ADJUVANTED INFLUENZA VACCINATION IN CHILDREN

CORRELATES OF VACCINE EFFICACY, EFFECTIVENESS AND OUTCOMES IN MF-59 INFLUENZA VACCINATED CHILDREN

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LAY ABSTRACT

Children are at high risk of complications related to influenza infections, and are recommended to receive seasonal vaccination. Identifying host and vaccine characteristics which are associated with protection against influenza in children would be valuable for the development of improved vaccines. Adjuvanted influenza vaccines have been assessed in clinical trials but are not yet licensed for children. In this thesis, we evaluate relationships between vaccine formulations, reactions, the magnitude of post-vaccination antibody responses, protection against influenza and presentation of symptoms in breakthrough infections. We aim to further explore these relationships by estimating the proportion of relative adjuvanted protection which is mediated by increased antibody responses to this vaccine. We explore the relationships between vaccine formulation and host factors, and test the associations between these relationships and adjuvanted vaccine efficacy and effectiveness in children.

ABSTRACT

Children are at high risk for influenza-related morbidity, including severe complications of illness and hospitalizations. Seasonal influenza vaccination is recommended as the primary method of prevention and protection. Vaccine efficacy has been shown to vary based on host demographics, immune history, influenza type and subtype, and seasonal match of the vaccine antigens to the circulating influenza strains. Adjuvanted vaccination has been shown to induce greater breadth, magnitude and longevity of antibody responses. Research characterizing correlations between host factors, vaccine formulation, antibody responses and influenza outcomes would provide insight on how these relationships contribute to adjuvant-mediated vaccine effectiveness in children.

In this thesis, I explored whether adjuvanted vaccination was associated with attenuated symptom severity in breakthrough influenza infections, as compared with non-adjuvanted vaccinees. I then explored the utility of vaccine reactions as predictors of post-vaccination antibody responses, accounting for the effect modification of the adjuvant. Finally, I used a causal mediation analysis to estimate the proportion of relative protection in adjuvanted vaccinees which is attributable to the increased antibody responses in this group.

We found that adjuvanted vaccination was associated with significant reductions in fever and systemic symptom severity in breakthrough influenza A infections. We observed that total, systemic and respiratory reactogenicity significantly interacted with adjuvanted vaccination, leading to enhanced antibody responses relative to non-adjuvanted

vaccinees. Finally, we found that adjuvanted vaccine protection was not significantly mediated by the increased antibody titers in this group.

Our findings provide insight on determinants of adjuvanted vaccine effectiveness in children. Our work may inform future research examining the adjuvant moderation of innate and adaptive immunity in children, which may help define new correlates of protection against influenza. Greater understanding of the network of these relationships and their causal contribution to vaccine protection would contribute to the fields of immunology, vaccinology, and epidemiology.

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LIST OF ALL ABBREVIATIONS

aTIV	Adjuvanted trivalent inactivated influenza vaccine
CI	Confidence intervals
HAI	Hemagglutination inhibition assay
HR	Hazard ratio
IQR	Interquartile range
IRR	Incidence rate ratio
PCR	Polymerase chain reaction
QIV	Quadrivalent inactivated influenza vaccine
SD	Standard deviation
VE	Vaccine efficacy

PREFACE

This thesis was intended to be a sandwich thesis, structured as a series of manuscripts following an introductory chapter which set the framework of the overall research plan. It is a secondary analysis of data collected as part of an industry-sponsored cluster-randomized controlled trial. The objectives of the trial were to investigate direct and indirect protection conferred by vaccinating children with adjuvanted trivalent influenza vaccine, relative to a non-adjuvanted quadrivalent vaccine. The original study idea was conceived by Dr. Mark Loeb, in collaboration with investigators from the Universities of Calgary and Saskatchewan, and with support from colleagues at McMaster University.

The idea for my thesis plan was conceived in January 2021, in which I intended to investigate effector-cell mediated functions of influenza antibodies as correlates of protection. Due to constrained sample sizes and event numbers, my thesis plan transitioned to apply the same methodologies to the data collected from the adjuvanted vaccine trial. I was responsible for drafting the research proposals and analytical plans for the three chapters, which were reviewed and approved by Dr. Loeb and my committee in January 2022.

Serological, symptom and demographic data collected from the trial were provided to me from Pardeep Singh, the research data custodian for the Mark Loeb Research Group. I was responsible for subsetting, merging and cleaning these datasets. I performed all analyses in this thesis, with input and support from Drs. Verschoor and Pullenayegum.

Valuable data commentary and analytical support was available from Pardeep Singh if issues arose pertaining to original datasets.

I am the first author of each proposed thesis manuscript. Due to the required shift in my thesis plan at a late stage in my PhD research, the manuscripts are yet unpublished. They remain structured in manuscript format; however, they include lengthier background information and interpretations in the discussion sections. They also include more supplementary and exploratory analyses which would not be relevant for journal publication. I feel that these extended formats support the comprehensiveness of my PhD research and analysis. It is my intent to abridge them and submit for publication following a successful thesis defense, and the incorporation of any additional feedback from examiners.

The manuscript chapters were first reviewed by Drs. Loeb and Verschoor, and then appraised by Dr. Pullenayegum. Since all chapters are derived from the same cohort, the reader should expect overlap in much of the methodology of each chapter, such as study design and laboratory methods. I drafted the introduction and conclusion, which were reviewed by Dr. Loeb and my thesis committee.

CHAPTER 1.

SPECIFIC AIMS OF THE THESIS

Children under the age of five are at high risk of complications and hospitalizations related to influenza infection. Seasonal influenza vaccination is recommended in this age group due to the high burden of disease; however, vaccine efficacy (VE) has been shown to vary [1–4]. Factors influencing VE include the vaccine formulation, host immune histories, influenza type and subtype, and seasonal match of the vaccine antigens to the circulating influenza strains[1,3–6]. Research investigating correlates of protection in children is valuable for the development and evaluation of improved vaccines for this group. The accepted correlate of protection against influenza is the hemagglutination inhibition assay (HAI)[7,8]. HAI titers of greater than 1:40, or a four-fold seroconversion, are considered protective titers and correlate with a 50% reduction in the risk of influenza illness. However, recent work has shown that this threshold does not consistently correlate with similar risk reduction in children, proposing thresholds of <1:110 [9,10]. Adjuvanted influenza vaccination, as with the MF-59 emulsion, has been shown to induce significantly superior antibody responses in children, enhancing vaccine immunogenicity [11–13]. It is widely held that the increased HAI titers mounted in adjuvant-vaccinated children will be responsible for improved protection, as observed in studies of adjuvanted vaccine effectiveness in adults[14–16]. However, studies on adjuvanted vaccine effectiveness in children are limited. Research characterizing correlations between host factors, vaccine formulation and influenza outcomes would provide insight on how these relationships contribute to vaccine effectiveness in children.

The objective of this thesis was to assess whether characteristics of host and vaccine factors were correlated with protection and disease outcomes in a pediatric cohort. Our specific aims were to:

1. Investigate whether vaccine formulation is associated with attenuated disease severity in breakthrough influenza infection
2. Assess whether vaccine reactogenicity is predictive of enhanced vaccine immunogenicity, as measured by magnitude of antibody induction
3. Quantify the proportion of relative vaccine protection in adjuvanted vaccinees which is mediated by the increased post-vaccination antibody titers

We expected that host characteristics—such as age, hypersensitivity, or antibody profiles—may shape immune responses after vaccination, and that these responses may be moderated by the formulation of the vaccine. We hypothesized that the moderation of host characteristics by adjuvanted vaccination may be associated with differences in immunogenicity, effectiveness, and presentation of influenza infection.

BACKGROUND AND SIGNIFICANCE

Seasonal influenza vaccines are produced in a number of formulations which activate the immune response via different pathways[17]. All influenza vaccines are designed to elicit an antibody response targeted at the hemagglutinin glycoprotein on the surface of influenza virus. While all vaccines are designed to generate an influenza antibody response, differences in formulation and host immune landscapes have been shown to impact the antibody response in other ways—such as isotype, longevity, and breadth or

cross-reactivity of antibodies[18–22]. Inactivated formulations contain either three or four antigens derived from influenza types A and B, and quadrivalent vaccination is generally recommended in children due to the high burden of influenza in this group [23,24]. Despite these vaccines being immunogenic, influenza virus undergoes both evolutionary change and novel reassortment events which can reduce vaccine protection [25,26]. Adjuvants have been proposed as a means of enhancing vaccine effectiveness by inducing more long-lasting and heterologous protection [11,12,21]. Adjuvanted vaccines act via various mechanisms to enhance the immune response, such as enriching cytokines which attract myeloid cells, activation of antigen-presenting cells, and upregulating T cell activities [13,18,21,27,28]. The MF59 adjuvant has been shown to activate immune cells and enhance antigen uptake. Further studies have demonstrated that it induces immunogenicity by the induction of chemokines, increased recruitment of immune cells, enhanced differentiation of monocytes into dendritic cells, and facilitating dendritic cell migration into the lymph nodes, thus triggering the adaptive immune response [13,21]. While numerous studies have shown the enhanced immunogenicity which is evoked by adjuvanted influenza vaccines, data on the effectiveness of adjuvanted vaccination in children is limited. Further work which examines the associations between host factors, influenza vaccine formulations and protection outcomes is necessary.

Adjuvanted Symptom Severity

Previous studies have evaluated the effect of influenza vaccination on symptom severity in children[2,29–31]. Further research has supported the hypothesis that robust antibody

levels might be associated with reduced symptom severity in children who develop influenza infections[32–34]. Vaccine adjuvants, particularly MF59, have been shown to enhance immune responses in children through a variety of mechanisms[11,13,35,36]. These include increased immune cell activation and antigen presentation, and studies have found that MF59 adjuvantation is associated with induction of higher titers antibodies against influenza infection[21,37–39]. Adjuvanted vaccines have generated interest for their ability to induce broader cross-protective antibody responses, which may influence the course of disease in breakthrough infections[11,35,40]. The profile of the immune response following vaccination may influence subsequent viral clearance and presentation of infection. Further study is required to evaluate whether adjuvanted vaccines are associated with attenuated severity of symptoms in children who go on to develop an influenza infection.

Reactogenicity as a Correlate of Immunogenicity

Reactogenicity encompasses the range of physical presentations of innate inflammatory responses to vaccine administration. Reactions may include pain, redness, swelling or erythema at the injection site; and systemic symptoms, such as fever, headache, chills, muscle aches, and disseminated rash[41]. Most reactions reported are mild, self-limiting and require minimal medical care[42]. Vaccines, particularly adjuvanted formulations, induces host inflammatory responses, which are hypothesized to influence reactions to immunization [43–46]. While the exact mechanism of action of the MF59 adjuvant is unclear, studies have shown that it increases engagement of innate immune

responses[43,47]. Innate immunity has been shown to be crucial to the activation of adaptive immune responses, triggering the activation of B and T lymphocytes, which facilitate antibody development [13,27,28,48–50]. The engagement of inflammatory innate immune activity may both mediate host reactions, and influence the quality and magnitude of subsequent adaptive immunity. Despite these relationships, limited data is available investigating the predictive value of reactions with vaccine immunogenicity. Moreover, our study cohort contains children serially vaccinated over multiple seasons of influenza. Data on increased reactogenicity following repeated vaccinations in children is sparse, but two available studies showed that revaccination was associated with greater incidence of reactions [51,52]. In one study of children vaccinated with a nonadjuvanted inactivated influenza vaccine, the authors found a clear dose response linking successive immunizations over the previous three seasons with increased reactogenicity [51]. A study evaluating children serially vaccinated found that incidence of reactions was higher in adjuvant-vaccinated children relative to non-adjuvanted controls [52]. Among adjuvanted vaccinees, local reactions were found to predominate reported responses to the first immunization; whereas subsequent vaccinations were associated with increased incidence of systemic reactions, namely fever [52]. Research correlating reactogenicity with vaccine responsiveness, accounting for potential interactions with adjuvantation, would guide a pragmatic clinical prediction rule for forecasting vaccine efficacy in children.

Proportion of Adjuvanticity Mediated by Antibodies

It is widely held that adjuvanted influenza vaccine confers superior protection by its increased induction of antibodies. Enhanced vaccine immunogenicity, as measured by the post-vaccination HAI titers, has consistently been demonstrated in adjuvant-vaccinated children [13,37,40]. To date, literature is sparse evaluating the effectiveness of adjuvanted influenza vaccines in children. Studies have shown that within clinical trials, adjuvanted vaccination may confer protection which is longer lasting and more heterologous than nonadjuvanted vaccines [11,37,52]. The contribution of antibody responses to relative vaccine protection in adjuvanted formulations has not been assessed. In the case of influenza vaccination, vaccine efficacy is measured by the robustness of post-vaccination HAI titers, which are assumed to correlate with protection against infection. Previous work has found that HAI titers account for the majority (57%) of vaccine induced protection against influenza B, in comparison to unvaccinated children [53]. We proposed a mediation framework, which allowed for investigation of causal relationships between vaccine formulation, antibody responses and protection against influenza. In this framework, vaccination would influence numerous immune mechanisms (including head-specific antibody titers, stalk-specific antibody titers, and T-cell cytotoxic immunity), which in turn mediate the outcome of influenza infection. To our knowledge, no study has yet quantified the proportion of adjuvant protection, relative to unadjuvanted influenza vaccines, which is mediated by its induction of superior HAI titers.

