

Seasons	N	NRI	OR	95% CI
Four and under				
Two years apart				
2017 to 2019	596	1	0.63	(0.02-3.80)
Three years apart				
2014 to 2017	494	2	0.94	(0.13-3.78)
Five years apart				
2014 to 2019	447	0	0.00	(0.00-0.09)
Five and over				
Two years apart				
2017 to 2019	989	1	0.46	(0.02-2.65)
Three years apart				
2014 to 2017	832	2	0.53	(0.08-1.96)
Five years apart				
2014 to 2019	571	1	0.31	(0.01-1.88)

Chapter 4 - Impact of Heterotypic and Heterosubtypic Repeat Influenza Infection Patterns in a Pediatric Cohort in Managua, Nicaragua

4.1 Author Summary

We examined patterns of heterotypic and heterosubtypic infection with influenza using 9 years of data from a prospective pediatric cohort. We did not observe protection from infection within the same season but did observe increased risk of heterotypic and heterosubtypic infections in subsequent seasons. This risk was not driven by age, healthcare seeking behaviors, or pre-existing differences in antibody titers. These findings indicate that there is some impact of repeat infections being driven by heterotypic interactions and suggest that it may be necessary to develop modeling techniques that incorporate the full immune history in order to expand on these repeat infection findings.

4.2 Abstract

Influenza poses a significant public health burden each year, and the effects of heterotypic and heterosubtypic influenza interactions across seasons have not been well-explored up to this point. Understanding these dynamics is critical to addressing future risk patterns for individuals and populations and will allow for improvements in influenza vaccine design. Using

data from a pediatric cohort study in Managua, Nicaragua, we explore the effects of heterotypic and heterosubtypic influenza infections both within and between seasons. Children aged 0-14 were followed from 2011-2019 with cases of influenza each year captured by RT-PCR. While we found no significant protection from heterotypic/heterosubtypic infection within influenza seasons, we did find that individuals infected with a given type or subtype of influenza in a season where two types/subtypes circulated were at increased risk for the other circulating type/subtype in the subsequent season. Risk of subsequent infection was particularly high for children who were first infected with A/H3N2 who were then exposed to A/H1N1pdm (OR 2.61, CI 1.42-4.78). This heightened risk was present for both older and younger children and held true even after adjustments were made for healthcare-seeking behavior and pre-exposure antibody titer levels.

4.3 Introduction

Despite the existence of a seasonal vaccine against influenza, there is still a significant annual global burden of disease [1-3]. The effectiveness of this seasonal vaccine varies annually but in the past decade has ranged from 10% to 60% effectiveness [93]. Because of this limited effectiveness and the need to constantly update the strains targeted by the vaccine as a result of ongoing antigenic drift, there is a great deal of ongoing interest in developing a more broadly protective or universal influenza vaccine [16, 73]. One of the major areas of research interest for developing a universal influenza vaccine involves assessing the effect of naturally occurring influenza infections on response to subsequent homotypic or heterotypic strain exposures [73].

As discussed in prior analyses of homotypic influenza infection effects [94], assessing the impact of repeat infections of influenza on protection and response has posed wide range of

challenges because of limited longitudinal data tracking annual influenza infections by subtype among a cohort of people followed over multiple influenza seasons. Homotypic infection dynamics have previously been explored in longitudinal studies [22, 26-27, 31, 33-34, 67-72, 95-96], but many of these studies were constrained by shorter study time periods or less accurate laboratory techniques than those that are currently available. A number of these studies were nonetheless critical in laying the framework for our present-day understanding of influenza, and with our work in Nicaragua focusing on homotypic and heterotypic infections we have sought to expand on this work.

In addition to the effect of repeated infections with the same subtype, which we have found to have a protective effect across seasons, we are also interested in exploring the heterotypic effects of repeated influenza infections – in other words, the effect of being infected with one influenza subtype on probability of infection with a different subtype in a subsequent season. The effects of heterotypic influenza interactions across seasons have not been well-explored up to this point but are clearly critical to understanding patterns of risk and protection given the integral role that each individual's lifetime history of exposure and infection plays in driving their susceptibility to a given strain. This concept of Original Antigenic Sin (OAS), first put forward by Thomas Francis in 1960 has been refined over the years to reflect the complexity of interactions between immune systems and ever-changing influenza viruses [18]. A recent review paper [17] discussed the current state of the literature with regards to heterotypic and heterosubtypic influenza relationships and how they are driven by immune history and viral change, and found that while there have been several studies examining the relationships between immune responses to different influenza strains [97-98], there has been minimal recent longitudinal work examining these associations within a defined cohort.

Earlier studies of heterotypic effects among humans [99] and ferrets [100] exist but have inconclusive findings regarding the association between infections with distinct influenza subtypes; additionally, the ferret findings established a decrease in shedding but not protection from infection [100]. Several more recent vaccine studies in humans have examined the production of heterotypic antibody production against historical strains stimulated by vaccination [8, 101-102]. These studies have found some evidence of heterosubtypic protection from infection driven by intranasal vaccination; however, all of these studies have been laboratory-based and have not examined longitudinal data focused on natural infections.

Here, using data from an ongoing pediatric cohort study in Managua, Nicaragua, we explore and model the dynamics of repeated infections with distinct influenza types across multiple seasons. These effects were explored by assessing whether infection in the current influenza season provided protection against an additional heterotypic infection within the same season; whether infection in one or more prior seasons resulted in increased risk of heterotypic infection in the subsequent season; whether the probability of subsequent heterotypic infection was impacted by age at the time of the initial infection; and what the duration of naturally occurring heterotypic protection was, if any.

4.4 Methods

4.4.1 Subject Selection

The structure of the Nicaraguan Pediatric Influenza Cohort Study (NPICS) has previously been described in depth [76, 94] but will be briefly detailed here. NPICS is conducted in District II of Managua, Nicaragua, at the Health Center Sócrates Flores Vivas (HCSFV). The study population comprises children aged 0-14 years residing in District II who are recruited into the study via house-to-house visits. The children's clinical history, sociodemographic information,

and household information are collected at enrollment; sampling is then conducted annually in order to collect blood, update the height and weight data, and re-administer surveys to note any changes from the previous year. Parental consent is obtained for all participants in the study, and verbal assent is obtained for all children six years and older. As part of study participation, all parents agree to bring participants into HCSFV at the first sign of fever; respiratory samples are then collected at HCSFV for all episodes of influenza-like illness (ILI), defined for the purposes of this study as meeting the testing criteria of feverishness or fever of ≥37.8°C with a cough, runny nose and/or sore throat or lower respiratory symptoms.

The specific selection of participants for these repeat infection analyses have been previously described [94], but briefly: for the repeat infection analyses within NPICS, participants were followed from the time the subject first enrolled in the study to subject's final exit date (or the date of data extraction if subject had not exited the study by that point). The total number of NPICS study participants included in this analysis was 2764. Subjects were coded for an influenza infection subtype (H1N1pdm or H3N2) for a specific year, if a swab taken in that year tested positive for the subtype. These participants were included in the analysis if the subject was present during the entirety of prior and subsequent flu seasons. In the logistic models that examined the effect of infection in multiple prior seasons on a subsequent infection, the subject was present during the entirety of at least one of the prior seasons. In these models, infection in multiple prior seasons was defined as having the flu subtype of interest in at least one of the multiple prior seasons. A subset of these children were frequency matched on age based on influenza positivity around the seasons where there were multiple influenza epidemics from different strains in a single season – these children then had annually collected blood

samples tested via HAI to assess antibody titer levels in the year before and after influenza exposure for A/H1N1pdm, A/H3N2, B/Yamagata, and B/Victoria.

Influenza vaccination levels are historically quite low in the study population; while in certain seasons children aged 6 months to 2 years of age were prioritized for influenza vaccination by the government, influenza vaccinations at the health center are dependent upon donations and annual vaccination percentages within the cohort were never above 6%, with an overall yearly vaccination rate among all children in the cohort of 2.7% [Table 3.3]. There were no significant differences in vaccination rates between the two age groups under comparison in the study. In models grouped by age (>4 years vs. ≤4 years, ≤2 years), the subject's age group was determined by age at the time of the prior infection.

4.4.2 Laboratory Methods

The specifics of sample collection and storage have been described elsewhere [76]. The primary outcome of interest for this study was laboratory-confirmed influenza, defined as a positive test for influenza A/H1N1pdm, influenza A/H3N2, or influenza/B by RT-PCR. For the RT-PCR testing, RNA was extracted from nasal/oropharyngeal swabs using the QIAamp® Viral RNA Mini Kit (Qiagen Corporation, Valencia, CA). Influenza viruses A and B were amplified and typed/subtyped according to the protocol of the Centers for Disease Control and Prevention (CDC).

4.4.3 Outcomes

Three different sets of models were run examining associations between: 1) symptomatic exposure and a symptomatic outcome (defined by meeting the testing criteria and a positive RT-PCR test), 2) symptomatic exposure and any outcome (defined by meeting the testing criteria and a positive RT-PCR test or a four-fold or higher rise in titer levels as measured by HAI), or 3)

any exposure and any outcome. Symptomatic exposure and outcome data was available for all seasons, serology-based exposure and outcome data was only available for 2012-2017 due to testing constraints.

4.4.4 Statistical Analysis

Effect of prior infection on risk of subsequent infection was assessed using logistic regression models to generate odds ratios and 95% confidence intervals. The exposure of interest was influenza infection in a prior season, with the outcome being repeated infection with a different influenza type or subtype in the same or subsequent season; models controlled for age and sex. Initial logistic models were constructed and run in SAS. To address the absence of repeat infections for certain comparison years (that caused convergence issues with frequentist models), we used Bayesian logistic models with non-informative priors of N(mean = 0, variance = 1000) on the beta coefficients. To address the potential impact of healthcare seeking behavior on likelihood of repeatedly testing positive for influenza, a dummy variable was constructed to account for an average of all non-ARI healthcare visits for each child by year; this variable was subsequently incorporated into all models to control for this behavior. To account for titer changes Wilcoxon signed-rank tests were conducted to examine pre- and post- titer measurements and assess whether comparison groups were meaningfully different in their pre- or post-influenza season titer levels. All Bayesian models and titer statistical analyses were conducted in R (version 4.0.2).

4.5 Results

4.5.1 Overall Descriptive Statistics

Between January 2011 and December 2019, we followed 2,764 participants aged 0-14 years who experienced 2,170 episodes of symptomatic, RT-PCR-confirmed influenza. We

identified 542 A/H1N1pdm symptomatic infections, 867 A/H3N2 symptomatic infections, and 798 influenza B symptomatic infections, with 37 symptomatic infections being co-infections. The dominant influenza A subtype fluctuated from year to year. A/H1N1pdm dominated in 2011, 2015, and 2018, and there were few if any cases in 2012, 2014, 2016 and 2017 [Table 3.3]. A/H3N2 was the dominant influenza A subtype in 2012-2014, 2016-2017, and 2019. Influenza B lineages fluctuated between B/Yamagata and B/Victoria, with B/Victoria being more dominant in recent years when lineage typing was routinely available.

4.5.2 Same Season Analysis

Within same-season analyses, we did not find significant evidence of protection from repeat heterotypic or heterosubtypic infection overall. We ran both individual season comparisons and collapsed same season models and found no evidence for protection. Collapsed same-season models [Figure 4.1] show that most models produced results centered around the null, with the exception of symptomatic H1N1pdm and H3N2, where there was a slightly protective trend. When these comparisons were broken down into specific seasons, the same pattern persisted, with a general centering around the null regardless of whether exclusively PCR data or combined PCR and serology data were utilized in the model [Figure 4.2]. The strongest result trending towards within-season protection was for individuals infected with either H1N1pdm or H3N2 in 2013 who were then slightly protected from repeat infection with the opposite subtype in the same season (OR 0.30, CI 0.04-2.22); however, this association was not statistically significant and was not consistent across other years or type/subtype comparisons [Table 4.1].

When stratified by age, this overall pattern of protection lacking statistical significance persisted for both children under 5 and for children aged 5 and over – older children had a

slightly less protective trend than was observed for younger children [Figure 4.6]. When we subset the analysis down to very young children (those aged under 2), there were several within-season comparisons that showed statistically significant protection, particularly those examining H1N1pdm/H3N2 in 2013 and H1N1pdm with B/Victoria in 2018. [See Appendix C] Once again these patterns were not consistent across all comparisons.

4.5.3 Subsequent Season Analysis

We found significant evidence of increased risk of infection with heterotypic/heterosubtypic influenza in a subsequent season following initial infection. This increased risk was particularly strong for heterotypic comparisons examining individuals who were initially infected with influenza A (H1N1pdm or H3N2) or influenza B in a given season where the two influenza types co-circulated, and who were subsequently re-exposed in the next season. Children who were initially infected with H1N1pdm or H3N2 were at significantly higher risk of infection with influenza B in the next season [Figure 4.3]; using the collapsed analysis, we also observed this heightened risk when serological data was incorporated into the analysis for children who were initially infected with H1N1pdm or influenza B and then exposed to H3N2 in the subsequent season. When the analysis was broken down to compare across all different seasons, this general pattern of increased heterotypic/heterosubtypic risk in the next season was still apparent [Figure 4.4]. Children infected with H1N1pdm in 2018 were at particularly heightened risk of infection with B/Victoria in 2019 (OR 3.57, CI 1.62-7.89); children infected with H3N2 in 2013 were at heightened risk of infection with H1N1pdm in 2015 (OR 2.61, CI 1.42, 4.78) [Table 4.2].