METHODOLOGICAL CONSIDERATIONS

The parent study vaccinated 424 children over the study period, for a total of 994 observations[54]. Hutterite colonies in Western Canada were randomized to receive either adjuvanted trivalent inactivated influenza vaccine (aTIV) or a quadrivalent inactivated influenza vaccine (QIV). The study period spanned three influenza seasons, with data collected from January 2017 to June 2019. Children were eligible to receive the study vaccine if they were between 6 months and 6 years of age and were otherwise healthy, regardless of their prior immunization or influenza infection status. Exclusion criteria included a previous anaphylactic reaction to influenza vaccines, known allergy or hypersensitivity to eggs, previous history of Guillain- Barré syndrome up to eight weeks subsequent to influenza vaccination, or used salicylate-containing products within 30 days prior to enrollment [54].

Our study cohort was composed of 330 unique children from 36 Hutterite colonies, across three consecutive influenza seasons. Many children were serially vaccinated and provided multiple observations over the study duration, resulting in a total of 542 paired serum samples from pre-and post-vaccination blood draws. Blood draws were tested by HAI assay for immunogenicity to the vaccine antigens each season. However, several factors contributed to missing data observations and inconsistent measurement in all participants:

- Children were enrolled for varying lengths of time in the study, with 13% enrolled for one season, 20% enrolled in two seasons and 67% enrolled in all three seasons

- Not all children supplied blood draws at each time point, resulting in either an incomplete serum pair during a given season, or a season with no serum pair taken for a given participant
- Not all blood draws were adequate for testing by HAI assay

Participants changed enrollment status due to varied reasons, including 'aging out' of the study inclusion criteria, or moving to a new colony not included within the study. Enrollment status also may have changed due to participation fatigue, causing parents to decline further involvement. This is likely to impact the participants of the parent study, as prior randomized controlled studies of influenza vaccination have been carried out in this same population over the past 18 years [55,56]. Blood draws were not available for all participants during at all enrolled time points due to parent declining to participate in blood draw collection as part of the study. Reasons for this varied but included needle phobia in the child and/or unavailable to attend the appointment with the phlebotomist. Finally, reasons why sera drawn were not suitable for testing by HAI included inadequate volume of blood, or sample degradation which prevented conclusive results. We included only complete paired serum samples, excluding observations missing HAI titer data at either baseline or post-vaccination. We assumed that the likelihood of a parent declining to participate in study serology was equal for all participants, and that the values of the missing HAI data were not related to the primary variables of interest (namely, adjuvanted vaccine intervention). We determined that missing data in our study may be

attributable to some measured factors, such as the child's age, and therefore could be accounted for using modelling techniques, and unlikely to bias our interpretations[57].

As a result of the cluster-randomized study design, colonies were randomized to receive either quadrivalent or adjuvanted trivalent influenza vaccines. We would therefore expect observations within colony clusters to resemble each other more closely, particularly in terms of the attack rates of influenza in each colony, and the magnitude of post-vaccination antibody responses. Post-vaccination titers would also be influenced by repeat observations of participants over multiple seasons, due to differences in antibody waning and boosting by vaccination in subsequent seasons. These clustering effects violate the assumptions of independence required for the statistical modelling methods used in this thesis. To account for the clustering of our predictors, several approaches were used:

Chapter Two

In Chapter Two, we assessed whether vaccine formulation was associated with severity of symptoms in breakthrough influenza infections. We expected clustering by participant to influence influenza severity outcomes (colony was not hypothesized to have a meaningful effect on symptoms reported). Recorded first infections of influenza for each participant were included. We proposed to include all influenza infections, and account for this using a mixed linear model with a random intercept for each participant. Due to the limited number of recurrent events (n=3), we chose to limit our analysis to first infections only.

Chapter Three

In Chapter Three, we explored whether reactogenicity to vaccination was predictive of immunogenicity. We expected clustering by participant to influence both reactions experienced, and magnitude of antibody responses observed. As participants in the study were serially vaccinated with the same formulations, we would expect repeated measurements of antibody titers to be correlated with titers from previous seasons [58–60]. Further, we hypothesize that participants experiencing reactions may be predisposed to hypersensitivity reactions, and thus more likely to report reactions in subsequent immunizations. We accounted for this variation using random intercepts for each participant, and evaluated the significance of intracluster correlations within participants using variance inflation factors and design effect calculations[61–64].

Chapter Four

In the fourth chapter, we investigated the proportion of relative vaccine protection in adjuvanted vaccinees which is mediated by increased antibody titers. We expected clustering by colony to influence the survival time to influenza infection, due to the similarities in immortal time in individuals within each colony. Due to the infectious nature of influenza, the timing of the first influenza infection within any colony would influence the survival times of all colony individuals. This would violate the assumption that all observations were independent. A robust sandwich variance estimator was used to adjust for this, which derives estimates of parameters and standard errors from a

covariance matrix of model parameters [65]. The robust sandwich variance estimator takes into account the residual variance at differing values of predictors, incorporating a heteroscedasticity consistent standard error. As a result, p-values and 95% confidence intervals computed using this method provide more valid estimates of regression coefficients, errors and hypothesis testing.

An alternative to robust cluster sandwich estimation is bootstrapping, which requires fewer assumptions about the distribution of data than traditional parametric modelling[66–70]. Ideally, we proposed to use bootstrapping to estimate parameters and confidence intervals for models within our causal mediation framework. We intended to use Cox proportional hazards models to estimate the time to influenza infection using a model weighted by scores derived from post-vaccination antibody titers. However, bootstrapping by cluster generated uninterpretable estimates, as the models were unable to account for the distribution of events across colonies. Clustering by colony was important to consider in our analysis, as the colony determined vaccine group allocation. Since we were unable to bootstrap by cluster, it was not possible to use the bootstrapping approach to estimate the causal mediation effects. As our sample size was sufficiently large (n=542), we concluded that the uncertainty in estimating the weights for each participant would be minimal. We opted to generate estimates of the weighted scores from models using the original data, which could account for the clustering effect, rather than the bootstrapped approach. We report the estimates, 95% confidence intervals, and p-values from the real data models for causal mediation effects.

THESIS OUTLINE

The remainder of this thesis includes three original research manuscripts, followed by a concluding chapter which speaks to the overall themes linking the chapters. Each of the three manuscript chapters investigates one of the aims set out in the introduction. In the first manuscript, I investigated the association between vaccine formulation and attenuated severity of disease in breakthrough influenza infections. I examined how characteristics of participants and vaccines were correlated with duration and severity of symptomatic infection, exploring associations with local and systemic symptoms. In the second manuscript, I determined whether increased reactogenicity to vaccines was predictive of post-vaccination immunogenicity, adjusting for the moderating adjuvant effect and potential dose response of serial vaccination using mixed linear models. In the third manuscript, I conducted a causal mediation analysis quantifying the proportion of relative vaccine protection which is conferred to adjuvanted vaccinees via increased post-vaccination antibody titers.

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PREFACE TO CHAPTER TWO.

This chapter investigates the association between vaccine formulation and symptom severity in breakthrough influenza infections in children. The study first characterizes clinical presentation of infection in children immunized with either adjuvanted or non-adjuvanted influenza vaccine, and then explores the relationship between vaccine and course of disease, including duration of symptoms, total severity reported, and incidence of systemic and respiratory symptoms.

The student contribution to this study included conception, design, data cleaning and preparation, analysis, and manuscript writing. This study was jointly conceived by myself and Dr. Loeb. The protocol and analysis plan were drafted by me, with input from Drs Loeb, Verschoor and Pullenayegum. Datasets from the parent study were provided to me by Pardeep Singh, who supervised my methods for some data cleaning issues. All other cleaning and data preparation was done by me. Dr. Verschoor and Dr. Pullenayegum supported statistical analysis by providing ongoing review and supervision of statistical software computation. All members of my thesis supervisory committee provided critical review of the chapter. Co-authors will include all listed above. Dr. Loeb provided funding support for the study.

The paper is proposed to be submitted to *Vaccine* or *Clinical Infectious Diseases* in August 2022 pending revisions recommended by the thesis supervisory committee.

CHAPTER TWO. ASSOCIATION OF INFLUENZA VACCINE FORMULATION ON SYMPTOM SEVERITY OF INFLUENZA INFECTIONS IN HUTTERITE CHILDREN

INTRODUCTION

Seasonal influenza is associated with significant morbidity and mortality among vulnerable populations, including children and the elderly [1–5]. Clinical presentation with children is variable, but is commonly characterized by fever, cough, sneezing, sore throat, rhinorrhea, headache, muscle and joint pain, malaise and chills[2,6–8]. Vaccination is the recommended strategy for protecting against influenza and reducing transmission[9–11]. Despite improving formulations of influenza vaccines, such as live attenuated or adjuvanted vaccines, which increase efficacy, features of both host immunity and vaccine formulation can result in breakthrough infections [12]. Previous studies have evaluated the association between influenza vaccination and symptom attenuation in breakthrough influenza infections following vaccination [8,10,12–16]. Vaccine adjuvants, particularly MF59, have been shown to enhance immune responses in children through a variety of mechanisms, and that MF59 adjuvantation is associated with greater breadth of antibody responses[17–23]. The profile of the immune response following vaccination may influence subsequent viral clearance and presentation of infection. To our knowledge, no study has evaluated the association between adjuvanted vaccination and influenza symptom severity in breakthrough infections during childhood.

METHODS

2.1 Setting and Study Design

This study used data from a cluster-randomized controlled trial (the Adjuvanted Inactivated Vaccine Versus Inactivated Influenza Vaccine in Hutterite Children Trial (NCT02871206)). In brief, children were enrolled from Hutterite colonies in Western Canada across the 2017 to 2019 influenza seasons. The parent study objective was to evaluate the incidence of influenza infections in community contacts of influenza-vaccinated children. Colonies were randomized to receive either trivalent MF59 adjuvanted vaccine (Fluad Pediatric™) (aTIV) or quadrivalent inactivated influenza vaccine (Fluzone®) QIV). Adjuvant vaccinated children received either a 0.25 ml or 0.5 ml dose intramuscularly (for ages <36 months and ≥ 36 months, respectively), with the same dose received four weeks after the first immunization. Quadrivalent vaccinated children received two 0.5 ml doses of the vaccine, administered four weeks apart. All vaccines contained the recommended antigenic components for each influenza season, per the guidance of the World Health Organization (Supplementary Table 1). Study vaccinated children were prospectively followed up across each influenza season by public health research nurses to monitor for development of respiratory symptoms. Respiratory symptoms recorded by standardized survey included fever (≥38.0°C), sneeze, cough, rhinorrhea, muscle aches, malaise, chills, sinus problems, earaches and ear infections, sore throat and headache. Children reporting ≥2 symptoms were sampled by nasopharyngeal swab for a multiplex respiratory pathogen panel and viral genotyping.

2.2 Inclusion criteria

The parent study was conducted over influenza seasons 2016-2019. Of originally enrolled participants, we identified a cohort of vaccinated children aged 6 months to 6 years who had a PCR-confirmed influenza infection over the course of the study period. We included all first influenza infections, excluding second or third infections in children over subsequent seasons. Influenza seasons were defined by a start date following $\geq$1 cases of PCR-confirmed flu over 2 consecutive weeks, and an end date determined by absence of any laboratory-confirmed flu for 2 consecutive weeks.

2.3 Outcomes

The primary outcome for this analysis was symptom severity in children diagnosed with influenza by PCR. Symptom severity was captured in real time during the follow-up period of the study by research nurses. Symptom severity was calculated as the total of all reported symptoms, cumulative across the days of reporting (total days, not required to be consecutive). Duration was calculated as the count of all days of a participant reporting symptoms. Both severity and duration were calculated during the time frame of -28 to +28 days from the date of the PCR-confirmed positive lab result. The total number of symptoms observed during this risk window were further divided into two categories: respiratory and systemic symptoms. For our analysis, symptoms categorized as respiratory were cough, sneeze, sore throat, rhinorrhea, sinus issues, earaches, and ear infections. Systemic symptoms were classified as fever, headache, muscle aches, chills, and fatigue.

2.4 Statistical Analysis

We summarized demographic characteristics of children included in our cohort using medians (IQR: interquartile ranges) and frequencies (%), as appropriate. We compared characteristics in children between vaccine allocation groups using Wilcoxon-Mann-Whitney tests and Fisher's exact tests, as appropriate, with 95% confidence intervals. Symptom severity outcomes were assessed as continuous variables. We compared the relationship between vaccine allocation and severity outcomes using multiple generalized negative binomial models with a log-link function. Severity outcomes (symptom severity scores and duration of symptoms) were regressed onto vaccine group, age, and sex. Model coefficients were exponentiated to report the incidence rate ratios (IRR) corresponding to a change in vaccine formulation, with quadrivalent vaccine as the reference level. We conducted a sensitivity analysis excluding outlier data using the same modelling approach. Outliers were defined as observations falling outside the interval between the 5^{th} and 95^{th} percentiles. To account for potential differences in clinical presentation by infecting influenza strain, we conducted strain-specific subgroup analyses for influenza A and B. A p-value of 0.05 were considered significant for all tests. All analyses were done in the R environment, version 4.0.2.[24]

RESULTS

Our cohort spanned three influenza seasons from January 2017 to June 2019, and included 424 unique participants aged 6 months to 6 years across 39 Hutterite colonies in

Western Canada. Characteristics of the original study population have been published elsewhere [25]. There were 201 participants who received adjuvanted trivalent influenza vaccine, and 223 children who received quadrivalent inactivated influenza vaccine. Of study-vaccinated children, we identified 49 PCR-confirmed influenza infections. Three of these were repeated infections and excluded from the analysis. The demographic characteristics of 46 unique children who developed influenza in our cohort can be found in Table 1. The median age at time of infection was 47.5 months (IQR: 26.25, 64.5). Males comprised 41.3 % of infections (n = 19). Demographic characteristics of children were similar when comparing vaccine groups (Table 1). There were seven infections in season one, 37 infections in season two, and three infections in season three. Infections were dominated by H3N2 strains, with 32 cases, as compared to one infection by H1N1, one case of untyped flu A and twelve infections by B lineages. (Table 2).

Of children who received the adjuvanted trivalent seasonal influenza vaccine, 12 went on to develop a laboratory-confirmed influenza infection during our cohort follow-up period, as compared to 34 who received the quadrivalent inactivated vaccine prior to the start date of a given influenza season. In unadjusted analyses, the median duration of symptoms in adjuvanted vaccinees was 4.50 days, and was not significantly different in QIV vaccinees (unadjusted: 4.50 days, x_0: -1.00, 95% CI: -3.00, 2.00, p=0.60) (Table 3a). We did not observe a significant difference in median total symptom severity based on vaccine formulation (9.25 in aTIV group, as compared to 11.5 in QIV (x_0: -2.00, 95% CI: -9.00, 5.00, p = 0.53). Respiratory symptom severity was similar between groups (median

8.00 in both aTIV and QIV vaccinees, x_0: -2.00, 95% CI: -8.00, 2.00, p =0.37). Systemic severity was similar between groups (median 3.00 in aTIV and QIV vaccinees, x_0: -3.10, 95% CI: -3.00, 2.00, p = 0.77). (Table 3a). Among individual symptoms, median severity of fever and muscle aches differed significantly in unadjusted tests (x_0: 1.00, 95% CI: 0.00, 2.00, p = 0.03, and x_0: -0.00, 95% CI: -3.85, -0.00, p = 0.01, respectively). No other symptoms were significantly different (Table 3a).

Multivariable models showed that, after accounting for age and sex, there was no evidence of a significant difference in the expected symptom severity between aTIV and QIV vaccinated children (Table 4.) Duration of symptoms were not significantly different between groups (in aTIV vs QIV: IRR = 1.04, 95% CI: 0.67, 1.60, p=0.87). Vaccine formulation was not significantly associated with a difference in total symptom severity (in aTIV: IRR = 1.16, 95% CI: 0.71, 1.90, p=0.55), nor respiratory severity (in aTIV vs QIV: IRR = 1.40, 95% CI: 0.79, 2.55, p=0.26). Systemic severity was reduced in adjuvanted vaccinees relative to QIV vaccinees, but not significantly so (IRR = 0.69, 95% CI: 0.30, 1.58, p=0.32). Consistent with the unadjusted trends, multivariable models found a significant reduction in fever severity in the aTIV group, as compared to QIV (IRR: 0.26, 95% CI: 0.07, 0.78, p = 0.02) (Table 4, Figure 1).

When stratifying breakthrough infections attributable to influenza A, adjuvanted vaccination was not found to be significantly associated with changes in the expected duration or total number of symptoms reported (IRR: 0.68, 95% CI: 0.35, 1.33, p = 0.26;

and IRR: 0.63, 95% CI: 0.32, 1.28, p = 0.11, respectively). The vaccine formulation was not observed to significantly attenuate respiratory symptom severity (in aTIV: IRR: 0.82, 95% CI: 0.36, 1.96, p = 0.53). Systemic symptom severity of influenza A infections was significantly reduced in aTIV vaccinees (IRR: 0.16, 95% CI: 0.03, 0.64, p = 0.01, Figure 2.) (Table 5a). Fever could not be modelled, as there were no reports of fever in adjuvanted vaccinees in influenza A infections. No significant associations were observed between vaccine formulations and symptom severity outcomes in influenza B infections (Table 5b).

In our sensitivity analysis, we included 43 influenza infections, excluding 3 outliers. We did not observe any significant association between vaccine group on total or respiratory symptom severity scores, nor on symptom duration (p>0.05 for all outcomes, Table 6). Receipt of adjuvanted vaccine was significantly associated with reduced systemic and fever severity (IRR: 0.35, 95% CI: 0.14, 0.87, p = 0.02, and IRR: 0.19, 95% CI 0.04, 0.73, p=0.02, respectively) (Table 6).

DISCUSSION

Children under age five are considered to be at high risk of influenza-related complications and hospitalization, with the greatest burden in children less than two years of age[26]. Vaccination is recommended in children to reduce the burden of disease; however, breakthrough infections still put children at risk of severe illness[26]. While there is a growing body of evidence on the effect of influenza vaccination on

symptomatic presentation in adults, data on vaccine-attenuated severity in children is more limited [27]. To our knowledge, no study has evaluated the association between adjuvanted vaccination on influenza symptom severity in breakthrough infections during childhood. Our study contributes unique insight on vaccine-attenuation of symptoms in adjuvanted vaccinees relative to quadrivalent controls; however, the mechanism of action and causality remains unclear. Adjuvanted vaccination may reduce severity through enriched antibody and cytokine profiles, which may reduce symptom presentation by preventing viral replication and enhancing clearance of infected cells [28–31]. The MF59 adjuvant increases engagement of innate immunity, increasing recognition of pathogen-specific molecular patterns and recruiting non-neutralizing effector cell functions [21,30,32–35]. These immunomodulatory effects may influence the magnitude and kinetics of immune responses in subsequent influenza exposures.

Vaccine formulation was not concluded to have a significant association with total symptom severity or duration of symptoms. Our study consistently found that aTIV vaccinees experienced significantly less fever severity in comparison to QIV vaccinated children. In all infections, adjuvanted vaccination was associated with a 74-81% reduction in fever severity. There were no reports of fever in aTIV vaccinees with breakthrough influenza A infections, which prevents us from modelling statistically but would suggest that fever severity is reduced. The absence of fever is likely a contributing factor to the significantly reduced overall systemic severity in A-type infections, estimated to be reduced by 84% in adjuvanted vaccinees relative to QIV. Our sensitivity

analysis demonstrated a similar trend, where breakthrough infections of any type were estimated to experience reduced systemic severity by 65%. Overall, our work suggests that adjuvanted vaccination is associated with attenuated systemic symptom severity in children, particularly by its relationship with reduced fever.

Our findings are consistent with existing studies which observed that influenza-vaccinated children experienced significantly reduced odds of fever as compared to unvaccinated children[8,10,36]. Point estimates in these studies ranged from 0.29 to 0.54, estimating that influenza vaccination reduces severe fever by 46% - 71%. One study has examined differing influenza vaccine formulations, finding that children who received a live attenuated vaccine reported significantly fewer total and respiratory symptoms than those receiving trivalent inactivated vaccine[14]. This study also stratified by influenza infecting strain, and observed that live-attenuated vaccinees who went on to develop an A/H1N1 infection had significantly reduced symptom severity in all outcomes. The authors found that severity influenza A/H3N2 infections were similar between vaccine groups, contrasting with the findings of our study. A study of vaccine-attenuated symptom severity of A/H3N2 infections observed significantly lower respiratory and total symptom severity scores, relative to unvaccinated controls [13]. However, this work was conducted in adults, which limits comparisons as adult immune responses are more robust and shaped by previous exposures[37–39].

Advantages of our study include the randomization and blinding of the original study design, its prospective surveillance and laboratory confirmation of suspected influenza infections. A potential drawback is that children were required to report at least two symptoms before being eligible for PCR testing, which precludes the detection of very mild or asymptomatic cases of influenza. As such, we cannot make any conclusions about whether vaccine formulation may be associated with attenuation of all clinical signs and symptoms. Moreover, our secondary analysis of data prevents us from gathering further information about symptoms reported, such as measurements of temperature in reported fever, which would be useful in defining severity of influenza illness. Due to the influenza vaccination of all study participants, we have a modest sample size which limits the conclusions we may make from our results. Due to the modest number of events, we have limited statistical power for strain-specific stratified analyses, which increases uncertainty in the interpretation of our results. Finally, influenza infections in our cohort were predominantly due to A/H3N2, which limits the generalizability of our findings to A/H1N1 infections.

Our findings support the emerging evidence linking influenza vaccination with reduced severity of clinical presentation, whilst contrasting vaccine formulations [13-16]. We demonstrated trends in systemic and febrile symptom attenuation of adjuvanted influenza vaccine in predominantly influenza A/H3N2 infections, which is associated with greater hospitalization and mortality [26,40,41]. Of note, most infections occurred during the 2017-2018 season (80%). Virus isolates from this season indicated that the vaccine

antigen against A/H3N2 was mismatched to the circulating strain [42–44]. Our observation that adjuvanted vaccine is associated with reduced systemic and fever severity in a season of vaccine mismatch is of unique value. It suggests that adjuvanted vaccination may be preferable for future seasons where mismatched antigens or novel antigenic shifts with pandemic potential may incur significant influenza burden and healthcare utilization. Further work to characterize adjuvant-specific attenuation of disease would be valuable for informing immunization recommendations, particularly in populations at high risk of influenza-related complications.

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Table 1. Demographic characteristics of children with PCR-confirmed influenza (n = 46)

	All children	**aTIV (n=12)**	**QIV (n=34)**	**P**
Age (years) (median, quantiles)	3.5 (2.0, 5.0)	4.0 (2.0, 5.0)	3.0 (2.0, 5.0)	0.96
Age (months) (median, quantiles)	47.5 (26.25, 64.5)	56.5 (26.75, 67)	46.0 (26.25, 64.0)	0.92
Sex (male)	19 (41.3%)	3 (25.0%)	16 (47.1%)	0.43

Table 2. Characteristics of first influenza infections (n = 46)

	All infections	**aTIV (n=12)**	**QIV (n=34)**
Season			
First season	7	2	5
Second season	37	8	29
Third season	2	2	0
Flu type			
All flu A	34	5	29
A H3N2	32	3 (25.0%)	29 (85.3%)
A H1N1	1	1 (8.3%)	0 (0.0%)
B	12	7 (58.3%)	5 (14.7%)
Unknown	1	1	0

Table 3a). Characteristics of symptom presentation in children with laboratory-confirmed influenza (n = 46) (median, IQR) (unadjusted)

	All children	**aTIV (n = 12)**	**QIV (n = 34)**	**(95% CI of estimated location shift x_0)**	***p***
Duration of Symptoms	4.5 (5.00)	4.5 (5.00)	4.5 (5.00)	(-3.00, 2.00)	0.60
Total symptoms	10.5 (14.25)	9.5 (17.25)	11.5 (14.5)	(-9.00, 5.00)	0.53
Respiratory Symptoms	8.0 (11.25)	8.0 (16.00)	8.0 (11.00)	(-8.00, 2.00)	0.37
Systemic Symptoms	3.0 (5.50)	3.0 (7.50)	3.0 (4.00)	(-3.00, 2.00)	0.77
Individual symptoms (median, IQR)					
Fever	1.0 (2.00)	0.0 (0.25)	1.0 (2.00)	(0.00, 2.00)	**0.03**
Cough	3.0 (5.00)	4.0 (5.00)	2.0 (5.00)	(-4.00, 1.00)	0.17
Runny Nose	2.0 (5.00)	3.5 (3.50)	2.0 (5.00)	(-3.00, 1.00)	0.25
Sore Throat	0.0 (2.75)	1.5 (3.75)	0.0 (2.75)	(-2.00, 0.00)	0.39
Headache	0.0 (0.00)	0.0 (0.50)	0.0 (0.00)	(0.00, 0.00)	0.76
Sinus Problems	0.0 (0.00)	0.0 (0.75)	0.0 (0.00)	(0.00, 0.00)	0.46
Muscle Aches	0.0 (0.00)	0.0 (2.25)	0.0 (0.00)	(-0.00, -0.00)	0.01
Fatigue	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	(0.00, 0.00)	0.70
Earache	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	(0.00, 0.00)	0.48
Ear Infection (n, %)†	1 (2.2%)	1 (8.3%)	0 (0.0%)	(0.07, Inf)	0.26
Chills	0.0 (2.00)	0.0 (1.25)	0.0 (2.00)	(0.00, 1.00)	0.55
Sneeze	0.0 (1.75)	0.0 (1.25)	0.0 (1.75)	(0.00, 0.00)	0.99