This pattern of increased risk in certain subsequent seasons was also apparent when children were stratified by age, with slightly higher odds of repeated infection for children aged

under 5 during the initial infection; there was still evidence of increased risk in subsequent seasons for older children, but this risk was slightly attenuated across all comparisons [Figure 4.7]. For children under 2 at age of initial infection, the general pattern trended closer towards the null [See Appendix C]. Overall, age did not appear to be a significant driver of this pattern of increased heterotypic risk across different seasons of influenza.

4.5.4 Influenza B

Comparisons between B/Yamagata and B/Victoria looked significantly different than either heterotypic or heterosubtypic analyses. In general, we found that protection across lineages from repeated infection persisted even across multiple seasons. There was strong same-season protection from repeat infection with a different lineage of influenza B [Figure 4.8]; additionally, there was a pattern of protection in subsequent seasons, particularly for children who were first infected with B/Yamagata. This association, while not significant, does look quite different than the subsequent seasons comparisons within influenza A subtypes or across influenza types.

4.5.5 Sensitivity Analyses

Models adjusted for healthcare seeking-behavior did not produce significantly different results than the overall models. Same-season comparisons showed a similar pattern of clustering around the null [See Appendix C] although again without any statistically significant protection from repeat infection. Next season models still showed a pattern of increased risk, indicating that it was not simply children with many healthcare center visits due to different patterns of parental care-seeking driving this observed risk [See Appendix C]. While there was a slight attenuation of the risk for certain associations, this was not true for most models, indicating that this association is unlikely to be driven solely by healthcare-seeking behavior.

In addition to incorporating serology data into the overall models, where we did not see significantly different results between those models utilizing serology and those focused solely on symptomatic PCR-positive individuals, we also wanted to confirm that children in the control and positive groups for the serology comparison analyses were not systematically different based on their pre-titer levels. For all three seasons where influenza B and H3N2 co-circulated, we compared pre- and post- titer levels for both influenza B and H3N2 by influenza B positivity (as influenza B circulated first in all three seasons) and found no significant differences in the H3N2 pre- or post- titers [Figure 4.5a-c]. Differences in influenza B pre- and post- titer values were present and expected as comparisons were being conducted on the bases of influenza B positivity.

4.6 Discussion

Overall, our findings regarding the effects of heterotypic and heterosubtypic infections were mixed. While we did not establish the presence of any within-season protection from heterotypic or heterosubtypic infection, we were able to show a slightly protective pattern and occasional protective associations among children. By comparison, the evidence for increased risk of heterotypic/heterosubtypic infection in subsequent seasons was demonstrated for both older and younger children and persisted even when adjusted for healthcare-seeking behavior patterns. These patterns do not appear to be driven by age or by pre-existing differences in titers. While these models primarily relied upon symptomatic data, those models where we were able to incorporate serology did not show significantly different findings to those utilizing solely PCR-based data.

While there has not been extensive exploration of these heterotypic dynamics in recent multi-year cohort studies among children, what has been shown in this analysis does not run

counter to what was found in older historical analyses [22-23, 26-27, 31, 33-34]. The period of increased heterotypic/heterosubtypic risk has not been previously demonstrated and is of particular interest given the potential implications for vaccine development [103]. While we hypothesized that a period of within-season heterotypic protection might be what was driving this subsequent risk, we were unable to demonstrate that protection through this analysis, either via PCR or serology-confirmed infections. This increased risk in subsequent reason also does not appear to have been driven by differences in healthcare-seeking behaviors among study participants, nor by pre-existing differences in relevant titer levels.

Lineage-based findings for influenza B did differ somewhat from the overall pattern, as there was evidence of persistent protection from repeated infection even in subsequent seasons and across different lineages. This finding is consistent with previous research surrounding influenza B however [90-91], and also mirrors some of what we had previously shown in our analysis of homotypic lineage-specific infection and protection patterns in this same cohort [94].

This analysis was somewhat limited to analysis of symptomatic influenza episodes due to the testing criteria for children in the cohort; however, serologic data was incorporated for a subset of children from 2012-2017. Because of the general reliance on PCR-confirmed influenza, the results of this study may not be generalizable to asymptomatic or paucisymptomatic individuals, who were not captured in this analysis. While our focus for this analysis was symptomatic individuals, we acknowledge that there may be a biasing effect due to not capturing all potentially infected individuals in the testing criteria for the study. One area of expansion for future research would be incorporating serologic data collected paired around the time of the influenza episode, rather than from annual sampling as was done for this analysis, as the annual surveying and sampling did not always align perfectly with the timing of each year's influenza

season and better incorporation of serology would allow for capturing asymptomatic and paucisymptomatic cases. In addition, while children are a vulnerable population for influenza infection and therefore of particular research interest, this study does exclude adult populations and therefore somewhat limit the generalizability of the findings. Additionally, it is worth noting that in seasons with two overlapping peaks of influenza, one of the two epidemics tended to be severely blunted; in seasons with two significant peaks, those peaks tended to be separated in time. In most seasons with two viruses co-circulating, one was clearly dominant, suggesting that there may be some level of ongoing interference that limits our ability to assess within-season influenza interactions using our current study approach.

This study was strengthened, however, by our ability to incorporate both PCR and serology-based data around a large number of naturally occurring influenza episodes spanning almost a decade across a cohort with very low rates of attrition or vaccination. Loss to follow-up was very low across the study period of NPICS (2011-2019) with the majority of children who exited the study doing so due to aging out of eligibility rather than non-response or dropping out. Influenza vaccination in Nicaragua remains fairly uncommon, with only approximately 5% of the study population receiving the seasonal influenza vaccine in a given study year.

These results provide valuable insight into the dynamics of heterotypic and heterosubtypic influenza infection and are indicative of the value of continued exploration of this area, potentially with techniques that pull together the entire life course history of infection for a given individual. This is an area that we are very interested in exploring moving forward as we continue to collect data on these children and their histories of influenza exposure. Overall, this study demonstrates that there is increased risk for repeated heterotypic/heterosubtypic influenza infection in subsequent seasons following initial infection, and that this risk is present

irrespective of children's age or patterns of healthcare-seeking behavior. These findings highlight the importance of understanding patterns of exposure and infection among populations and how these can drive both individual risk and overall dynamics of influenza across years of virus circulation.

Table 4.1: Same Season Heterotypic/Heterosubtypic Infection Odds

Comparison	OR	LL	UL	Cases
H1N1pdm2013/H3N22013	0.304	0.042	2.218	Symptomatic
H1N1pdm2013/H3N22013	1.06	0.134	8.536	Mixed
H1N1pdm2013/H3N22013	0.794	0.101	6.325	Any
H1N1pdm2018/BVictoria2018	1.007	0.351	2.884	Symptomatic
H3N22012/BVictoria2012	0.632	0.151	2.649	Symptomatic
H3N22012/BVictoria2012	0.598	0.143	2.508	Mixed
H3N22012/BVictoria2012	1.259	0.472	3.357	Any
H3N22014/BYamagata2014	1.062	0.559	2.019	Symptomatic
H3N22014/BYamagata2014	0.986	0.519	1.871	Mixed
H3N22014/BYamagata2014	1.451	0.821	2.566	Any
H3N22017/Bcombined2017	2.715	1.788	4.124	Symptomatic
H3N22019/Bcombined2019	1.487	0.848	2.606	Symptomatic

*All models were adjusted for the age and sex of participants

Table 4.2: Subsequent-Season Heterotypic/Heterosubtypic Infection Odds

Comparison	OR	LL	UL	Cases
H1N1pdm2013/H3N22014	0.654	0.157	2.759	Symptomatic
H1N1pdm2013/H3N22014	2.234	0.899	5.547	Mixed
H1N1pdm2013/H3N22014	2.385	1.052	5.406	Any
H1N1pdm2018/BVictoria2019	3.573	1.618	7.887	Symptomatic
H3N22013/H1N1pdm2015	2.605	1.419	4.784	Symptomatic
H3N22013/H1N1pdm2015	2.158	1.183	3.938	Mixed
H3N22013/H1N1pdm2015	0.985	0.219	4.442	Any
H3N22012/BVictoria2016	0	0	1.17	Symptomatic
H3N22012/BVictoria2016	0	0	0.58	Mixed
H3N22012/BVictoria2016	0	0	0.38	Any
H3N22014/BYamagata2017	2.205	0.91	5.341	Symptomatic
H3N22017/BVictoria2018	0.796	0.241	2.648	Symptomatic
H3N22017/BYamagata2019	1.091	0.512	2.323	Symptomatic
BVictoria2012/H3N22013	0.573	0.229	1.431	Symptomatic
BVictoria2012/H3N22013	1.277	0.686	2.38	Mixed
BVictoria2012/H3N22013	2.023	0.767	5.337	Any
BYamagata2014/H3N22016	1.186	0.584	2.41	Symptomatic
BYamagata2014/H3N22016	1.43	0.815	2.51	Mixed
BYamagata2014/H3N22016	1.671	0.991	2.819	Any
Bcombined2017/H3N22019	1.144	0.65	2.013	Symptomatic