†: tested by Fisher's exact test

Table 3b) Characteristics of symptom presentation in children with laboratory-confirmed influenza type A (n = 34) (median, IQR) (unadjusted)

	All children	**aTIV (n = 5)**	**QIV (n = 29)**	**(95% CI of estimated location shift x_0)**	***p***
Duration of Symptoms	4.0 (5.00)	3.0 (3.00)	4.0 (5.00)	(-2.00, 5.00)	0.64
Total symptoms	9.5 (14.50)	8.0 (1.00)	10.0 (15.00)	(-5.00, 13.00)	0.64
Respiratory Symptoms	7.5 (10.75)	8.0 (5.00)	7.0 (11.00)	(-7.00, 9.00)	0.92
Systemic Symptoms	2.0 (3.75)	0.0 (0.00)	2.0 (4.00)	(0.00, 5.00)	**0.03**
Individual symptoms (median, IQR)					
Fever	0.0 (2.00)	0.0 (0.00)	1.0 (2.00)	(0.00, 2.00)	**0.04**
Cough	2.0 (4.75)	3.0 (3.00)	2.0 (5.00)	(-3.00, 2.00)	0.77
Runny Nose	2.0 (5.75)	3.0 (3.00)	2.0 (7.00)	(-3.00, 3.00)	0.88
Sore Throat	0.0 (2.00)	0.0 (1.00)	0.0 (2.00)	(-1.00, 2.00)	0.62
Headache	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	(0.00, 0.00)	0.87
Sinus Problems	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	(0.00, 0.00)	0.29
Muscle Aches	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	(0.00, 0.00)	0.59
Fatigue	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	(0.00, 0.00)	0.97
Earache	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	(0.00, 0.00)	0.19
Ear Infection (n, %)	1 (2.9%)	1 (20.0%)	0 (0.0%)	(0.15, Inf)	0.15
Chills	0.0 (1.00)	0.0 (0.00)	0.0 (2.00)	(0.00, 2.00)	0.09
Sneeze	0.0 (1.00)	0.0 (1.00)	0.0 (1.00)	(0.00, 0.00)	0.74

†: *tested by Fisher's exact test*

Table 3c) Characteristics of symptom presentation in children with laboratory-confirmed influenza type B (n = 12) (median, IQR) (unadjusted)

	All children	**aTIV (n =7)**	**QIV (n = 5)**	**P**
Duration of Symptoms	5.5 (5.25)	6.0 (5.00)	5.0 (3.00)	0.67
Total symptoms	13.5 (17.25)	15.0 (36.50)	12.0 (5.00)	0.62
Respiratory Symptoms	8.5 (12.50)	8.0 (24.50)	9.0 (12.00)	0.51
Systemic Symptoms	5.5 (6.25)	7.0 (10.00)	4.0 (4.00)	0.62
Individual symptoms (median, IQR)				
Fever	1.5 (2.00)	0.0 (1.50)	2.0 (1.00)	**0.03**
Cough	4.5 (6.75)	4.0 (5.50)	5.0 (6.00)	0.87
Runny Nose	2.0 (3.25)	4.0 (4.50)	1.0 (2.00)	0.08
Sore Throat	2.0 (6.75)	2.0 (8.00)	2.0 (3.00)	0.50
Headache	0.0 (0.75)	0.0 (4.00)	0.0 (0.00)	0.67
Sinus Problems	0.0 (0.75)	0.0 (6.00)	0.0 (0.00)	0.13
Muscle Aches	0.0 (2.25)	2.0 (6.00)	0.0 (0.00)	0.07
Fatigue	0.0 (2.25)	0.0 (0.00)	0.0 (1.00)	0.52
Earache	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	-
Ear Infection (n, %)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	-
Chills	1.5 (2.25)	1.0 (2.00)	2.0 (3.00)	0.73
Sneeze	0.0 (2.00)	0.0 (1.00)	2.0 (2.00)	0.52

Table 4. Multivariable generalized linear regression model output for PCR-confirmed influenza infections among children from 2017 to 2019, adjusting for age and sex (n=46)

Predictors	**Incidence Rate Ratio (95% CI), p-value**
	Vaccine [aTIV]
Duration of symptoms	1.04 (0.67, 1.60), 0.87
Total symptoms reported	1.16 (0.71, 1.90), 0.55
Number of respiratory symptoms	1.40 (0.79, 2.55), 0.26
Number of systemic symptoms	0.69 (0.30, 1.58), 0.324
Fever	0.26 (0.07, 0.78), **0.022**
Notes	
1. Incidence rate ratio is the exponentiated coefficient from the negative binomial regression model, and corresponds to the expected ratio of symptom score per one-unit increase in the exposure (where vaccine = 0 for QIV, and = 1 for aTIV, and all other predictors are held constant)	

Table 5a) Stratified regression model outputs for influenza A infection symptom severity outcomes, adjusting for age and sex (n=34)

Predictors	**Incidence Rate Ratio (95% CI), p-value**
	Vaccine [aTIV]
Duration of symptoms	0.68 (0.35, 1.33), 0.26
Total symptoms reported	0.63 (0.32, 1.28), 0.11
Number of respiratory symptoms	0.82 (0.36, 1.96), 0.53
Number of systemic symptoms	0.16 (0.03, 0.64), **0.012**
Fever	0.00 (0.00, Inf)
Notes	
1. Incidence rate ratio is the exponentiated coefficient from the negative binomial regression model, and corresponds to the expected ratio of symptom score per one-unit increase in the exposure (where vaccine = 0 for QIV, and = 1 for aTIV, and all other predictors are held constant)	

Table 5b) Stratified regression model outputs for influenza B infection symptom severity outcomes, adjusted for age (n=12)

Predictors	**Incidence Rate Ratio (95% CI), p-value**
	Vaccine [aTIV]
Duration of symptoms	1.71
Total symptoms reported	1.71
Number of respiratory symptoms	1.88
Number of systemic symptoms	1.76
Fever	0.42
Notes 1. Incidence rate ratio is the exponentiated coefficient from the negative binomial regression model, and corresponds to the expected ratio of symptom score per one-unit increase in the exposure (where vaccine = 0 for QIV, and = 1 for aTIV, and all other predictors are held constant) 2. 95% confidence intervals and p-values are not shown here, due to the small sample size	

Table 6. Sensitivity analysis of regression model outputs for all PCR-confirmed influenza infections, excluding outliers and adjusting for age and sex (n=43)

Predictors	**Incidence Rate Ratio (95% CI), p-value**
	Vaccine [aTIV]
Duration of symptoms	0.73 (0.44, 1.20), 0.226
Total symptoms reported	0.72 (0.44, 1.17), 0.184
Number of respiratory symptoms	0.91 (0.50, 1.72), 0.764
Number of systemic symptoms	0.35 (0.14, 0.87), **0.017**
Fever	0.19 (0.04, 0.73), **0.021**
Notes 1. Incidence rate ratio is the exponentiated coefficient from the negative binomial regression model, and corresponds to the expected ratio of symptom score per one-unit increase in the exposure (where vaccine = 0 for QIV, and = 1 for aTIV, and all other predictors are held constant)	

Figure 1. Predicted fever severity, by vaccine allocation. *This figure shows the real data points of observations and the predicted fever severity (smoothed line) for each vaccine group, in all influenza infections.*

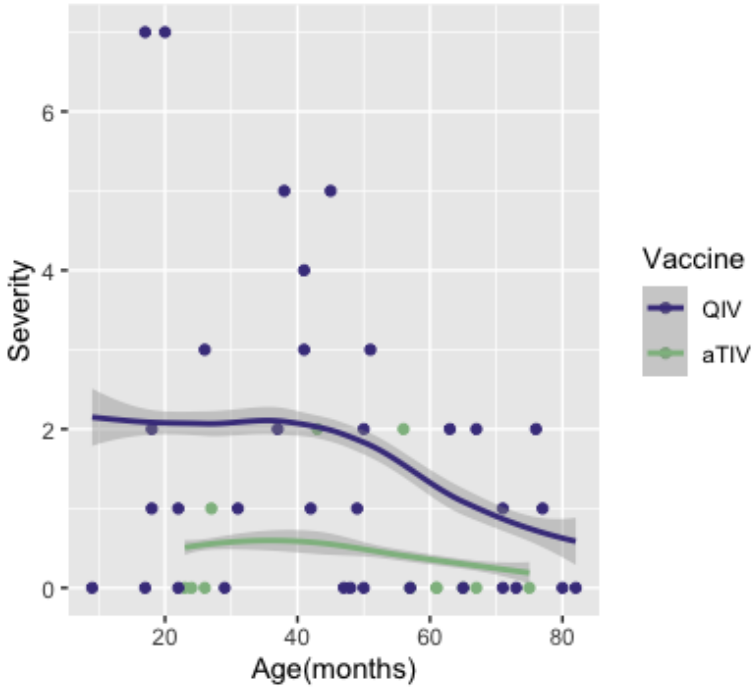
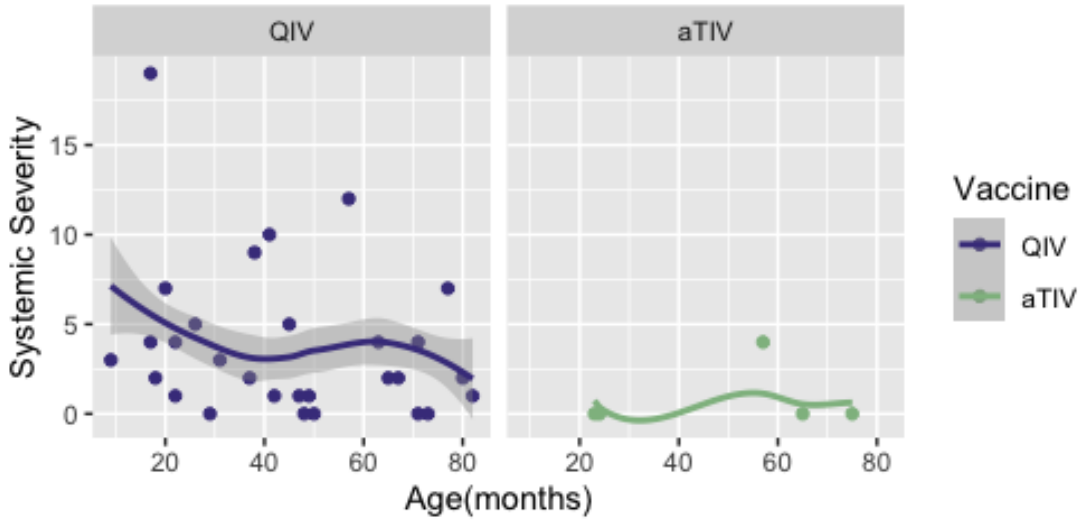


Figure 2. Predicted systemic symptom severity in influenza A type infections, by vaccine. *This figure shows the real data points of observations and the predicted systemic severity (smoothed line) for each vaccine group.*



Supplementary Table 1. Vaccine antigen components used in the study vaccines and hemagglutination inhibition assays for vaccinated children

Year	A/H1N1	A/H3N2	B/Victoria	B/Yamagata
2016-2017	A/California/7/2009	A/Hong Kong/4801/2014	B/Brisbane/60/2008	B/Phuket/3073/2013
2017-2018	A/Michigan/45/2015	A/Hong Kong/4801/2014	B/Brisbane/60/2008	B/Phuket/3073/2013
2018-2019	A/Michigan/45/2015	A/Singapore/INFIMH-16-0019/2016	B/Colorado/06/2017	B/Phuket/3073/2013

PREFACE TO CHAPTER THREE.

In this chapter, we assessed reactogenicity as a predictor of vaccine immunogenicity using mixed linear modelling. We explored whether the experience of any reaction, as a binary predictor, was significantly associated with changes in post-vaccination HAI titers. We further examined whether increasing reactogenicity severity was correlated with subsequent increases in HAI titers. We examined interactions between vaccine formulations and reported reactions, investigating reactogenicity as a correlate of immunogenicity in the presence of the moderating effect of adjuvantation.

The student contribution to this study included conception, design, data cleaning and preparation, analysis, and manuscript writing. The study was conceived by myself, with input from Dr. Loeb and support from Dr. Matthew S Miller. Co-authors include Drs. Loeb, Verschoor and Pullenayegum. All members of the thesis supervisory committee provided critical review of the research protocol, analysis plan, and draft manuscript. Raw datasets were provided to me by Pardeep Singh, who will be named as a co-author. Drs Verschoor and Pullenayegum provided statistical modelling support. Laboratory serology for HAI titers was done by Dr. Brian Ward's research group at the McGill Center for Viral Diseases; however, no additional testing was required for this study. Dr. Verschoor aided in data interpretation. The paper is proposed to be submitted to *Vaccine, The Lancet,* or *The Journal of the American Medical Association* in August 2022 pending revisions recommended by the thesis supervisory committee.

CHAPTER THREE. REACTOGENICITY AS A PREDICTOR OF HAI IMMUNOGENICITY IN VACCINATED CHILDREN

BACKGROUND

Vaccine reactogenicity encompasses the range of physical presentations of innate inflammatory responses to vaccine administration. Reactions are important information which inform the benefit-risk profile of the vaccine for licensure. Moreover, they may be clinical presentations of inflammatory immunity, which is associated with the adaptive immune response[1]. Reactions may include pain, redness, swelling or erythema at the injection site; and systemic symptoms, such as fever, headache, chills, muscle aches, and disseminated rash[2]. Most reactions reported occur within days of vaccination; and are mild, self-limiting and require minimal medical care[3].

Seasonal influenza vaccines activate the immune response via different pathways[4]. Adjuvanted influenza vaccines act via various mechanisms to enhance the immune response, such as inducing chemokines which attract myeloid cells, activating antigen-presenting cells, and upregulating T cell activities [5–8]. By improving antigen-presentation, the MF59 adjuvant enhances the activation of B and T lymphocytes, which facilitate antibody development [5–9]. Studies have shown that influenza vaccination induces host inflammatory responses, which are thought to be mediators of vaccine reactions[10,11]. Vaccination has been associated with increased local and systemic serum levels of pyrogenic cytokines (including tumor necrosis factors and interleukins), histamines, and C-reactive proteins[10–13]. Adjuvanted vaccines in particular have been

shown to induce inflammatory immune activity which may elicit stronger host reactions [10,13,14]. Given the relationship to innate immunity, vaccine reactions may be predictive of subsequent adaptive immune responses.

Despite the pathways by which host immune mechanisms may contribute to vaccine reactions, studies which link reactogenicity with the magnitude of the immune response are limited. Studies have examined transcriptomic profiles and candidate genes as biomarkers of influenza vaccine reactions in mice [10,11,15]. One study of children vaccinated with pandemic influenza vaccines found that immune response was increased in children who experienced post-vaccination fever, and that more reactions were observed in children vaccinated with the adjuvanted formulation [16]. A study of adults immunized against hepatitis B with various adjuvant formulations evaluated innate inflammatory markers (including interleukin-6, C-reactive protein, and interferon levels), reported reactions, and the magnitutde of adaptive immune responses 44 days post-vaccination [13]. The authors found that the profiles of innate immune responses were similar across different adjuvant doses, and that increases in inflammatory markers correlated with greater antibody responses post-vaccination.

Further studies have correlated vaccine reactogenicity with enriched humoural and T-cell mediated immune responses in recipients of mRNA vaccines against SARS-Cov-2[17–19], finding weak or inconsistent results. One study in older adults receiving adjuvanted recombinant zoster vaccine found a small but statistically significant correlation between

reactogenicity scores and gE-specific CD4+ T-cell mediated immune responses [20]. Interestingly, this study used IgE antibodies as the endpoint for assessing humoural immunity in response to the vaccine, which are of limited value—while these antibodies correlate with hypersensitivity reactions in the host, they are not considered an endpoint for vaccine efficacy[21,22]. The study found that reactogenicity score was significantly associated with increased antibody responses but did not significantly influence CD4+ T-cell responses.

The most common method to evaluate the immunogenicity of influenza vaccines is the hemagglutination inhibition assay (HAI)[22]. HAI titers are associated with induction of the humoral immune response, primarily via antibody production and its coinciding processes[23,24]. The literature which correlates reactogenicity with HAI seroconversion is limited. Studies have examined the impact of adjuvantation on vaccine reactions, and the potential link between reactions and the adaptive immune responses [6,7,25,26]. However, limited data is available examining influenza vaccine reactions as a predictor for immunogenicity in children, particularly after adjusting for the adjuvant effect. We sought to examine influenza vaccine reactions as a predictor of immunogenicity, accounting for differences in vaccine formulation, in children aged six months to six years. Our study would have pragmatic implications for the prediction of adequate immune responses to vaccination in children. Influenza vaccine efficacy is known to vary across seasons, formulations, demographics and types/ subtypes [27–29]. Symptomatic presentations, such as reactogenicity, which correlate strongly with vaccine response

would inform a useful clinical prediction rule for forecasting vaccine efficacy, potentially identifying individuals at risk of vaccine failure, and focusing efforts for booster doses or revaccination campaigns.

METHODS

2.1 Setting and study design

Our study was a cohort, which made use of serum samples from a cluster randomized controlled trial (the Adjuvanted Inactivated Vaccine Versus Inactivated Influenza Vaccine in Hutterite Children Trial (NCT02871206)[30]. In brief, children aged 6 months to 72 months were enrolled from Canadian Hutterite colonies from January 2017 to June 2019. Children within randomized colonies were vaccinated with either trivalent MF59 adjuvanted vaccine (Fluad Pediatric™) (aTIV) or quadrivalent inactivated influenza vaccine (Fluzone®) (QIV), per the immunization recommended guidelines [31–33]. Adjuvant vaccinated children received either a 0.25 ml or 0.5 ml dose intramuscularly (for ages <36 months and ≥ 36 months, respectively), with the same dose received four weeks after the first immunization. Quadrivalent vaccinated children received two 0.5 ml doses of the vaccine, administered four weeks apart. All vaccines contained the recommended antigenic components for each influenza season, per the guidance of the World Health Organization (Supplementary Table 1). Following the administration of vaccines, participants were observed for the onset of any reaction symptoms within 15 minutes following vaccine administration and were followed up for 5 days thereafter for

other symptom onset. We included study-vaccinated children for whom there was both pre- and post-vaccination data on serum hemagglutination inhibition assay results.

2.2 Vaccine reactions

Signs and symptoms of reactions in this study included: pain / swelling / redness at injection site, loss of movement, sore throat, runny nose, headache, myalgia, chills, nausea / vomiting / diarrhea, fever, conjunctivitis, shortness of breath, loss of appetite, fainting, and others. We generated vaccine reactogenicity scores based on the sum of reactions reported within the 5-day risk window. Subgroup scores were summed from the total of systemic, local, and respiratory reaction scores. Systemic symptoms included in the score were headaches, appetite loss, muscle aches, chills, nausea, vomiting, diarrhea, rash, and fever. Local injection site reactions were the sum of limb pain, redness, swelling and loss of movement reported. Respiratory scores were the sum of conjunctivitis, shortness of breath, sore throat and runny nose [34–36]. Reactions categorized as "Other" were included in total reactogenicity scores, but no subgroup scoring component, due to the limited data available to characterize the reaction.

2.3 Laboratory Testing

Serum samples were collected at baseline from study vaccinated children, and four weeks post-vaccination. Sera were assessed by hemagglutination inhibition assay per the standard protocol[24]. In brief, serum from each patient was serially diluted in two-fold dilutions across ten wells and incubated with erythrocytes and a stock titer of virus of a

known concentration. Virus antigens used in this study were the four components of the quadrivalent influenza vaccine for each study year (Supplementary Table 1). The reciprocal of the lowest serial dilution at which hemagglutination inhibition was observed was determined to be the HAI titer for that serum sample. Titers which were undetectable by the assay were imputed as 1:5. Titers were log-transformed for analysis.

2.4 Statistical Analysis Plan

The primary outcome for this analysis was vaccine immunogenicity, measured by the post-vaccination HAI titer, against each vaccine antigen. The log-transformed HAI titers against each vaccine antigen was assessed as a continuous outcome, and geometric mean titers were reported with geometric standard deviation factors (GSD) and 95% confidence intervals [37]. We tested for differences in the mean post-vaccination titers to each vaccine antigen in children who experienced a reaction, compared to those who did not, using Welch's t-test, assuming unequal variances. We modelled the experience of any reaction, as a categorical predictor, using linear mixed models regressing log-transformed post-vaccination titers onto baseline titers, reaction, vaccine, and age. We included random intercepts for each participant and for season. The association between vaccine reactogenicity and immunogenicity was assessed using linear mixed models regressing log-transformed HAI titers onto reactogenicity score, baseline titers, vaccine, and age. Since the MF59 adjuvanted vaccine has been shown to have a higher incidence of reported reactions, as well as enhancing immunogenicity, we expected to see effect

modification by the vaccine group[38,39]. We included an interaction term for the relationship between vaccine group and reactogenicity predictors in the models.

For associations which were found to be significant, we examined the assumptions of linearity in the model using plots to assess the independence of observations; linear relationships between variables; and equal variance of residuals in the predictors. For significant associations which were found to be non-linear, we adjusted the model to better reflect the relationship using non-linear regression and factored predictors fit using locally weighted smoothing regression. Reactogenicity scores were converted to factored categorical variables for these models, which followed all other methods of the primary modelling analysis.

We estimated that we had 80.3% power (alpha = 0.05) to detect a mean difference of 0.25 standard deviations (small to medium effect) in the means of post-vaccination titers between children who experienced reactions, as compared to those who did not, and 99% powered to detect a difference of 0.5 standard deviations in the means of post-vaccination titers between children. A p-value of 0.05 was considered significant for all tests. All analyses were done in the R environment, version 4.0.2.[40]

RESULTS

The original trial vaccinated 424 unique children across three influenza seasons, for a total of 994 observations[30]. Data on HAI titers were available for 542 pairs of serum

samples from 330 unique children over three study seasons. In serum samples tested by HAI assays, there was no missing data—participants had both pre- and post-vaccination titers completed. There were 176 observations available in season one, 203 from season two, and 163 from season three. The characteristics of the study cohort can be found in Table 1. The mean age of children was 54.4 months (SD: 17.9), or approximately 4.5 years. Male sex accounted for 52.8% of the observations (n=286). Adjuvant-vaccinated children comprised 44.1% of observations (n=239). Of 542 instances of study-vaccinated children, 203 experienced a reaction to the vaccine (37.5%). The mean total reactogenicity score was 2.78 (SD: 2.09). Mean subgroup scores for local, systemic and respiratory reactogenicity were 1.09 (SD: 0.98), 1.46 (SD: 1.59) and 0.16 (SD: 0.40), respectively. Among reactions experienced, the most frequent reported was limb pain (n = 130, 64%), followed by fever (n=66, 32.5%), muscle ache (n=55, 27.1%), and chills (n=47, 23.3%). The frequencies and proportions of events are summarized in Table 1 and shown in Figure 1. The pre- and post-vaccination titers against vaccine antigens are shown in Figure 2. The geometric mean post-vaccination titers, pooled across seasons, were 211.72 (GSD: 5.53) against A/H1N1, 259.12 (GSD: 4.46) against A/H3N2, 111.99 (GSD: 4.56) against B/Victoria; and 148.37 (GSD: 4.84) against B/Yamagata. Post-vaccination titers were not significantly different between children who experienced a reaction as compared to those that did not (Figure 3), except for A/H1N1 (geometric mean: 368.1 (GSD: 5.1), versus 247.2 (GSD: 5.9) in non-reactive children, $p<0.001$).

3.1 Any vaccine reaction and immunogenicity

We modelled the association between any reaction to vaccination and immunogenicity, adjusting for relevant covariates (Table 2). Post-vaccination titers against influenza A types were not significantly different in children who experienced a reaction as compared to those who did not (A/H1N1: β: 0.09, 95% CI: -0.26, 0.44; A/H3N2: β: -0.06, 95% CI: -0.31, 0.19, where betas represent the log change in post-vaccination titers when the reaction variable is increased from zero to one). Post-vaccination titers against influenza B types were found to be lower in children who experienced reactions, with B/Victoria responses being significantly diminished in children with reactions as compared to non-reactive children (B/Victoria: β: -0.48, 95% CI: -0.80, -0.16, p = 0.004; B/Yamagata: β: -0.23, 95% CI: -0.54, 0.08). No significant interactions were observed between the categorical experience of a reaction and the vaccine formulation which affected immunogenicity against any antigen.