*All models were adjusted for the age and sex of participants

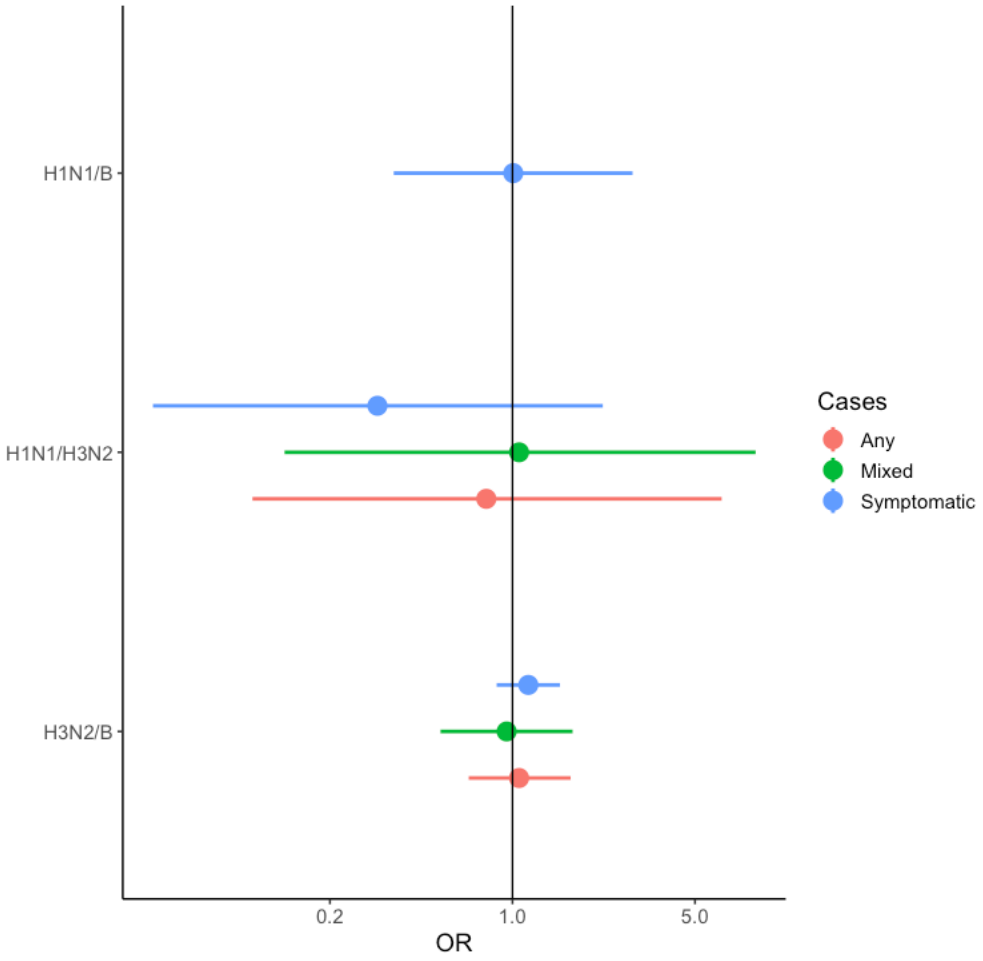

Figure 4.1 Within-Season Heterotypic/Heterosubtypic Infection Odds, Collapsed

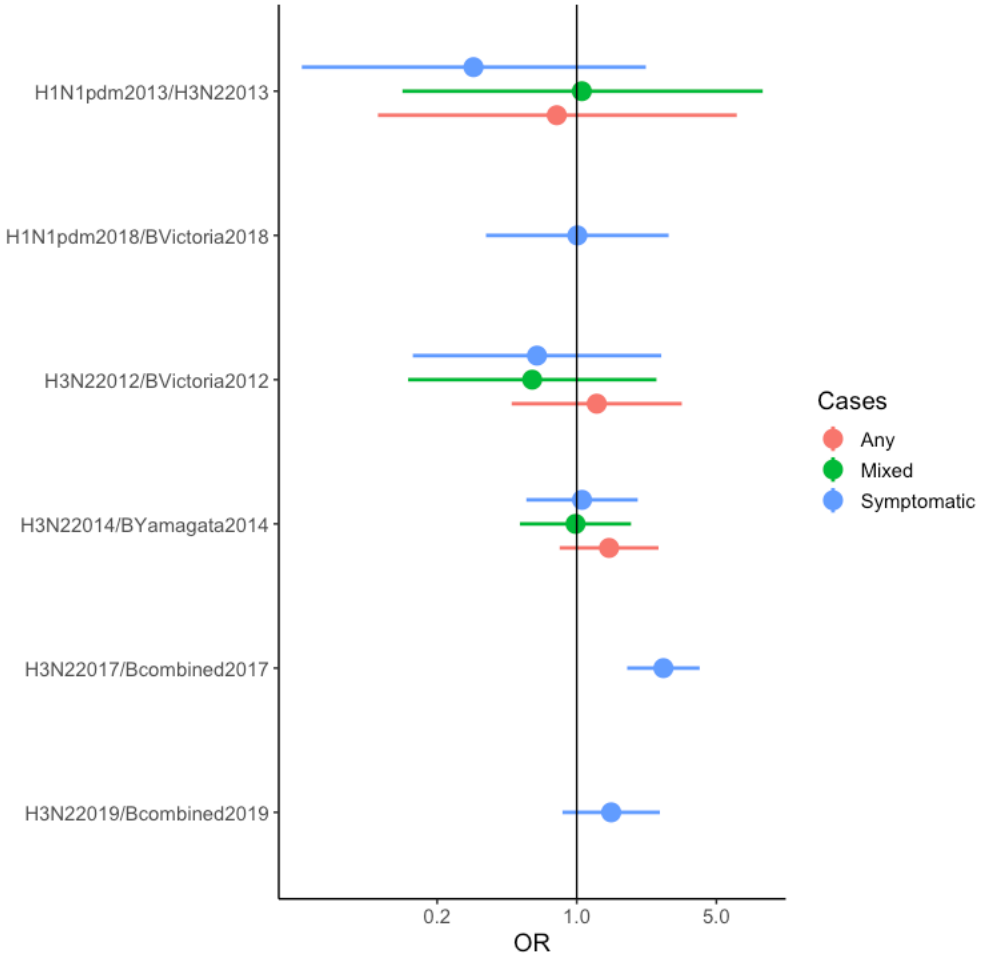

Figure 4.2 Within-Season Heterotypic/Heterosubtypic Infection Odds, All Comparisons

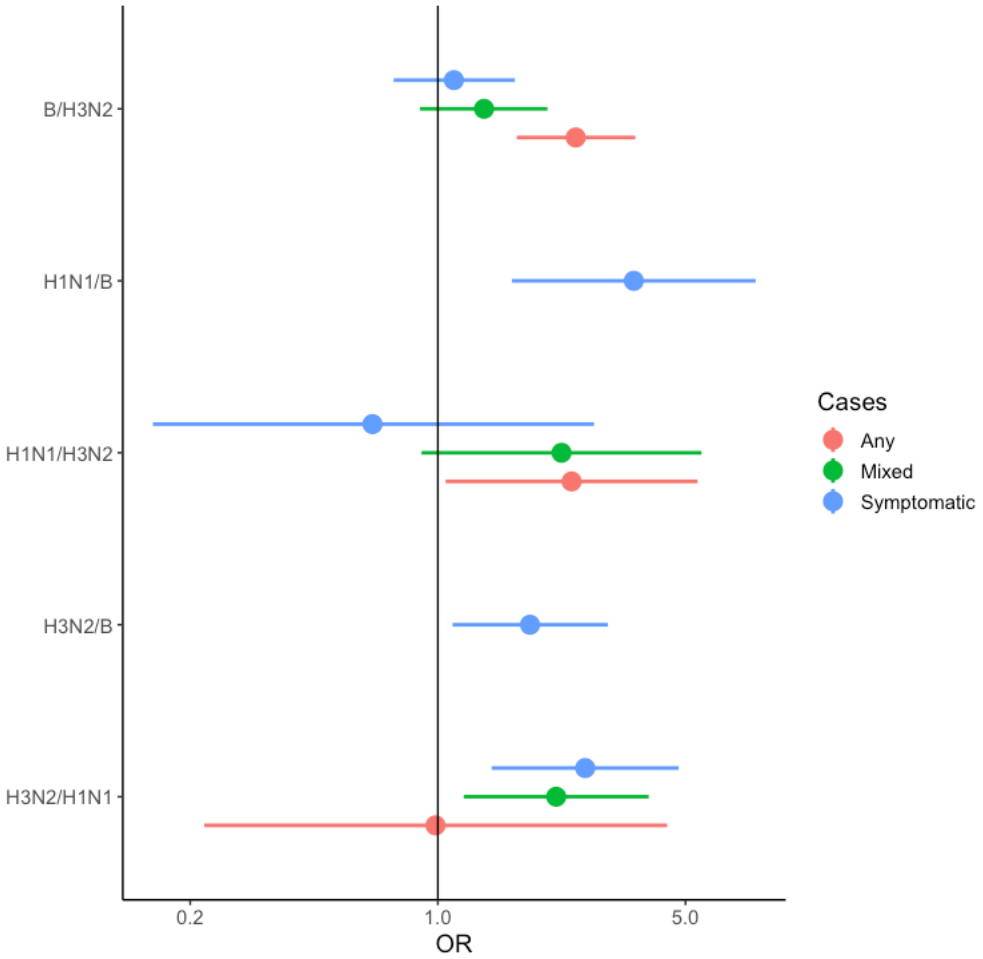

Figure 4.3 Subsequent-Season Heterotypic/Heterosubtypic Infection Odds, Collapsed

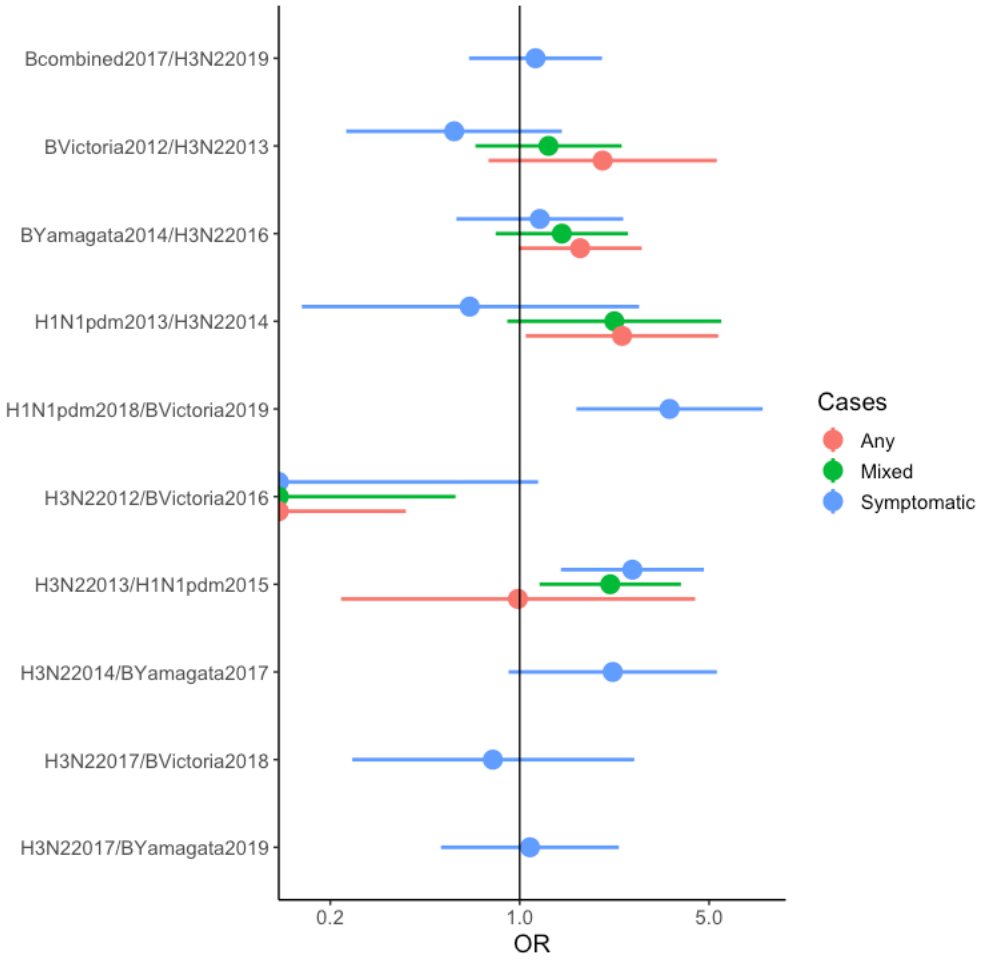

Figure 4.4 Subsequent-Season Heterotypic/Heterosubtypic Infection Odds, All Comparisons

Figure 4.5 Pre- and post-titer comparisons between influenza B and A/H3N2

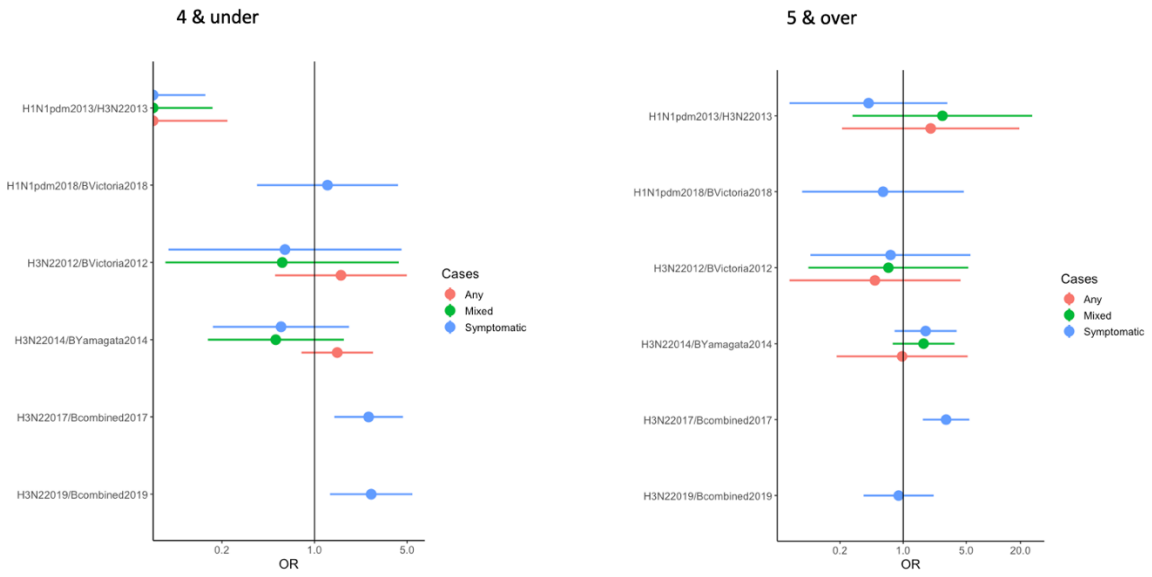

Figure 4.6: Age-stratified same-season comparisons

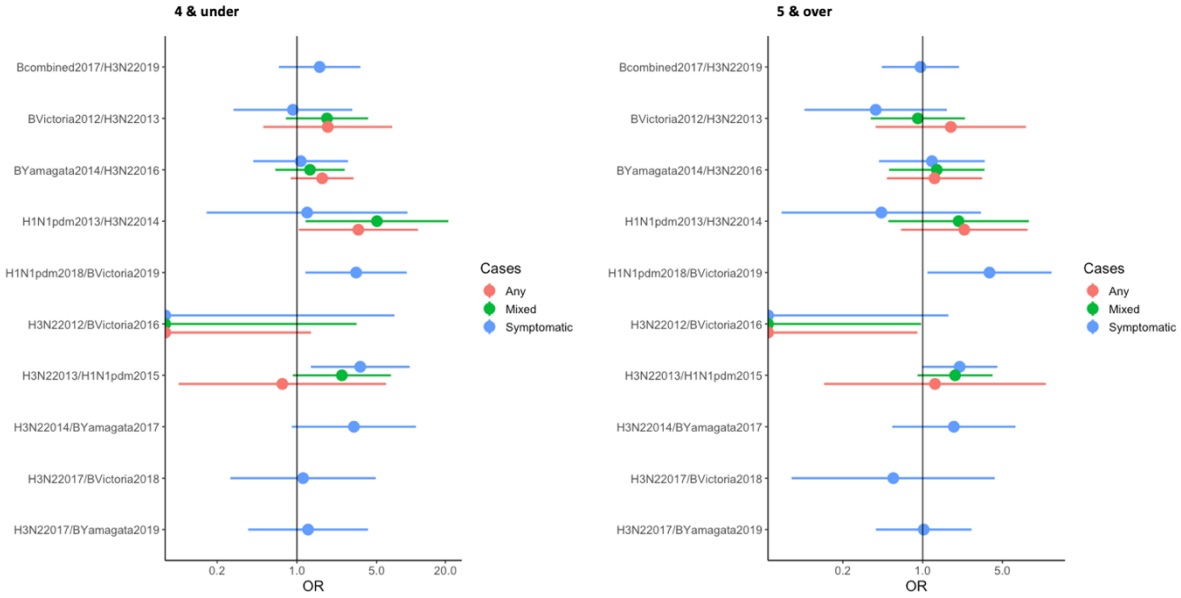

Figure 4.7: Age-stratified subsequent season comparisons

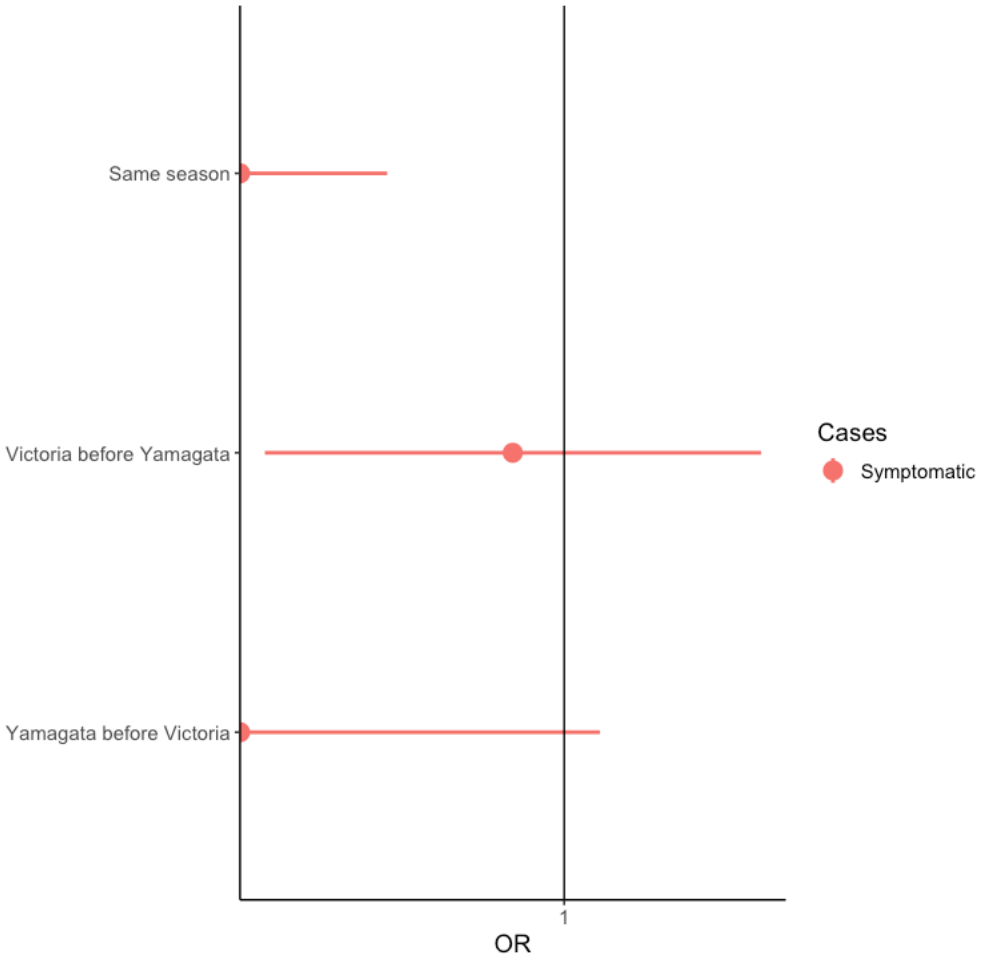

Figure 4.8: Influenza B comparisons, both same and subsequent seasons, collapsed

Chapter 5 – Knowledge Added and Future Directions

The clear synthesis and demonstration of value added, and potential future steps is a key component of any research process, and particularly those that have involved human subjects research in a critical area of public health and infectious disease investigation. The importance, now more than ever, of unpacking respiratory infectious dynamics and how they impact vaccination, goes without saying. In this chapter, I seek to unpack the ways in which this dissertation has contributed to the overall influenza literature, and the possible routes forward for each of these projects.

5.1 Aim 1

In aim 1 of this dissertation, we sought to examine the immunological responses of individuals to influenza infection, with a particular focus on correlates of protection and those participants who generated alternate responses to infection. Exploring alternate correlates of protection against influenza is a key avenue to improving our vaccination development strategies; while HAI has been regarded as the only acceptable correlate of protection against influenza, recent research suggests that alternate correlates may be appropriate for inclusion. There has been evidence that not all individuals develop a strong HAI antibody response to influenza infection, but what proportion of the population falls into this category and how those individuals respond to alternate correlates of protection is not well established in the literature.

In exploring non-HAI responders and alternate responses to infection among a household transmission study in Nicaragua we were able to observe several key details. Firstly, we established that there is a major population of individuals who, while RT-PCR positive for influenza, did not generate a ≥4-fold HAI antibody response; these individuals did not differ on the basis of age, sex, or index patient status, but were significantly less symptomatic and had a shorter period of shedding duration. Secondly, we observed that more than half of those individuals who did not generate a ≥4-fold HAI antibody response generated a ≥4-fold antibody response to one of the alternate correlates of protection (HA stalk, full-length HA, and/or NA). These alternate responders were less symptomatic and had shorter duration of symptoms than HAI responders, but otherwise were very similar in all aspects with the exception of their HAI pre-titers.

Our findings regarding alternative responses to influenza infection and HAI non-response are similar to those observed in other studies, but prior to this study there had not been in-depth investigation of how these two areas of immune response coincide; in this study we were able to establish that there is a meaningful population of individuals who fail to respond to HAI but respond significantly to alternate markers. The findings of this study suggest that these alternative correlates should be included as serological markers of infection and should additionally be incorporated into influenza vaccine design.

Important future steps with this research include expanding it to include other subtypes/types of influenza, as this analysis was focused on influenza A/H1N1pdm since it was the only subtype circulating during the study time period. Initial work has already been conducted to replicate this analysis with A/H3N2 in our research group and established similar levels of non-response and alternate correlate response as was observed here. Additionally, it

would be beneficial to explore these responses in a larger study population, as one of the key limitations of this study was the lowered power due to the smaller numbers of participants. More broadly speaking, the key area of interest building from these findings is their implications for novel influenza vaccine approaches. In the time since this and other papers exploring NA and HA stalk as correlates of protection were published, there have been exciting advances in developing vaccines that target either HA stalk [104] or NA [105-106], suggesting that incorporating these components may indeed lead to a more broadly protective influenza vaccine. Continued exploration of these alternate correlates will enhance our understanding of immunologic responses to infection and support the future development of a universal influenza vaccine.

5.2 Aim 2

In aim 2 of this dissertation, we explored the effects of repeated homotypic infection and the strength and duration of protection conferred by these infections. While there has generally been a historical understanding that homotypic protection from influenza infection occurs for at least some period of time, this relationship has not been well-explored with modern laboratory techniques and not over a nearly decade-long period in a tropical, low vaccination population. In a cohort of Nicaraguan children from 2011-2019 we were able to explore these dynamics and establish the duration of homotypic protection and how those associations were driven by both waning protection over time and patterns of antigenic changes in viruses from season to season.

We observed homotypic protection from repeat infection among children infected with A/H1N1pdm, A/H3N2, and B/Victoria (although not with B/Yamagata, largely owing to the lower overall prevalence of Yamagata among pediatric populations). We found that this protection was stronger among older children, but broadly consistent regardless of age or the

season under consideration. Thanks to sequencing data collected over the study period we were able to establish via phylogeny the dominant clades and patterns of antigenic drift and sequence change and found that these generally were aligned with global patterns. Broadly, we found that children were well-protected for a period of two to three years post-infection, but that this protection waned over time and as antigenic distance grew.

This study provides strong evidence that homotypic protection against influenza infection occurs, and that this protection is robust regardless of changes in season, subtype, lineage, or age of children under consideration. This finding is broadly important in its own right, as it fills a key gap in the literature; homotypic protection has not been well-observed based on naturally occurring influenza infection (confirmed via RT-PCR) in a population with high levels of infection followed over an extensive time period. These results highlight the ways in which establishing prior patterns of infection can predict subsequent risk for individuals and also support the development of more specifically targeted vaccine development based on population patterns of influenza virus circulation.

Our findings from aim 2 also provide a range of avenues for future research into this topic of repeat homotypic infection impacts. This analysis was focused on symptomatic infections and thus did not incorporate questions of asymptomatic or paucisymptomatic effects, as those were not captured within the testing criteria of NPICS. The collection of serology data for these cohort studies has been expanded recently and would provide an interesting additional window into exploring these effects. Additionally, whether the findings of this study are fully generalizable to other populations is not entirely clear; while we believe that our findings among children should be broadly applicable and are critical public health findings given the vulnerability of children to influenza, it would nonetheless be interesting to explore these

patterns of homotypic protection in an adult population. An additional avenue of research based off of these findings would be to explore the effects of a child's full course of infection, starting with their initial infection and modeling how that informs subsequent responses to infection; such an approach would allow a much more nuanced examination of imprinting and have stronger implications for how OAS truly influences influenza outcomes. While the structure of NPICS did not allow for such analyses – as first exposure events could not be conclusively established – newer studies led by our group in Nicaragua are designed to explore precisely these questions. Continuing to build on this work and establish the ways in which repeated influenza infection events drive immunity is critical to understanding both the dynamics of the viral burden from season to season and also how to develop an appropriate vaccine response to the ever-shifting landscape of influenza.

5.3 Aim 3

In aim 3 of this dissertation, we expanded our analysis of repeated influenza infections to assess the impacts of heterotypic and heterosubtypic exposures and infections both within and between seasons. While there has been significant research in exploring these dynamics, assessing their impact has been challenging due to the limited longitudinal data available focused on naturally occurring influenza infections. By exploring these associations using data from the same period of NPICS as for aim 2 we worked to build on our findings from the previous aim and continue to develop the available knowledge in the literature surrounding repeated influenza infections.

As in aim 2, we explored associations between repeated infections – however, for this study, we were focused on heterotypic and heterosubtypic comparisons, and examined outcomes both within and between seasons. We did not observe any significant protection from

heterotypic/heterosubtypic repeat infection within the same season, either using RT-PCR confirmed infection or serology-confirmed infections. We did, however, observe heightened risk of heterotypic/heterosubtypic infection in the season following initial infection for certain associations; this heightened risk was strongest among individuals infected with either influenza A or influenza B in a season where those two influenza types co-circulated, and who were subsequently re-exposed in the next season to the other influenza type in the subsequent season. This association was present both when using RT-PCR infections and combined serology/RT-PCR findings in modeling approaches. When we controlled these models for healthcare-seeking behavior we found no significant changes in association, indicating that this increased risk was not simply an artifact of more frequent clinic attendance. Additional comparisons of pre-exposure titer values confirm that these periods of heightened risk were not being driven by pre-existing differences in titer levels. Age-stratification of the models showed that these associations were consistent across different age groups of children in the study.

While our findings for heterotypic and heterosubtypic repeat infections were certainly more mixed than the very clear picture painted by our results from aim 2, they nonetheless provide evidence for increased risk of heterotypic infection in seasons after a season in which two influenza types co-circulate; these dynamics have not been extensively explored in pediatric cohorts, and our findings here support what was shown in the historical literature on this subject. This period of increased risk has not been well-documented and is highly relevant given the potential vaccine development implications, as individuals protected in one season of virus co-circulation may be at heightened risk of infection in the next season. It is also possible that this risk is driven by some pattern of influenza infection history established early in the life course, which would also contribute to our understanding of how to improve vaccine development. Our

findings around influenza B lineage also suggest that cross-lineage protection may last longer than a single season for seasons in which B/Victoria and B/Yamagata co-circulate.

As with aim 2, incorporating additional serological data into this analysis might expand the findings beyond what can be captured with a focus on symptomatic influenza. Additionally, as this study was unable to establish any period of temporary protection from heterotypic/heterosubtypic infection within the same season, one avenue of future research is to establish what mechanism is driving the heightened risk in subsequent seasons. It is possible that the period of heterotypic protection is shorter than what was able to be captured in this study (i.e. a matter of days rather than weeks or months) although how this would drive a later period of risk is somewhat unclear. Alternatively, this subsequent season is being caused by something other than protection within the original season of co-circulation; while we were able to establish that it was unlikely to be driven by healthcare-seeking behaviors or pre-exposure titer differences, it is possible that there is some other population difference driving this risk. One area of particular interest is developing modeling techniques that are better able to capture and incorporate an entire life history of exposure – we are currently working with partner researchers to explore this approach, as it would greatly enhance our ability to unpack some of these more complicated questions of influenza dynamics.