3.2 Reactogenicity scores

We tested the association between total, systemic, local and respiratory reactogenicity scores and post-vaccination HAI titers, adjusting for relevant covariates (Table 2). We found that total reactogenicity was significantly associated with changes in post-vaccination titers against B/Victoria, after adjustment. Immunogenicity was significantly reduced with increasing total reactogenicity scores (β: -0.19, 95% CI: -0.29, -0.08, p = 0.001); however, adjuvanted vaccination moderated this effect, and the coefficient of total reactogenicity was significantly increased in this group (β: 0.22, 95% CI: 0.09, 0.35, p = 0.001, where β represents the difference in the coefficient in the aTIV group relative to

QIV controls) (Figure 4A). Similarly, systemic reactogenicity was significantly associated with reduced post-vaccination titers against A/H1N1(β: -0.36, 95% CI: -0.54, -0.18, p = <0.001). The interaction between systemic reactogenicity and adjuvanted vaccination was shown to moderate immunogenicity, with a significant difference in the coefficient of systemic reactogenicity, relative to controls, in models predicting post-vaccination titers against A/H1N1 and B/Victoria (β: 0.38, 95% CI: 0.17, 0.60, p = <0.001; and β: 0.44, 95% CI: 0.24, 0.64, p = <0.001, respectively, where β represents the difference in the coefficient in the aTIV group relative to QIV controls) (Figures 4B and 4C). Local reactogenicity was associated with significant increases in antibody titers against A/H1N1 (β: 0.25, 95% CI: 0.04, 0.47, p = 0.02), but no significant interaction was observed between local reactions and vaccine formulation. Respiratory reactogenicity was negatively associated with immunogenicity against A/H3N2 (β: -0.65, 95% CI: -1.17, -0.13, p = 0.02). The interaction between adjuvanted vaccination and respiratory reactions was significant in models predicting immunogenicity against A/H3N2 (β: 0.78, 95% CI: 0.13, 1.43, p = 0.02, where β represents the difference in the coefficient in the aTIV group relative to QIV controls) (Figure 4D). No other associations between total or subgroup reactogenicity scores and vaccine immunogenicity were observed.

When assessing nonlinear models, we observed results consistent with the findings of the primary linear analysis (above). We found that while total reactogenicity $\geq$2 was associated with significantly reduced immunogenicity against B/Victoria, the interaction between adjuvanted vaccination and total reactogenicity range was associated with

improvements in B/Victoria antibody responses, relative to QIV vaccinees (Table 3, Figure 5A). Total reactogenicity of 2-4 or 4+ were associated with reduced B/Victoria titers (β: -0.56, 95% CI: -1.06 – -0.06; and β: -0.87, 95% CI: -1.53 – -0.21, respectively, $p<0.05$ for both outcomes). In adjuvanted vaccinees, the coefficient of total reactogenicity scores $\geq$4 was significantly increased in models predicting B/Victoria titers, as compared to controls (β: 0.94, 95% CI: 0.15 – 1.73, $p = 0.02$, where β represents the difference in the coefficient in the aTIV group relative to QIV). When assessing systematic scores as non-linear predictors of immunogenicity, these effects were amplified: for A/H1N1 immunogenicity, children with systemic scores $\geq$ 2 mounted significantly lower titers relative to children with lower systemic scores, (β: -1.12, 95% CI: -1.73, -0.52; as compared to β: 0.56, 95% CI: -0.13, 1.24 in children with a score of 1). In aTIV vaccinated children relative to QIV controls, the coefficient of systemic reactogenicity range $\geq$2 was significantly increased in models predicting titers against A/H1N1 (β: 1.26, 95% CI: 0.54, 1.99, where β represents the difference in the coefficient in the aTIV group relative to QIV) (Table 4, Figure 5B). The same trend persisted in titers against B/Victoria. Post-vaccination titers in children with scores $\geq$ 2 were significantly diminished (β: -1.38, 95% CI: -1.94, -0.82; as compared to β: 0.09, 95% CI: -0.74, 0.55 in children with a score of 1). In adjuvanted vaccinees relative to QIV controls, the coefficient of a systemic reactogenicity range $\geq$2 was significantly increased (β: 1.58, 95% CI: 0.91, 2.25, where β represents the difference in the coefficient in the aTIV group relative to QIV controls) (Table 4, Figure 5C).

The nonlinear relationship between respiratory reactogenicity and post-vaccination titers was significant and moderated by the effect of adjuvanted vaccination (Table 5). We observed that any respiratory reactogenicity ≥ 1 was associated with reduced titers against A/H3N2 (β: -0.66, 95% CI: -1.26, -0.07), whereas the coefficient of respiratory reactogenicity in aTIV vaccinees was significantly increased, relative to QIV controls, in the model predicting A/H3N2 titers (β: 0.85, 95% CI: 0.11, 1.59, where β represents the difference in the coefficient in the aTIV group relative to QIV) (Table 5, Figure 5D).

DISCUSSION

Current guidance for assessing influenza vaccine immunogenicity relies on serological correlates of protection, namely the four-fold seroconversion of HAI titers [41]. New computational vaccinology approaches have made use of epitope mapping, immunoinformatics and systems biology models to identify molecular and cellular predictors of influenza vaccine immunogenicity[42–44]. Other recent studies have evaluated the association between vaccine reactions and humoural immunogenicity [17,19,20,45]. Ours is the first study to evaluate reactogenicity as a predictor of the immune response to influenza vaccination, taking into consideration the effect of adjuvantation. As adjuvants are known to increase reactions, it is important to investigate whether the reactions experienced are a by-product of immunostimulation, or whether they might be predictors of adjuvant-enhanced immunogenicity. Further work to identify adequate vaccine responses from clinical presentation of reactions would have practical implications for evaluating novel vaccine candidate efficacy, identifying individuals at

greater risk of vaccine failure, and targeting populations for booster doses or enhanced safety surveillance monitoring.

Our study evaluated the correlation between reactions and immunogenicity in influenza-vaccinated children. We found that vaccine reactions were significantly associated with decreased immune responses to the B/Victoria strain. The experience of any respiratory reaction was associated with reduced titers against A/H3N2; but was significantly moderated by vaccine group (Figure 5D). We hypothesize that this may be attributable to previous immune histories in our cohort, and adjuvant-moderated boosting of memory responses. Literature has shown that the initial exposure to influenza shapes subsequent antibody responses, becoming biased towards immunological memory antibody proliferation [46–48]. Studies of memory responses have demonstrated that influenza-specific cytotoxic and helper T cells and tissue resident B cells are rapidly mobilized in the lungs and airways upon re-exposure [49–52]. Proinflammatory cytokines secreted by effector cells during this rapid proliferation may contribute to the respiratory inflammation and reactions observed [53–56]. Given the age distribution of our cohort, it is plausible that the primary exposure of many participants may have been to A/H3N2, as it was the predominant strain in recent prior seasons driving influenza epidemics in Canada [57]. We propose that preferential memory immune responses, enhanced by the adjuvant effect, may have contributed to the association between increasing respiratory reactions and immunogenicity against A/H3N2.

In contrast, the interaction between vaccine and increasing reactogenicity scores was consistently significant in immunogenicity against B/Victoria. In QIV vaccinees, increasing total and systemic reactogenicity were associated with significantly poorer immunogenicity relative to adjuvanted vaccinees with comparable reaction scores. Systemic reactogenicity in this group was also predictive of diminished titers against A/H1N1. Of note, the interaction between adjuvanted vaccine and systemic reactogenicity of > 2 was associated with significantly increased titers against A/H1N1, but significantly diminished titers in quadrivalent vaccinated children within the same systemic reactogenicity range (Figure 5B). This might suggest that systemic reactions (in the absence of adjuvant) may be indicative of immune interference, whereby proinflammatory cytokines may be diverting immune activity and blunting the adaptive response.

It has been shown that the innate immune response elicits proinflammatory mediators which contribute to the engagement of the adaptive immune response[58–61]. These same cytokines have been implicated in the immunopathology of inflammatory reactions [59,62]. Systemic reactogenicity may support the inflammatory immunomodulation of adjuvanted vaccines through increased cytokine activity [14,63]. MF59 has been shown to upregulate the innate immune response, characterized by the increased expression of cytokine levels, which may contribute to systemic proinflammatory responses [7,10,63]. Studies have shown that MF59 increases the magnitude and duration of antibody responses in children [7,64]. One study evaluated the influence of MF59 on the cytokine

profiles of vaccine-specific CD4+ T helper cells [7]. This study found the cytokine expression levels to be comparable in aTIV vaccinees as compared to trivalent vaccinated children, with predominantly IL-2 and TNF-α secreted. Interleukin-2 has been shown to influence T cell differentiation and fate-determination via an inflammatory signalling pathway [15,65]. The complex interactions between IL-2 signals and inflammation regulate the development of both effector and memory cells [15,65]. Similarly, TNF-α has been well-established as both an immune mediator and an endogenous pyrogen, capable of inducing systemic inflammatory reactions [66–70]. In sum, the literature presents possible pathways by which systemic inflammation and adaptive immunity might interact, supporting the findings observed in our study. The cross-talk between proinflammatory mediators of innate immunity and subsequent adaptive responses may explain why moderate improvements were observed in influenza A/H1N1 and B/Victoria titers for adjuvanted children, but significant negative associations in non-adjuvanted vaccinees. Further work to characterize systemic reactogenicity as a potential biomarker of post-vaccination responses is necessary.

An alternate hypothesis may be that systemic reactogenicity, driven by the inflammatory immunomodulation of cytokines discussed above, may in fact be a correlate of vaccine adjuvanticity. In adjuvanted children, we observed significant associations between systemic scores greater than two in terms of post-vaccination responses to A/H1N1 and B/Victoria. Vaccine immunogenicity and efficacy against influenza A/H3N2 is known to be more modest [29,71–73], and as adjuvanted children did not receive a B/Yamagata

antigen, it is not surprising that these titers were relatively unchanged in our analysis. In more immunogenic vaccine antigens, the adjuvant may induce systemic reactions as a by-product of the stimulation of innate immunity[7,63]. A transcriptomic study of immune responses in MF59-adjuvant influenza vaccinated children demonstrated that the rapid increase in cytokine expression following a first vaccine dose correlated with enhanced antibody responses following the second dose [42]. By contrast, non-adjuvanted vaccinees demonstrated slower and less robust inflammatory engagement of innate immunity, which was associated with weaker post-vaccination immunogenicity. This would suggest that early and dramatic facilitation of innate immune responses are a necessary step for induction of greater adaptive immunity.

Across the influenza seasons of our study, A/H3N2 and B/Yamagata lineage were the predominant circulating strains[74–76]. It is possible that systemic reactions may have been induced by the engagement of proinflammatory immunomodulators in adjuvanted vaccinees, as proposed above. However, it is also possible that immune responses against influenza B in QIV vaccinees were biased by the circulating predominant lineage. Both influenza B lineages have co-circulated globally since 2001, prompting the rationale for a quadrivalent vaccine[77,78]. While the Yamagata lineage diversifies more slowly than B/Victoria, it has evolved two antigenically distinct clades [79,80]. In 2016, clade 3 of Yamagata (containing B/Wisconsin/2/2010 and P/Phuket/3073/2013-like viruses) emerged as the predominant Yamagata lineage[79,80]. For QIV-vaccinated children, it is entirely possible that the study vaccine was their first exposure to B/Phuket-like viruses

of the Yamagata lineage. Studies have shown that primary immune responses to influenza antigens are greater than subsequent exposures[46,48,81]. As such, it may be that upon encountering the novel B/Phuket antigen, QIV vaccinees mounted robust responses to the Yamagata lineage, at the expense of immune resources being allocated to the B/Victoria strain.

Our study is subject to some limitations, particularly in the measurement and evaluation of vaccine reactions. Reactions were assessed within a 15-minute window with follow-up relying on self-reporting, which is subject to recall bias. Self-reporting undertaken by parents on behalf of a child may not fully characterize symptoms experienced, particularly in younger children. The standardized questionnaire of symptoms used to assess reactogenicity is limited in that it was not able to comprehensively capture reaction severity during the risk window. Severity data would have contributed to a more fulsome understanding of the relationship between reactogenicity and immunogenicity outcomes; as our method of capturing reaction severity was based on the cumulative sum of events reported, rather than clinical characteristics. We investigated variables under the assumptions of linearity, and examined non-linear models only in total and systemic reactogenicity outcomes as these models had significant signals. While we conducted multiple exploratory analyses, we did not adjust for multiple testing. Our study evaluates immunogenicity by post-vaccination HAI titers only, which are known to be only a subset of the immune response to influenza. Measures of cell mediated immunity may be more appropriate for assessing adaptive immunity against influenza, and would provide a more

complete picture of the adaptive immune response if analyzed alongside the conventional HAI titers. Finally, our study cohort includes many serially vaccinated children, or children who may have been vaccinated in prior seasons due to previous study enrollment of the Hutterite communities. Repeated influenza vaccinations have been shown to influence both vaccine efficacy and immunogenicity[82–85]. Studies have shown blunting of antibody responses in serial vaccinees, particularly against A/H3N2 [57,72]. As such, evaluating immunogenicity in our study cohort may be impacted by both repeated vaccinations during the study period, and prior season vaccinations. This could potentially reduce the observed vaccine immunogenicity and dilute the strength of our observed effects.

Our study found two signals warranting further research. The first is the potential use of systemic reactogenicity as a correlate of adjuvanticity in seasonal influenza vaccines. Further work to characterize the association between systemic reactions and adjuvant-mediated immunogenicity would be a powerful contribution to research on influenza vaccines, as it would drive improved vaccine benefit-risk assessments and regulatory determinations in the assessment of vaccine safety. It would also enhance vaccine acceptance and risk tolerance for systemic reactions, were they shown to be conclusively associated with improved vaccine immunogenicity and efficacy outcomes. Finally, the exact mechanism of action by which the MF59 adjuvant enhances immunogenicity remains unclear, and studies linking systemic inflammatory immunomodulation with the adjuvant effect would be meaningful for further design of novel vaccine adjuvants.