5.4 Conclusions

Throughout this dissertation, we sought to explore the dynamics of influenza infection and assess the potential impacts of those effects on the development of a more broadly effective influenza vaccine. By describing patterns of HAI non-response and establishing the existence of alternate responders to influenza infection, we have added knowledge surrounding the complexities of human immunological response to influenza infection and highlighted the value

of considering novel correlates of infection when developing both surveillance and vaccine strategies in response to influenza. Through our characterization of patterns of homotypic protection within a long-standing pediatric cohort in Nicaragua we have provided valuable insight into the duration of protection induced by natural infection and underlined the importance of understanding patterns of exposure and how these exposures in conjunction with antigenic changes in the virus drive infection dynamics from season-to-season. Our exploration of the heterotypic and heterosubtypic infection patterns among that same cohort established that there is a period of heightened risk following seasons with co-circulation of two viruses and reinforce the complexities of influenza infection dynamics. This work with longitudinal pediatric data in a low-resource setting helps to fill in some critical gaps in the current literature while also opening up a number of areas for valuable future research. Our hope is that the research presented here, and the work to come that will build upon it, will continue to shed light on the many remaining questions surrounding influenza and contribute to the development of more effective protective strategies to reduce the massive global burden that continues to be posed by influenza virus infections.

Appendices

Appendix A: Supplemental Materials for Chapter 2

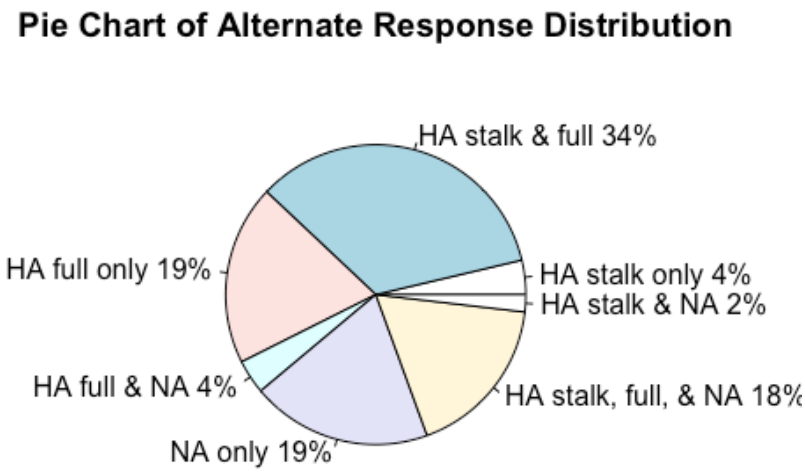

Figure A.1 Pie Chart of Alternate Response Distribution

A pie chart depicting the overlap in 4-fold or greater responses to full-length hemagglutinin (HA full), hemagglutinin stalk (HA stalk), and neuraminidase (NA) among all PCR-positive individuals in the study population who exhibited an alternate response (n=108).

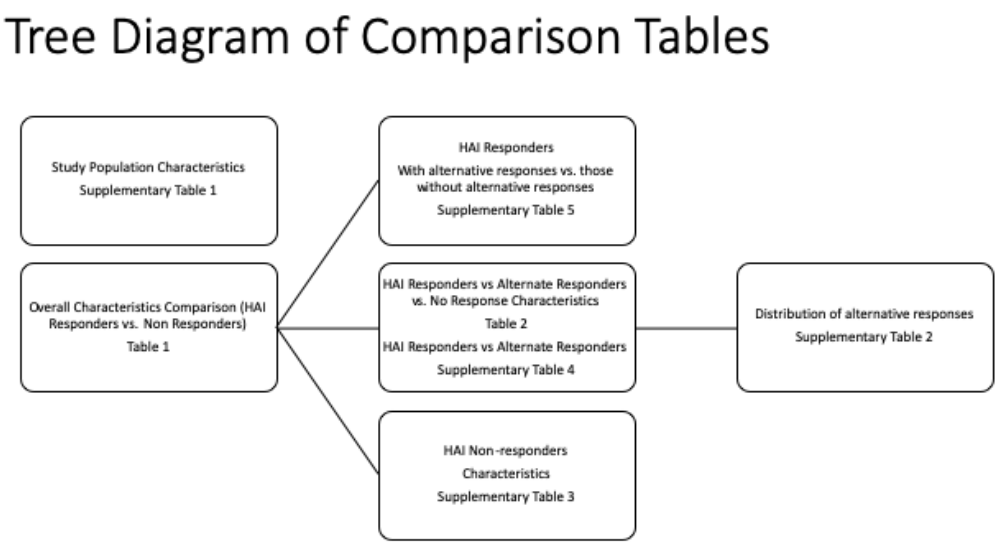

Figure A.2 Tree Diagram of Comparison Tables

A tree diagram demonstrating which subsets of the study population are included within each table included in the main and supplementary materials, to assist in understanding each of the generated comparisons.

Appendix B: Supplemental Materials for Chapter 3

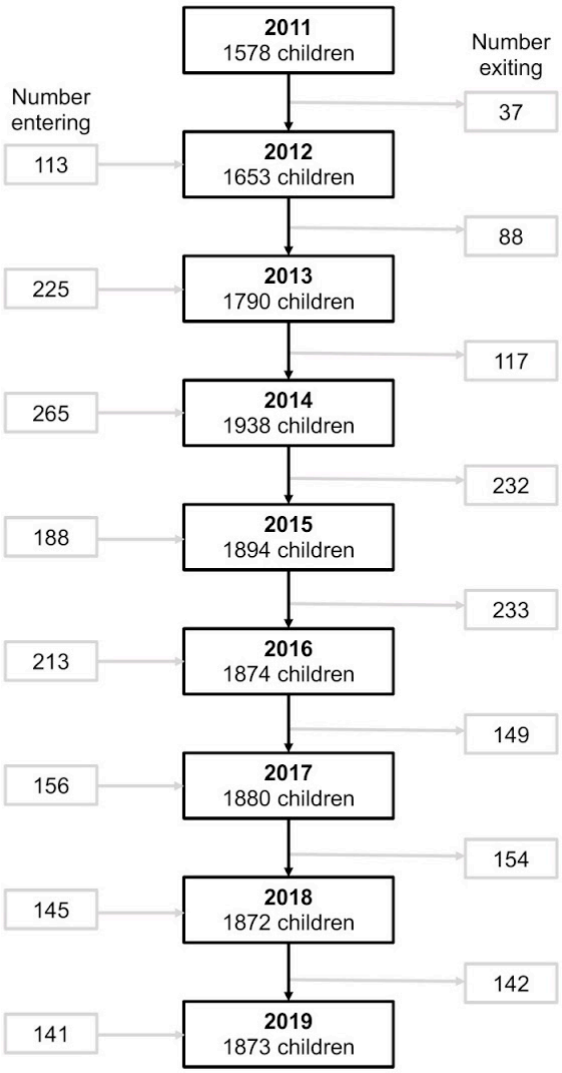

Figure B.1 NPICS Flowchart

Flowchart of study participation

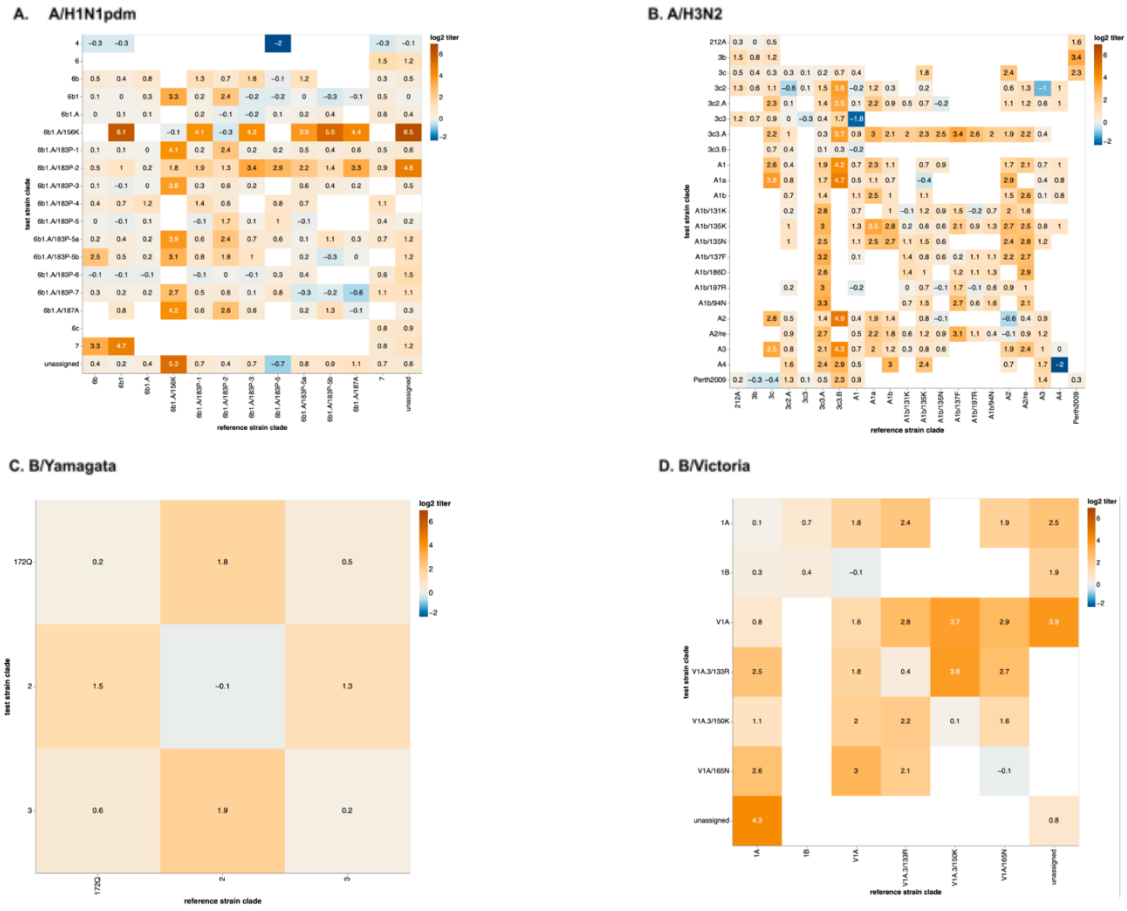

Figure B.2 Antigenic Distance Heatmaps

A. Heatmap depicting the antigenic distances between globally circulating clades of A/H1N1pdm between 2011 and 2019. B. Heatmap depicting the antigenic distances between globally circulating clades of A/H3N2 between 2011 and 2019. C. Heatmap depicting the antigenic distances between globally circulating clades of B/Yamagata between 2011 and 2019. D. Heatmap depicting the antigenic distances between globally circulating clades of B/Victoria between 2011 and 2019.

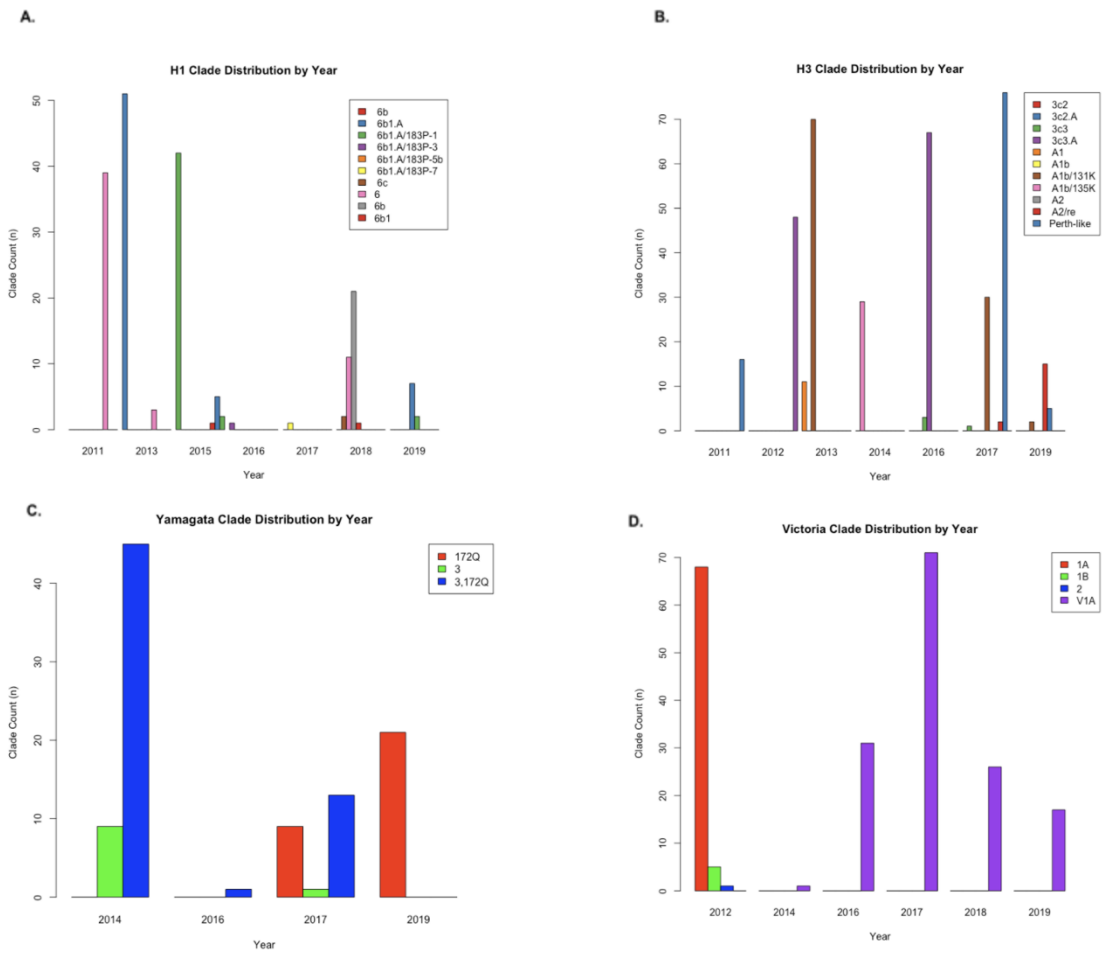

Figure B.3 Clade Distributions by Year

A. Counts of clade distributions by year for sequencing data for all H1 viruses from Nicaragua during the study time period. B. Counts of clade distributions by year for sequencing data for all H3 viruses from Nicaragua during the study time period. C. Counts of clade distributions by year for sequencing data for all Yamagata viruses from Nicaragua during the study time period. D. Counts of clade distributions by year for sequencing data for all Victoria viruses from Nicaragua during the study time period.

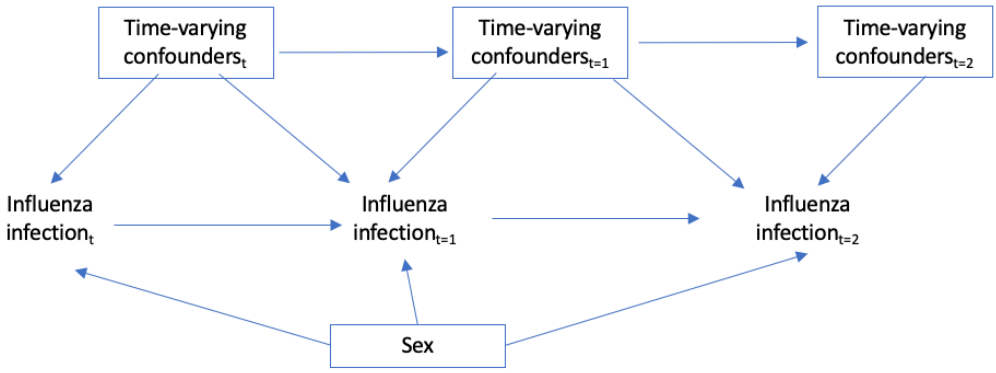

Figure B.4 Directed Acyclic Graph of Repeat Infection Analysis

Appendix C: Supplemental Materials for Chapter 4

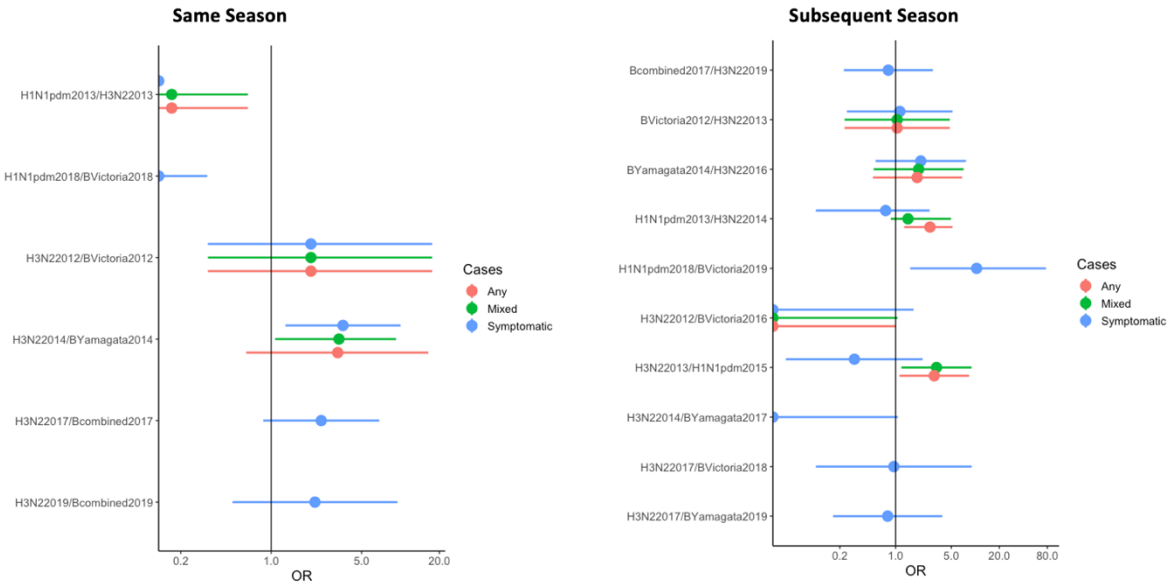

Figure C.1 Under 2 Repeat Infection Odds

Under-two odds of heterotypic/heterosubtypic repeat infection in both same and subsequent seasons of influenza.

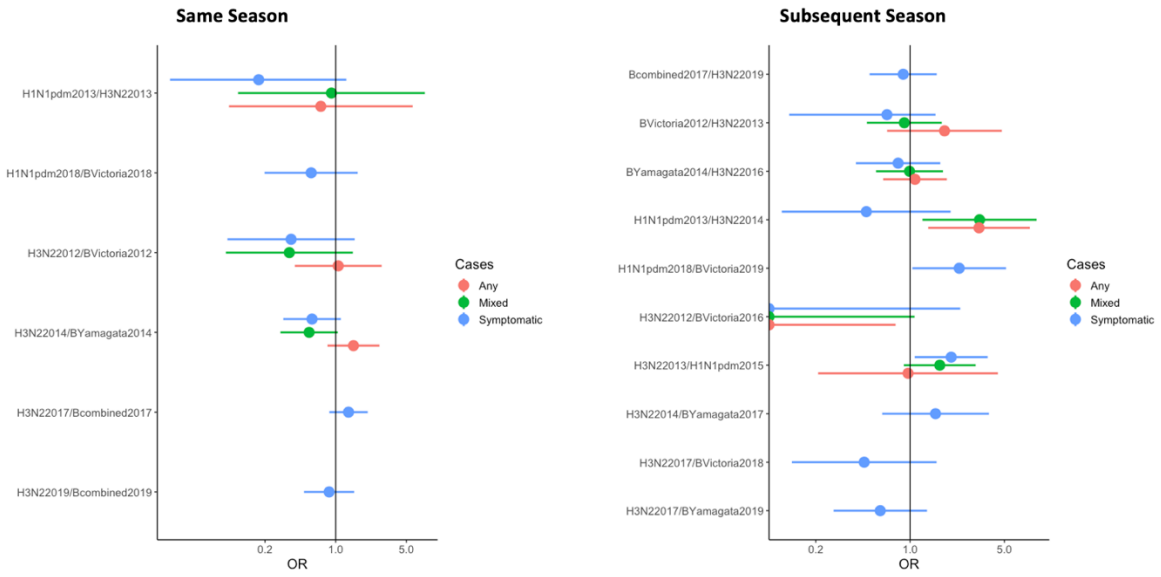

Figure C.2 Healthcare-seeking behavior adjusted odds

Same and subsequent season odds of heterotypic/heterosubtypic influenza repeat infection, adjusted for healthcare-seeking behavior.

Bibliography

1. Iuliano, A. D., Roguski, K. M., Chang, H. H., Muscatello, D. J., Palekar, R., Tempia, S., Cohen, C., Gran, J. M., Schanzer, D., Cowling, B. J., Wu, P., Kyncl, J., Ang, L. W., Park, M., Redlberger-Fritz, M., Yu, H., Espenhain, L., Krishnan, A., Emukule, G., van Asten, L., … Global Seasonal Influenza-associated Mortality Collaborator Network (2018). Estimates of global seasonal influenza-associated respiratory mortality: a modelling study. *Lancet (London, England)*, *391*(10127), 1285–1300. https://doi.org/10.1016/S0140-6736(17)33293-2
2. Lozano, R., Naghavi, M., Foreman, K., Lim, S., Shibuya, K., Aboyans, V., Abraham, J., Adair, T., Aggarwal, R., Ahn, S. Y., Alvarado, M., Anderson, H. R., Anderson, L. M., Andrews, K. G., Atkinson, C., Baddour, L. M., Barker-Collo, S., Bartels, D. H., Bell, M. L., Benjamin, E. J., … Memish, Z. A. (2012). Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet (London, England)*, *380*(9859), 2095–2128. https://doi.org/10.1016/S0140-6736(12)61728-0
3. Paget, J., Spreeuwenberg, P., Charu, V., Taylor, R. J., Iuliano, A. D., Bresee, J., Simonsen, L., Viboud, C., & Global Seasonal Influenza-associated Mortality Collaborator Network and GLaMOR Collaborating Teams* (2019). Global mortality associated with seasonal influenza epidemics: New burden estimates and predictors from the GLaMOR Project. *Journal of global health*, *9*(2), 020421. https://doi.org/10.7189/jogh.09.020421
4. Wang, X., Li, Y., O'Brien, K. L., Madhi, S. A., Widdowson, M. A., Byass, P., Omer, S. B., Abbas, Q., Ali, A., Amu, A., Azziz-Baumgartner, E., Bassat, Q., Abdullah Brooks, W., Chaves, S. S., Chung, A., Cohen, C., Echavarria, M., Fasce, R. A., Gentile, A., Gordon, A., … Respiratory Virus Global Epidemiology Network (2020). Global burden of respiratory infections associated with seasonal influenza in children under 5 years in 2018: a systematic review and modelling study. *The Lancet. Global health*, *8*(4), e497–e510. https://doi.org/10.1016/S2214-109X(19)30545-5
5. Coleman, B. L., Fadel, S. A., Fitzpatrick, T., & Thomas, S. M. (2018). Risk factors for serious outcomes associated with influenza illness in high- versus low- and middle-

income countries: Systematic literature review and meta-analysis. *Influenza and other respiratory viruses*, *12*(1), 22–29.
6. Krammer F. (2019). The human antibody response to influenza A virus infection and vaccination. *Nature reviews. Immunology*, *19*(6), 383–397. https://doi.org/10.1038/s41577-019-0143-6
7. Taubenberger, J. K., & Morens, D. M. (2010). Influenza: the once and future pandemic. *Public health reports (Washington, D.C. : 1974)*, *125 Suppl 3*(Suppl 3), 16–26.
8. Pedersen J. C. (2014). Hemagglutination-inhibition assay for influenza virus subtype identification and the detection and quantitation of serum antibodies to influenza virus. *Methods in molecular biology (Clifton, N.J.)*, *1161*, 11–25. https://doi.org/10.1007/978-1-4939-0758-8_2
9. Peteranderl, C., Herold, S., & Schmoldt, C. (2016). Human Influenza Virus Infections. *Seminars in respiratory and critical care medicine*, *37*(4), 487–500. https://doi.org/10.1055/s-0036-1584801
10. Krammer, F., Smith, G.J.D., Fouchier, R.A.M. *et al.* Influenza. *Nat Rev Dis Primers* **4,** 3 (2018). https://doi.org/10.1038/s41572-018-0002-y
11. Rajendran, M., Nachbagauer, R., Ermler, M. E., Bunduc, P., Amanat, F., Izikson, R., Cox, M., Palese, P., Eichelberger, M., & Krammer, F. (2017). Analysis of Anti-Influenza Virus Neuraminidase Antibodies in Children, Adults, and the Elderly by ELISA and Enzyme Inhibition: Evidence for Original Antigenic Sin. *mBio*, *8*(2), e02281-16. https://doi.org/10.1128/mBio.02281-16
12. Labella, A. M., & Merel, S. E. (2013). Influenza. *The Medical clinics of North America*, *97*(4), 621–x. https://doi.org/10.1016/j.mcna.2013.03.001
13. Webster, R. G., & Govorkova, E. A. (2014). Continuing challenges in influenza. *Annals of the New York Academy of Sciences*, *1323*(1), 115–139. https://doi.org/10.1111/nyas.12462
14. McLean, H. Q., & Belongia, E. A. (2021). Influenza Vaccine Effectiveness: New Insights and Challenges. *Cold Spring Harbor perspectives in medicine*, *11*(6), a038315. https://doi.org/10.1101/cshperspect.a038315
15. Tenforde, M. W., Talbot, H. K., Trabue, C. H., Gaglani, M., McNeal, T. M., Monto, A. S., Martin, E. T., Zimmerman, R. K., Silveira, F. P., Middleton, D. B., Olson, S. M., Garten Kondor, R. J., Barnes, J. R., Ferdinands, J. M., Patel, M. M., & Hospitalized Adult Influenza Vaccine Effectiveness Network (HAIVEN) Investigators (2021). Influenza Vaccine Effectiveness Against Hospitalization in the United States, 2019-2020. *The Journal of infectious diseases*, *224*(5), 813–820. https://doi.org/10.1093/infdis/jiaa800
16. Erbelding, E. J., Post, D. J., Stemmy, E. J., Roberts, P. C., Augustine, A. D., Ferguson, S., ... & Fauci, A. S. (2018). A universal influenza vaccine: the strategic plan for the National Institute of Allergy and Infectious Diseases. *The Journal of infectious diseases*, *218*(3), 347-354.
17. Krammer F. (2015). Emerging influenza viruses and the prospect of a universal influenza virus vaccine. *Biotechnology journal*, *10*(5), 690–701. https://doi.org/10.1002/biot.201400393
18. Francis T.J. On the Doctrine of Original Antigenic Sin. *Proc. Am. Philos. Soc.* 1960;104:572–578.