The second signal of interest which we found in our study was the potential for inflammatory immune interference in non-adjuvant vaccinated children. Greater systemic reactions in this group were associated with poor immunogenicity against the non-dominant antigens, A/H1N1 and B/Victoria. This finding may suggest that systemic inflammatory reactions may blunt immune responses in non-adjuvanted children, potentially inhibiting antibody boosting against historical exposure antigens. Further research to correlate systemic reactogenicity with blunting would be particularly important to conduct in previously unvaccinated children, as diminished immunogenicity could be concluded to be unrelated to repeat vaccination effects. Systemic reactogenicity as a biomarker of poor immunogenicity in non-adjuvanted children would be useful for identifying target populations for a booster dose of vaccine. It would be a meaningful prognostic indicator of vaccine failures, burden of influenza infections and subsequent health economic analyses.

Whether systemic reactogenicity serves as a correlate of adjuvanticity, or a biomarker of vaccine blunting in non-adjuvanted vaccinees, remains unclear. Our study identifies a significant interaction effect between systemic reactions, vaccine formulation and adaptive immunity to influenza vaccination. We present some hypotheses on the potential pathways by which inflammatory immunomodulation may either enhance or inhibit immune responses. Further work to examine this association would contribute to

enhanced understanding of host-pathogen-adjuvant interactions which shape vaccine immunogenicity, efficacy, and future design.

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Table 1. Characteristics of children with paired serum samples included in the study cohort (*n* = 542 observations) (*n*, %, except where otherwise indicated)

Demographics		
Age (months) (mean, SD)	54.4	17.9
Sex (Male = 1)	286	52.8
Vaccine (aTIV = 1)	239	44.1
Experienced reaction	203	37.5
Proportion of children experiencing reactions who received adjuvanted vaccine	128	63.1
Total reactogenicity score (mean, SD)	2.8	2.1
Local reactogenicity score (mean, SD)	1.1	1.0
Limb Pain	130	64.0
Limb Redness	28	13.8
Limb swelling	31	15.3
Limb loss of movement	33	16.3
Systemic reactogenicity score (mean, SD)	1.5	1.6
Headache	33	16.3
Appetite loss	30	14.8
Muscle ache	55	27.1
Chills	47	23.2
Nausea	18	8.9
Vomiting	22	10.8
Diarrhea	13	6.4
Rash	4	2.0
Fever	66	32.5
Respiratory reactogenicity score (mean, SD)	0.2	0.4
Conjunctivitis	9	4.4
Shortness of breath	3	1.5
Sore throat	11	5.4
Runny nose	18	8.9
Other events†	14	6.9
† Other events included fainting, but as data was not consistently captured to characterize the events, we included them only in total reactogenicity scoring.		

Table 2. Multivariable linear regression model coefficients for association of reactogenicity predictors and post-vaccination HAI titers (adjusted for age, baseline titers, vaccine group and reaction) (summary of effects from all models)

Reactogenicity Predictor	**Antigen**	**Estimate**	**95% CI**	**p-value**
Any reaction [1+]	*A/ H1N1*	0.09	-0.26 – 0.44	0.601
	A/ H3N2	-0.06	-0.31 – 0.19	0.627
	B/ Victoria	-0.48	-0.80 – -0.16	**0.004**
	B/ Yamagata	-0.23	-0.54 – 0.08	0.145
Total reactogencity score	*A/ H1N1*	-0.07	-0.18 – 0.05	0.260
	A/ H3N2	-0.04	-0.12 – 0.05	0.401
	B/ Victoria	-0.19	-0.29 – -0.08	**0.001**
	B/ Yamagata	-0.07	-0.18 – 0.03	0.157
Total reactogenicity * Vaccine [aTIV]	*B/ Victoria*	0.22	0.09 – 0.35	**0.001**
Systemic reactogencity score	*A/ H1N1*	-0.36	-0.54 – -0.18	**<0.001**
	A/ H3N2	-0.04	-0.17 – 0.10	0.572
	B/ Victoria	-0.37	-0.54 – -0.20	<0.001
	B/ Yamagata	-0.16	-0.33 – 0.00	0.056
Systemic reactogenicity * Vaccine [aTIV]	*A/ H1N1*	0.38	0.17 – 0.60	**<0.001**
	B/ Victoria	0.44	0.24 – 0.64	**<0.001**
Local reactogencity score	*A/ H1N1*	0.25	0.04 – 0.47	**0.021**
	A/ H3N2	-0.00	-0.16 – 0.15	0.952
	B/ Victoria	-0.13	-0.33 – 0.07	0.215
	B/ Yamagata	-0.06	-0.26 – 0.13	0.511
Respiratory reactogencity score	*A/ H1N1*	-0.08	-0.79 – 0.63	0.821
	A/ H3N2	-0.65	-1.17 – -0.13	**0.015**
	B/ Victoria	-0.27	-0.94 – 0.41	0.440
	B/ Yamagata	0.12	-0.54 – 0.78	0.728
Respiratory reactogencity * Vaccine [aTIV]	*A/ H3N2*	0.78	0.13 – 1.43	**0.018**
1. Log-transformed titers were used for analysis. Coefficients estimate the mean log-change in post-vaccination HAI titers.				

Table 3. Multivariable non-linear regression model coefficients for significant interaction effects of total reactogenicity predictors and post-vaccination HAI titers against (adjusted for age, baseline titers, vaccine and child)(where the reference category is QIV vaccinated children with no reactions)

Antigen	Predictors	Estimate	95% CI	p-value
B/ Victoria				
Total reactogenicity (1]		-0.26	-0.70 – 0.17	0.230
Total reactogenicity (2-4]		-0.56	-1.06 – -0.06	**0.029**
Total reactogenicity (4-12]		-0.87	-1.53 – -0.21	**0.010**
Total reactogenicity (0] * Vaccine [aTIV]		-0.13	-0.41 – 0.16	0.386
Total reactogenicity (1] * Vaccine [aTIV]		-0.05	-0.60 – 0.49	0.849
Total reactogenicity (2-4] * Vaccine [aTIV]		0.45	-0.15 – 1.06	0.141
Total reactogenicity (4-12] * Vaccine [aTIV]		0.94	0.15 – 1.73	**0.019**
Notes				
1. Log-transformed titers were used for analysis. Coefficients estimate the mean log-change in post-vaccination HAI titers.				

Table 4. Multivariable non-linear regression model coefficients for significant interaction effects of systemic reactogenicity predictors and post-vaccination HAI titers against (adjusted for age, baseline titers, vaccine and child)(where the reference category is QIV vaccinated children with no reactions)

Antigen	Predictors	Estimate	95% CI	p-value
A/ H1N1				
Systemic reactogenicity score (1)		0.56	-0.13 – 1.24	0.113
Systemic reactogenicity score (2-8)		-1.12	-1.73 – -0.52	**<0.001**
Systemic reactogenicity score (1) * Vaccine [aTIV]		-0.52	-1.36 – 0.32	0.223
Systemic reactogenicity score (2-8) * Vaccine [aTIV]		1.26	0.54 – 1.99	**0.001**
B/ Victoria				
Systemic reactogenicity score (1)		-0.09	-0.74 – 0.55	0.774
Systemic reactogenicity score (2-8)		-1.38	-1.94 – -0.82	**<0.001**
Systemic reactogenicity score (1) * Vaccine [aTIV]		0.23	-0.56 – 1.01	0.571
Systemic reactogenicity score (2-8) * Vaccine [aTIV]		1.58	0.91 – 2.25	**<0.001**
Notes **1.** Log-transformed titers were used for analysis. Coefficients estimate the mean log-change in post-vaccination HAI titers.				

Table 5. Multivariable non-linear regression model coefficients for significant interaction effects of respiratory reactogenicity predictors and post-vaccination HAI titers against (adjusted for age, baseline titers, vaccine and child) (where the reference category is QIV vaccinated children with no reactions)

Antigen	**Predictors**	**Estimate**	**95% CI**	**p-value**
A/ H3N2				
Respiratory reactogenicity (1+]		-0.66	-1.26 – -0.07	**0.03**
Respiratory reactogenicity (0] * Vaccine [aTIV]		0.41	0.23 – 0.59	**<0.001**
Respiratory reactogenicity (1+] * Vaccine [aTIV]		0.85	0.11 – 1.59	**0.02**
Notes **1.** Log-transformed titers were used for analysis. Coefficients estimate the mean log-change in post-vaccination HAI titers.				

Figure 1. Reported vaccine reactions, by subgroup and frequency. *This figure shows the relative contributions of reported reactions.*

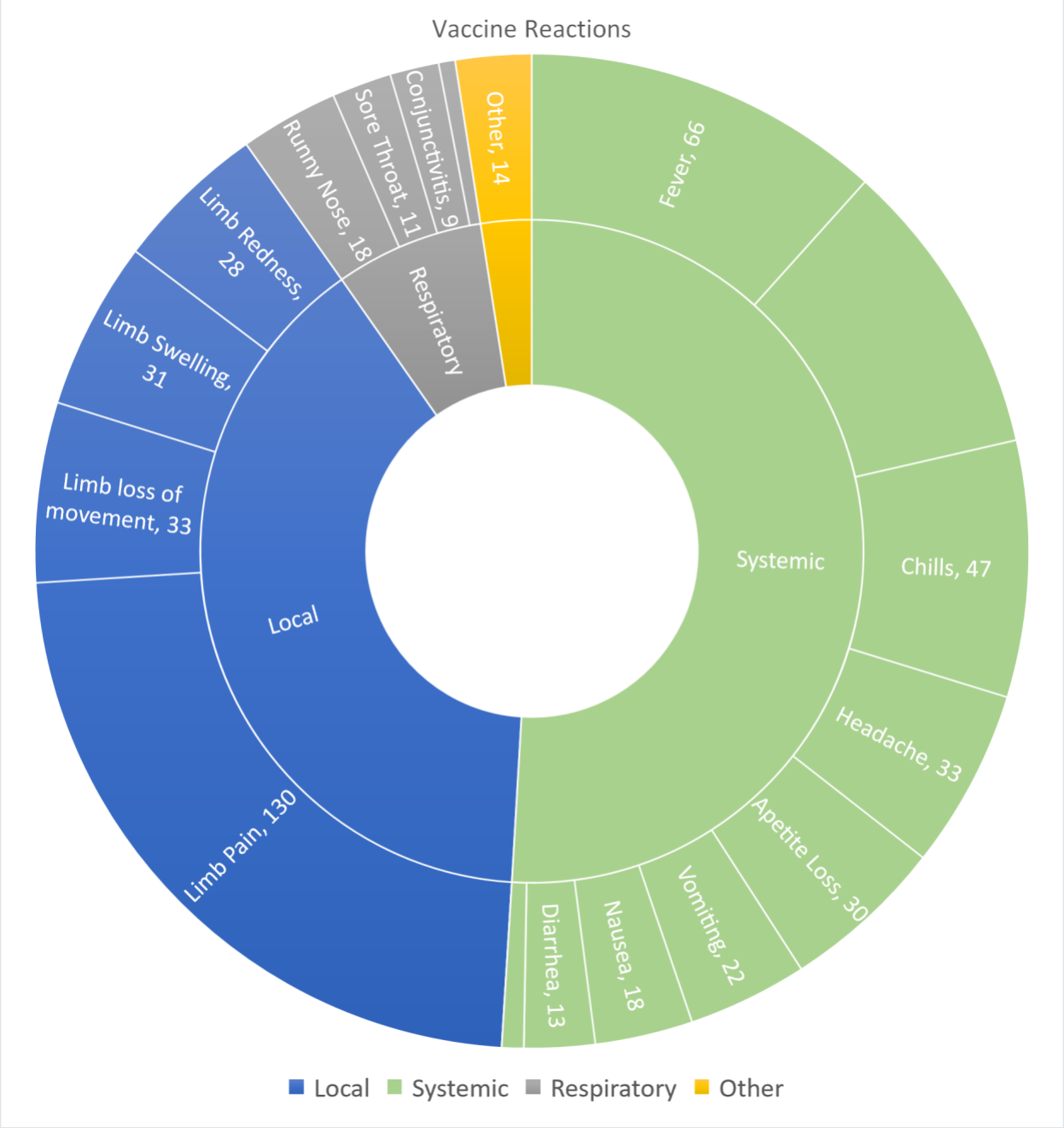


Figure 2. Geometric mean pre- and post-vaccination titers (geometric standard deviation factor), by vaccine antigen