19. Zhang, A., Stacey, H. D., Mullarkey, C. E., & Miller, M. S. (2019). Original Antigenic Sin: How First Exposure Shapes Lifelong Anti-Influenza Virus Immune Responses. *Journal of immunology (Baltimore, Md. : 1950)*, *202*(2), 335–340. https://doi.org/10.4049/jimmunol.1801149
20. Vatti, A., Monsalve, D. M., Pacheco, Y., Chang, C., Anaya, J. M., & Gershwin, M. E. (2017). Original antigenic sin: A comprehensive review. *Journal of autoimmunity*, *83*, 12–21. https://doi.org/10.1016/j.jaut.2017.04.008
21. Brown, E. L., & Essigmann, H. T. (2021). Original Antigenic Sin: the Downside of Immunological Memory and Implications for COVID-19. *mSphere*, *6*(2), e00056-21. https://doi.org/10.1128/mSphere.00056-21
22. Monto, A.S., Ullman, B.M. (1974). Acute Respiratory Illness in an American Community: The Tecumseh Study. *JAMA, 227*(2), 164–169. doi:10.1001/jama.1974.03230150016004
23. Monto, A. S., Koopman, J. S., & Longini, I. M., Jr (1985). Tecumseh study of illness. XIII. Influenza infection and disease, 1976-1981. *American journal of epidemiology*, *121*(6), 811–822. https://doi.org/10.1093/oxfordjournals.aje.a114052
24. Monto, A. S., Koopman, J. S., & Bryan, E. R. (1986). The Tecumseh Study of Illness. XIV. Occurrence of respiratory viruses, 1976-1981. *American journal of epidemiology*, *124*(3), 359–367. https://doi.org/10.1093/oxfordjournals.aje.a114406
25. Monto, A. S., Malosh, R. E., Evans, R., Lauring, A. S., Gordon, A., Thompson, M. G., Fry, A. M., Flannery, B., Ohmit, S. E., Petrie, J. G., Martin, E. T., & HIVE Study Research Staff (2019). Data resource profile: Household Influenza Vaccine Evaluation (HIVE) Study. *International journal of epidemiology*, *48*(4), 1040–1040g. https://doi.org/10.1093/ije/dyz086
26. Frank, A. L., & Taber, L. H. (1983). Variation in frequency of natural reinfection with influenza A viruses. *Journal of medical virology*, *12*(1), 17–23. https://doi.org/10.1002/jmv.1890120103
27. Frank, A. L., Taber, L. H., & Porter, C. M. (1987). Influenza B virus reinfection. *American journal of epidemiology*, *125*(4), 576–586. https://doi.org/10.1093/oxfordjournals.aje.a114571
28. Taber, L. H., Paredes, A., Glezen, W. P., & Couch, R. B. (1981). Infection with influenza A/Victoria virus in Houston families, 1976. *The Journal of hygiene*, *86*(3), 303–313. https://doi.org/10.1017/s0022172400069059
29. Frank, A. L., Taber, L. H., Glezen, W. P., Paredes, A., & Couch, R. B. (1979). Reinfection with influenza A (H3N2) virus in young children and their families. *The Journal of infectious diseases*, *140*(6), 829–836. https://doi.org/10.1093/infdis/140.6.829
30. JORDAN, W. S., Jr, BADGER, G. F., & DINGLE, J. H. (1958). A study of illness in a group of Cleveland families. XVI. The epidemiology of influenza, 1948-1953. *American journal of hygiene*, *68*(2), 169–189. https://doi.org/10.1093/oxfordjournals.aje.a119961
31. Epstein S. L. (2006). Prior H1N1 influenza infection and susceptibility of Cleveland Family Study participants during the H2N2 pandemic of 1957: an experiment of nature. *The Journal of infectious diseases*, *193*(1), 49–53. https://doi.org/10.1086/498980
32. Fragaszy, E. B., Warren-Gash, C., Wang, L., Copas, A., Dukes, O., Edmunds, W. J., Goonetilleke, N., Harvey, G., Johnson, A. M., Kovar, J., Lim, M. S., McMichael, A., Millett, E. R., Nazareth, I., Nguyen-Van-Tam, J. S., Tabassum, F., Watson, J. M., Wurie, F., Zambon, M., Hayward, A. C., … Flu Watch Group (2017). Cohort Profile: The Flu

Watch Study. *International journal of epidemiology*, *46*(2), e18. https://doi.org/10.1093/ije/dyv370
33. Hayward, A. C., Fragaszy, E. B., Bermingham, A., et al. (2014). Comparative community burden and severity of seasonal and pandemic influenza: results of the Flu Watch cohort study. *The Lancet. Respiratory medicine*, *2*(6), 445–454. https://doi.org/10.1016/S2213-2600(14)70034-7
34. Hayward, A. C., Wang, L., Goonetilleke, N., et al. (2015). Natural T Cell-mediated Protection against Seasonal and Pandemic Influenza. Results of the Flu Watch Cohort Study. *American journal of respiratory and critical care medicine*, *191*(12), 1422–1431. https://doi.org/10.1164/rccm.201411-1988OC
35. Cowling, B. J., Perera, R. A., Fang, V. J., et al. (2014). Incidence of influenza virus infections in children in Hong Kong in a 3-year randomized placebo-controlled vaccine study, 2009-2012. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 59(4), 517–524. https://doi.org/10.1093/cid/ciu356
36. Ranjeva, S., Subramanian, R., Fang, V. J., et al. (2019). Age-specific differences in the dynamics of protective immunity to influenza. *Nature communications*, *10*(1), 1660. https://doi.org/10.1038/s41467-019-09652-6
37. Cowling, B. J., Peiris, J., & Kwok, K. O. (2019). Influenza virus infections in Hong Kong in 2013-14: a community-based longitudinal seroepidemiological study. *Hong Kong medical journal = Xianggang yi xue za zhi*, *25 Suppl 7*(5), 23–26.
38. Yang, W., Lau, E., & Cowling, B. J. (2020). Dynamic interactions of influenza viruses in Hong Kong during 1998-2018. *PLoS computational biology*, *16*(6), e1007989. https://doi.org/10.1371/journal.pcbi.1007989
39. Hsu, J. P., Zhao, X., Chen, M. I., et al. (2014). Rate of decline of antibody titers to pandemic influenza A (H1N1-2009) by hemagglutination inhibition and virus microneutralization assays in a cohort of seroconverting adults in Singapore. *BMC infectious diseases*, *14*, 414. https://doi.org/10.1186/1471-2334-14-414
40. Virk, R. K., Tambyah, P. A., Inoue, M., Lim, E. A., Chan, K. W., Chua, C., & Tan, B. H. (2014). Prospective surveillance and molecular characterization of seasonal influenza in a university cohort in Singapore. *PloS one*, *9*(2), e88345. https://doi.org/10.1371/journal.pone.0088345
41. Lee, V., Yap, J., Cook, A. R., Chen, M., Tay, J., Barr, I., Kelso, A., Tan, B., Loh, J. P., Lin, R., Cui, L., Kelly, P. M., Leo, Y., Chia, K., Kang, W. L., Tambyah, P., & Seet, B. (2010). Effectiveness of public health measures in mitigating pandemic influenza spread: a prospective sero-epidemiological cohort study. *The Journal of infectious diseases*, *202*(9), 1319–1326. https://doi.org/10.1086/656480
42. Goh, E. H., Jiang, L., Hsu, J. P., Tan, L., Lim, W. Y., Phoon, M. C., Leo, Y. S., Barr, I. G., Chow, V., Lee, V. J., Lin, C., Lin, R., Sadarangani, S. P., Young, B., & Chen, M. I. (2017). Epidemiology and Relative Severity of Influenza Subtypes in Singapore in the Post-Pandemic Period from 2009 to 2010. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, *65*(11), 1905–1913. https://doi.org/10.1093/cid/cix694
43. Chen, M. I., Cook, A. R., Lim, W. Y., Lin, R., Cui, L., Barr, I. G., Kelso, A., Chow, V. T., Leo, Y. S., Hsu, J. P., Shaw, R., Chew, S., Yap, J. K., Phoon, M. C., Koh, H. W., Zheng, H., Tan, L., & Lee, V. J. (2013). Factors influencing infection by pandemic

influenza A(H1N1)pdm09 over three epidemic waves in Singapore. *Influenza and other respiratory viruses*, *7*(6), 1380–1389. https://doi.org/10.1111/irv.12129
44. Cauchemez, S., Ferguson, N. M., Fox, A., et al. (2014). Determinants of influenza transmission in South East Asia: insights from a household cohort study in Vietnam. *PLoS pathogens*, *10*(8), e1004310. https://doi.org/10.1371/journal.ppat.1004310
45. Horby, P., Mai, l., Fox, A., Thai, P. Q., Thi Thu Yen, N., Thanh, l., Le Khanh Hang, N., Duong, T. N., Thoang, D. D., Farrar, J., Wolbers, M., & Hien, N. T. (2012). The epidemiology of interpandemic and pandemic influenza in Vietnam, 2007-2010: the Ha Nam household cohort study I. *American journal of epidemiology*, *175*(10), 1062–1074. https://doi.org/10.1093/aje/kws121
46. Vinh, D. N., Nhat, N., de Bruin, E., Vy, N., Thao, T., Phuong, H. T., Anh, P. H., Todd, S., Quan, T. M., Thanh, N., Lien, N., Ha, N., Hong, T., Thai, P. Q., Choisy, M., Nguyen, T. D., Simmons, C. P., Thwaites, G. E., Clapham, H. E., Chau, N., … Boni, M. F. (2021). Age-seroprevalence curves for the multi-strain structure of influenza A virus. *Nature communications*, *12*(1), 6680. https://doi.org/10.1038/s41467-021-26948-8
47. Lee VJ, Ho ZJ, Goh EH, Campbell H, Cohen C, Cozza V, et al. Advances in measuring influenza burden of disease. Influenza Other Respi Viruses 2018; 12:3-9. doi:10.1111/irv.12533
48. Reber A, Katz J. Immunological assessment of influenza vaccines and immune correlates of protection. Expert Review of Vaccines 2013; 12(5), 519-36, doi:10.1586/erv.13.35
49. Jacobsen H, Rajendran M, Choi A, Sjursen H, Brokstad KA, Cox RJ, et al. Influenza virus hemagglutinin stalk-specific antibodies in human serum are a surrogate marker for in vivo protection in a serum transfer mouse challenge model. MBio 2017; 8. doi:10.1128/mBio.01463-17.
50. Katz JM, Hancock K, Xu X. Serologic assays for influenza surveillance, diagnosis and vaccine evaluation. Expert Review of Anti-infective Therapy 2011; 9(6), 669-83. doi:10.1586/eri.11.51
51. Hermans D, Webby RJ, Wong S. Atypical antibody responses to influenza. Journal of Thoracic Disease 2018; 10(S9). doi:10.21037/jtd.2017.12.122
52. Ng S, Nachbagauer R, Balmaseda A, Stadlbauer D, Ojeda S, Patel M, et al. Novel correlates of protection against pandemic H1N1 influenza A virus infection. Nature Medicine 2019; 25, 962-7. doi: 10.1038/s41591-019-0463-x
53. Gordon A, Tsang TK, Cowling BJ, Kuan G, Ojeda S, Sanchez N, et al. Influenza transmission dynamics in urban households, Managua, Nicaragua, 2012–2014. Emerging Infectious Diseases 2018; 24(10), 1882-88. doi: 10.3201/eid2410.161258
54. Cauchemez S, Horby P, Fox A, Mai LQ, Thanh LT, Thai PQ, et al. Influenza infection rates, measurement errors and the interpretation of paired serology. PLoS Pathog 2012; 8:e1003061.
55. Tete SM, Krammer F, Lartey S, Bredholt G, Wood J, Skrede S, Cox RJ. Dissecting the hemagglutinin head and stalk-specific IgG antibody response in healthcare workers following pandemic H1N1 vaccination. Npj Vaccines 2016; 1(1). doi: 10.1038/npjvaccines.2016.1
56. Mcelhaney JE, Ewen C, Zhou X, Kane KP, Xie D, Hager WD, et al. Granzyme B: Correlates with protection and enhanced CTL response to influenza vaccination in older adults. Vaccine 2009; 27(18), 2418-25. doi:10.1016/j.vaccine.2009.01.136

57. Forrest BD, Pride MW, Dunning AJ, Capeding MR, Chotpitayasunondh T, Tam JS, et al. Correlation of cellular immune responses with protection against culture-confirmed influenza virus in young children. Clinical and Vaccine Immunology 2008; 15(7), 1042-53. doi:10.1128/cvi.00397-07
58. Christensen SR, Toulmin SA, Griesman T, Lamerato LE, Petrie JG, Martin ET, et al. Assessing the protective potential of H1N1 influenza virus hemagglutinin head and stalk antibodies in humans. Journal of Virology 2019; 93(8). doi:10.1128/jvi.02134-18
59. Couch RB, Atmar RL, Franco LM, Quarles JM, Wells J, Arden N, et al. Antibody correlates and predictors of immunity to naturally occurring influenza in humans and the importance of antibody to the neuraminidase. The Journal of Infectious Diseases 2013; 207(6), 974-981. doi:10.1093/infdis/jis935
60. Krammer F, Fouchier R, Eichelberger M, Webby RJ, Shaw-Saliba K, Wan H, et al. NAction! how can neuraminidase-based immunity contribute to better influenza virus vaccines? mBio 2018; 9 (2) e02332-17. doi: 10.1128/mBio.02332-17
61. Zarnitsyna VI, Lavine J, Ellebedy A, Ahmed R, Antia R. Multi-epitope models explain how pre-existing antibodies affect the generation of broadly protective responses to Influenza. PLoS pathogens 2016; 12(6), e1005692. doi:10.1371/journal.ppat.1005692
62. Huang QS, Bandaranayake D, Wood T, Newbern EC, Seeds R, Ralston J, et al. Southern Hemisphere Influenza and Vaccine Effectiveness Research and Surveillance (SHIVERS) Investigation Team, Risk Factors and Attack Rates of Seasonal Influenza Infection: Results of the Southern Hemisphere Influenza and Vaccine Effectiveness Research and Surveillance (SHIVERS) Seroepidemiologic Cohort Study. The Journal of Infectious Diseases 2019; 219(3), 347-57. doi: 10.1093/infdis/jiy443
63. Gordon, A., & Reingold, A. (2018). The Burden of Influenza: A Complex Problem. *Current Epidemiology Reports, 5*(1), 1-9. doi:10.1007/s40471-018-0136-1
64. Fraaij, P. L., & Heikkinen, T. (2011). Seasonal influenza: The burden of disease in children. Vaccine,29(43), 7524-7528. doi:10.1016/j.vaccine.2011.08.010
65. Donikian MA, McKee J, Greene LC. (1977). Challenge versus natural infection as an index or protection after influenza immunization. *Dev Biol Stand. 39*, 149-54.
66. Tannock GA, Paul JA. (1987). Homotypic and heterotypic immunity of influenza A viruses induced by recombinants of the cold-adapted master strain A/Ann Arbor/6/60-ca. *Arch Virol. 92*(1-2):121-33.
67. Wilkinson, T. M., Li, C. K., Chui, C. S., et al. (2012). Preexisting influenza-specific CD4 T cells correlate with disease protection against influenza challenge in humans. *Nature Medicine,18*(2), 274-280. doi:10.1038/nm.2612
68. Chan, J., Babb, R., David, S. C., Mccoll, S. R., & Alsharifi, M. (2016). Vaccine-Induced Antibody Responses Prevent the Induction of Interferon Type I Responses Upon a Homotypic Live Virus Challenge. *Scandinavian Journal of Immunology,83*(3), 165-173. doi:10.1111/sji.12410
69. Endo, A., Itamura, S., Iinuma, H., et al. (1991). Homotypic and heterotypic protection against influenza virus infection in mice by recombinant vaccinia virus expressing the haemagglutinin or nucleoprotein gene of influenza virus. *Journal of General Virology,72*(3), 699-703. doi:10.1099/0022-1317-72-3-699
70. Johansson, B. E., & Brett, I. C. (2008). Recombinant Influenza B virus HA and NA antigens administered in equivalent amounts are immunogenically equivalent and induce

equivalent homotypic and broader heterovariant protection in mice than conventional and live influenza vaccines. *Human Vaccines,4*(6), 420-424. doi:10.4161/hv.4.6.6201
71. Kaye, H. S., Dowdle, W. R., & Mcqueen, J. L. (1969). Studies On Inactivated Influenza Vaccines. *American Journal of Epidemiology,90*(2), 162-169. doi:10.1093/oxfordjournals.aje.a121060
72. Couch, R. B., & Kasel, J. A. (1983). Immunity to influenza in man. *Annual review of microbiology*, *37*, 529–549.
73. Paules, C. I., Marston, H. D., Eisinger, R. W., Baltimore, D., & Fauci, A. S. (2017). The Pathway to a Universal Influenza Vaccine. *Immunity,47*(4), 599-603. doi:10.1016/j.immuni.2017.09.007
74. Sautto, G. A., Kirchenbaum, G. A., & Ross, T. M. (2018). Towards a universal influenza vaccine: different approaches for one goal. *Virology journal*, *15*(1), 17. https://doi.org/10.1186/s12985-017-0918-y
75. Lynch, G. W., Selleck, P., Church, W. B., & Sullivan, J. S. (2012). Seasoned adaptive antibody immunity for highly pathogenic pandemic influenza in humans. *Immunology and cell biology*, *90*(2), 149–158.
76. Gordon, A., Kuan, G., Aviles, W., et al. (2015). The Nicaraguan pediatric influenza cohort study: Design, methods, use of technology, and compliance. *BMC Infectious Diseases,15*(1). doi:10.1186/s12879-015-1256-6
77. Katoh K., Standley DM, MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability, Molecular Biology and Evolution, Volume 30, Issue 4, April 2013, Pages 772–780, https://doi.org/10.1093/molbev/mst010
78. Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ, IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies, Molecular Biology and Evolution, Volume 32, Issue 1, January 2015, Pages 268–274, https://doi.org/10.1093/molbev/msu300
79. Bedford T, Suchard MA, Lemey P, et al. (2014). Integrating influenza antigenic dynamics with molecular evolution. *Elife, 3*(e01914). doi: 10.7554/eLife.01914.
80. Neher RA, Bedford T, Daniels RS, Russell CA, Shraiman BI. (2016). Antigenic phenotypes of seasonal influenza viruses. *Proceedings of the National Academy of Sciences, 113*(12) E1701-E1709; DOI: 10.1073/pnas.1525578113
81. Belongia, E. A., Skowronski, D. M., McLean, H. Q., Chambers, C., Sundaram, M. E., & De Serres, G. (2017). Repeated annual influenza vaccination and vaccine effectiveness: review of evidence. *Expert review of vaccines*, *16*(7), 1–14.
82. Bedford T, Riley S, Barr IG, et al. (2015). Global circulation patterns of seasonal influenza viruses vary with antigenic drift. *Nature, 523*(7559):217-20. doi: 10.1038/nature14460.
83. Cobey S. Hensley S.E. (2017). Immune history and influenza virus susceptibility. *Curr Opin Virol*. 22, 105-111. doi: 10.1016/j.coviro.2016.12.004.
84. Arevalo, C. P., Le Sage, V., Bolton, M. J., et al. (2020). Original antigenic sin priming of influenza virus hemagglutinin stalk antibodies. *Proceedings of the National Academy 117*(29) 17221-17227.
85. Lewnard, J. A., & Cobey, S. (2018). Immune History and Influenza Vaccine Effectiveness. *Vaccines*, *6*(2), 28. https://doi.org/10.3390/vaccines6020028
86. Arevalo, P., McLean, H. Q., Belongia, E. A., & Cobey, S. (2020). Earliest infections predict the age distribution of seasonal influenza A cases. *eLife*, *9*, e50060.

87. Vieira, M.C., Donato, C.M., Arevalo, P., et al. (2020). Lineage-specific protection and immune imprinting shape the age distributions of influenza B cases. medRxiv 2020.09.30.20204909; doi: https://doi.org/10.1101/2020.09.30.20204909
88. Caini, S., Spreeuwenberg, P., Kusznierz, G. F., et al. (2018). Distribution of influenza virus types by age using case-based global surveillance data from twenty-nine countries, 1999-2014. *BMC infectious diseases*, *18*(1), 269. https://doi.org/10.1186/s12879-018-3181-y
89. Gouma, S., Kim, K., Weirick, M. E., et al. (2020). Middle-aged individuals may be in a perpetual state of H3N2 influenza virus susceptibility. *Nature communications*, *11*(1), 4566. https://doi.org/10.1038/s41467-020-18465-x
90. Yang, J., Lau, Y. C., Wu, P., et al. (2018). Variation in Influenza B Virus Epidemiology by Lineage, China. *Emerging infectious diseases*, *24*(8), 1536–1540. https://doi.org/10.3201/eid2408.180063
91. Caini, S., Kusznierz, G., Garate, V. V., et al (2019). The epidemiological signature of influenza B virus and its B/Victoria and B/Yamagata lineages in the 21st century. *PloS one*, *14*(9), e0222381. https://doi.org/10.1371/journal.pone.0222381
92. Vijaykrishna, D., Holmes, E. C., Joseph, U., et al. (2015). The contrasting phylodynamics of human influenza B viruses. *eLife*, *4*, e05055. https://doi.org/10.7554/eLife.05055
93. CDC Seasonal Flu Vaccine Effectiveness Studies | CDC. (n.d.). Retrieved from https://www.cdc.gov/flu/vaccines-work/effectiveness-studies.htm
94. Steph Wraith, Angel Balmaseda, Fausto Andres Bustos Carrillo, Guillermina Kuan, John Huddleston, John Kubale, Roger Lopez, Sergio Ojeda, Amy Schiller, Brenda Lopez, Nery Sanchez, Richard Webby, Martha I. Nelson, Eva Harris, Aubree Gordon. Homotypic protection against influenza in a pediatric cohort in Managua, Nicaragua. (2022). *Nature Communications.*
95. Donikian MA, McKee J, Greene LC. (1977). Challenge versus natural infection as an index or protection after influenza immunization. *Dev Biol Stand. 39*, 149-54.
96. Tannock GA, Paul JA. (1987). Homotypic and heterotypic immunity of influenza A viruses induced by recombinants of the cold-adapted master strain A/Ann Arbor/6/60-ca. *Arch Virol. 92*(1-2):121-33.
97. Francis, M., King, M., & Kelvin, A. (2019). Back to the Future for Influenza Preimmunity—Looking Back at Influenza Virus History to Infer the Outcome of Future Infections. *Viruses,11*(2), 122. doi:10.3390/v11020122
98. Gostic, K. M., Ambrose, M., Worobey, M., & Lloyd-Smith, J. O. (2016). Potent protection against H5N1 and H7N9 influenza via childhood hemagglutinin imprinting. *Science,354*(6313), 722-726. doi:10.1126/science.aag1322
99. Xie, H., Li, L., Ye, Z., Li, X., Plant, E. P., Zoueva, O., . . . Wan, X. (2017). Differential Effects of Prior Influenza Exposures on H3N2 Cross-reactivity of Human Postvaccination Sera. *Clinical Infectious Diseases,65*(2), 259-267. doi:10.1093/cid/cix269
100. McLaren, C.L. & Potter, C.W. (1974). Effect of previous infection with heterotypic and heterologous influenza viruses on the response of ferrets to inactivated influenza virus vaccines. J Hyg (Lond). 72(1): 91–100.
101. Mendez-Legaza, J. M., Ortiz de Lejarazu, R., & Sanz, I. (2019). Heterotypic Neuraminidase Antibodies Against Different A(H1N1) Strains are Elicited after Seasonal Influenza Vaccination. *Vaccines*, *7*(1), 30.

102. Powell, T. J., Rijal, P., McEwen-Smith, R. M., Byun, H., Hardwick, M., Schimanski, L. M., Huang, K. A., Daniels, R. S., & Townsend, A. (2019). A single cycle influenza virus coated in H7 haemagglutinin generates neutralizing antibody responses to haemagglutinin and neuraminidase glycoproteins and protection from heterotypic challenge. *The Journal of general virology*, *100*(3), 431–445.
103. Powell, T. J., Silk, J. D., Sharps, J., Fodor, E., & Townsend, A. R. (2012). Pseudotyped Influenza A Virus as a Vaccine for the Induction of Heterotypic Immunity. *Journal of Virology,86*(24), 13397-13406. doi:10.1128/jvi.01820-12
104. Nachbagauer, R., Feser, J., Naficy, A. *et al.* A chimeric hemagglutinin-based universal influenza virus vaccine approach induces broad and long-lasting immunity in a randomized, placebo-controlled phase I trial. *Nat Med* **27,** 106–114 (2021). https://doi.org/10.1038/s41591-020-1118-7
105. Strohmeier, S., Amanat, F., Zhu, X., McMahon, M., Deming, M. E., Pasetti, M. F., Neuzil, K. M., Wilson, I. A., & Krammer, F. (2021). A Novel Recombinant Influenza Virus Neuraminidase Vaccine Candidate Stabilized by a Measles Virus Phosphoprotein Tetramerization Domain Provides Robust Protection from Virus Challenge in the Mouse Model. *mBio*, *12*(6), e0224121. https://doi.org/10.1128/mBio.02241-21
106. Gao, J., Wan, H., Li, X., Rakic Martinez, M., Klenow, L., Gao, Y., Ye, Z., & Daniels, R. (2021). Balancing the influenza neuraminidase and hemagglutinin responses by exchanging the vaccine virus backbone. *PLoS pathogens*, *17*(4), e1009171. https://doi.org/10.1371/journal.ppat.1009171