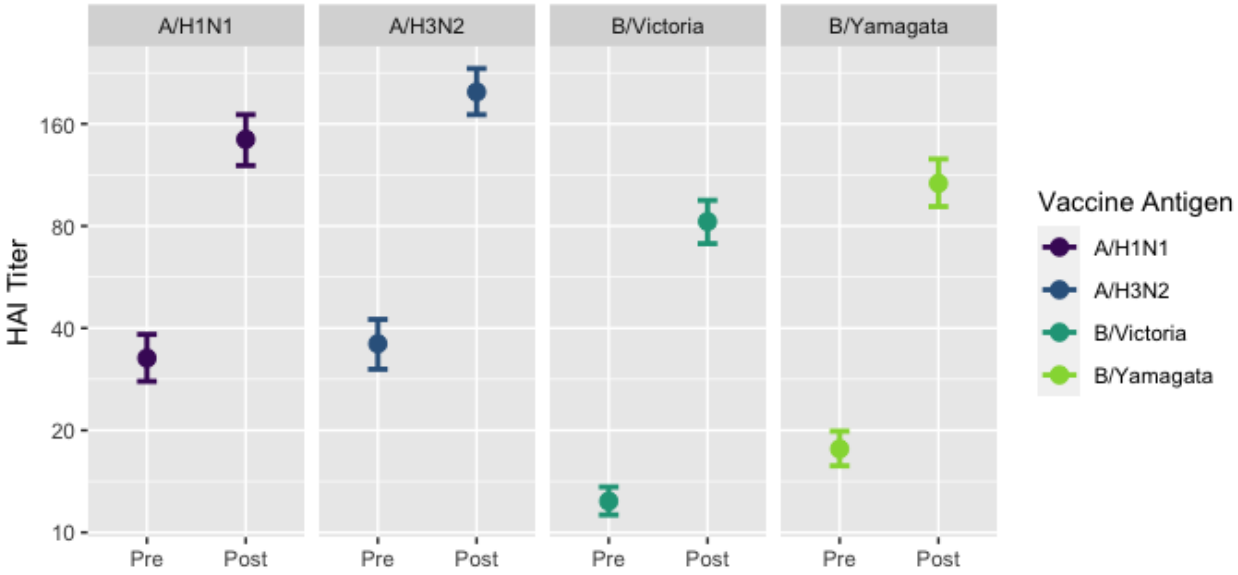


Figure 3. Geometric mean post-vaccination titers, by reaction status (children who reported no reactions, as compared to those who reported ≥1 reaction) (* : p <0.05, ** : p < 0.01, *** : p <0.001)

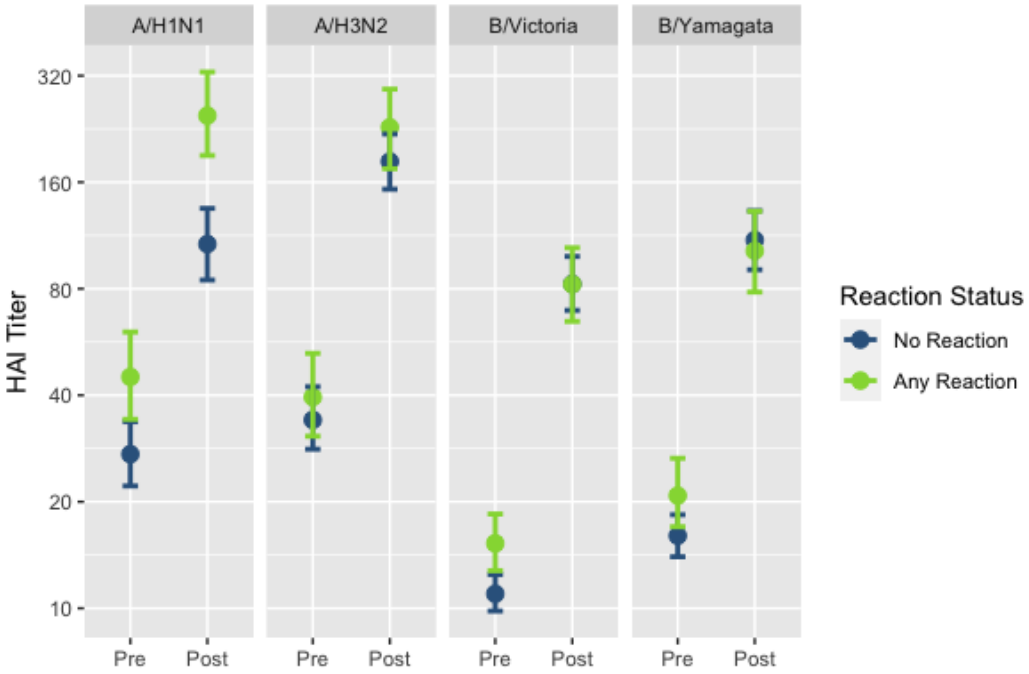


Figure 4. Interaction effects of reactogenicity scores, by vaccine group. *This figure shows the post-vaccination titers for each significant reactogenicity association, with the interaction between vaccine group and reactogenicity accounted for in the model.*

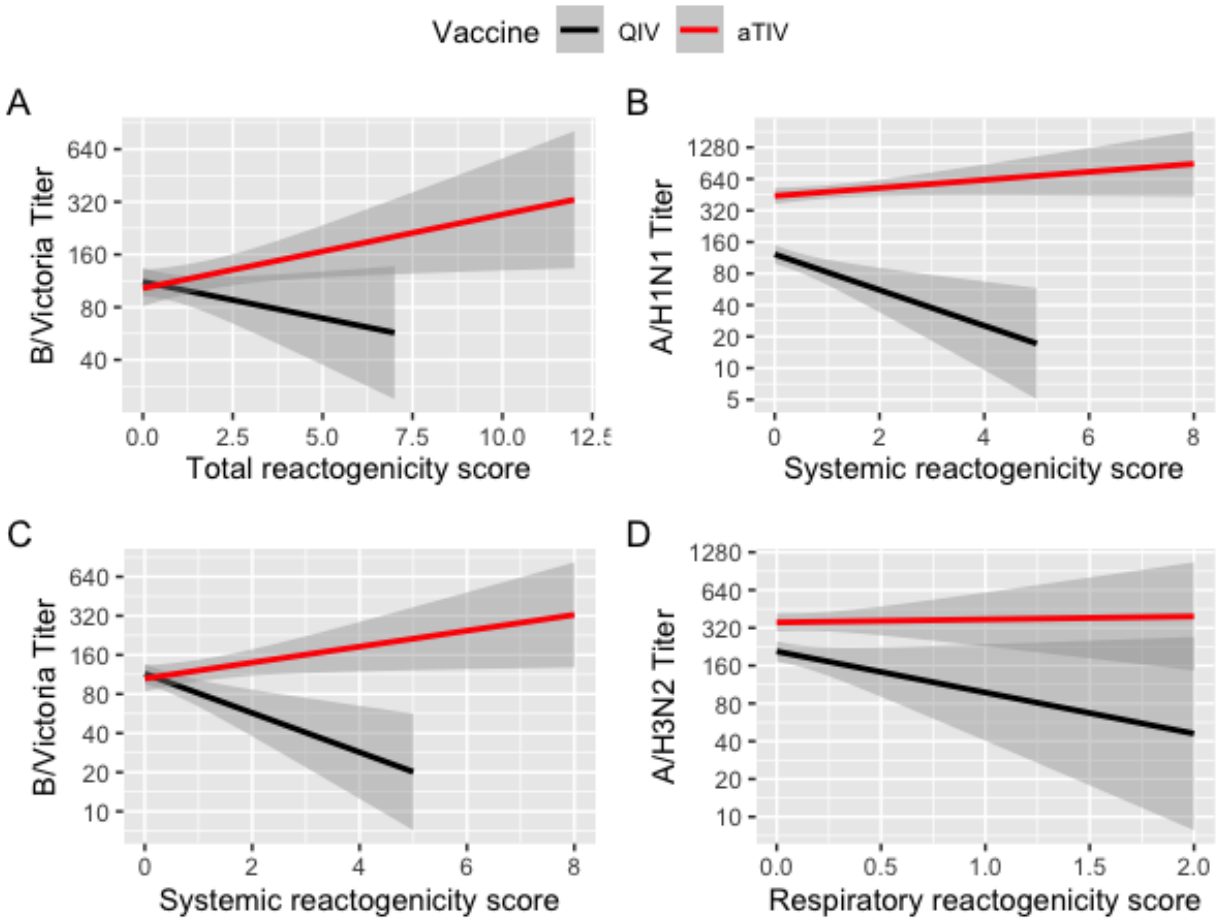
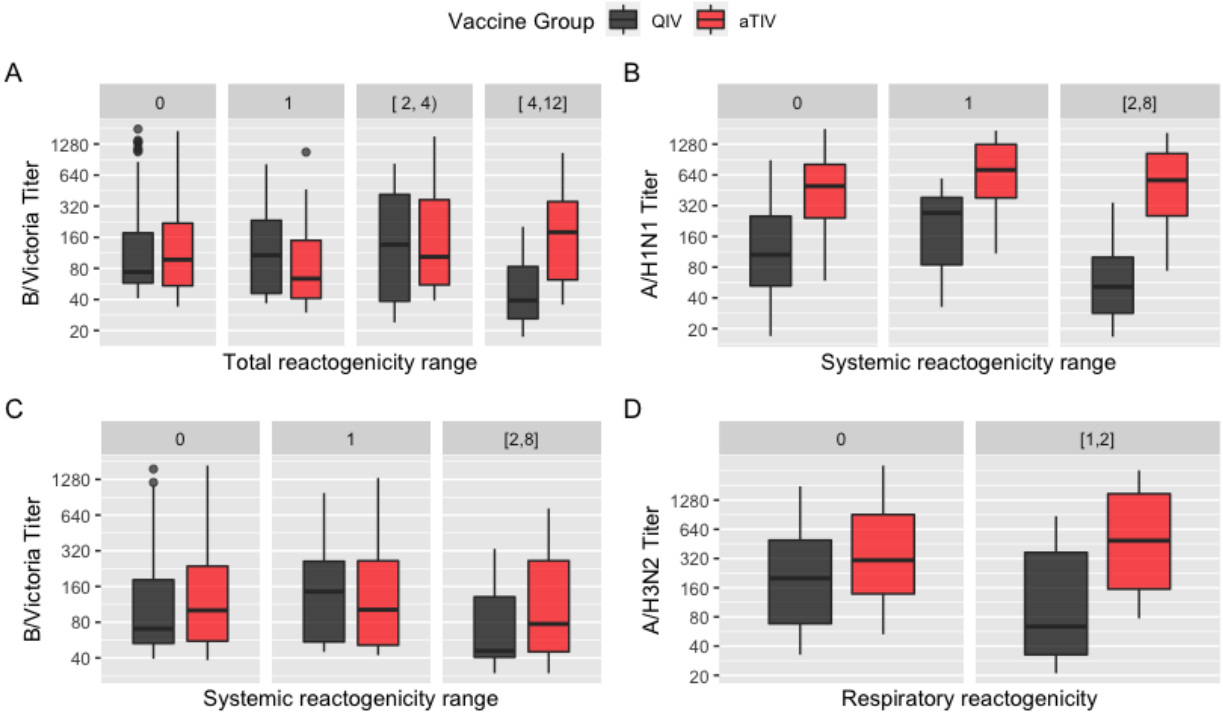


Figure 5. Interaction effects of non-linear reactogenicity factors, by vaccine group. *This figure shows the post-vaccination titers for each significant reactogenicity range association, with the interaction between vaccine group and reactogenicity accounted for in the model.*



Supplementary Tables

Supplementary Table 1. Vaccine antigen components used in the study vaccines and hemagglutination inhibition assays for vaccinated children

Year	A/H1N1	A/H3N2	B/Victoria	B/Yamagata
2016-2017	A/California/7/2009	A/Hong Kong/4801/2014	B/Brisbane/60/2008	B/Phuket/3073/2013
2017-2018	A/Michigan/45/2015	A/Hong Kong/4801/2014	B/Brisbane/60/2008	B/Phuket/3073/2013
2018-2019	A/Michigan/45/2015	A/Singapore/INFIMH-16-0019/2016	B/Colorado/06/2017	B/Phuket/3073/2013

PREFACE TO CHAPTER FOUR.

In this chapter, we used a causal mediation framework to assess relationships between vaccine formulation, antibody responses, and influenza infection. Mediation analysis allows us to investigate causal relationships between the predictor variables which influence the dependent variable. Using inverse odds ratio weighted mediation analysis, we estimated the proportion of relative vaccine efficacy in adjuvanted vaccinees which is mediated by the rise in post-vaccination HAI titers.

The student contribution to this study included conception, design, data cleaning and preparation, analysis, and manuscript writing. The study was conceived by myself, with input from Dr. Loeb. Co-authors include Drs. Loeb, Verschoor and Pullenayegum. All members of the thesis supervisory committee provided critical review of the research protocol, analysis plan, and draft manuscript. Raw datasets were provided to me by Pardeep Singh, who will be named as a co-author. Statistical modelling methods were based on code available from a causal mediation analysis evaluating antibody titers in a randomized clinical trial [1]. Drs Verschoor and Pullenayegum provided statistical modelling support. Laboratory serology for HAI titers was done by Dr. Brian Ward's research group at the McGill Center for Viral Diseases; however, no additional testing was required for this study. Dr. Verschoor aided in data interpretation. Dr. Loeb provided funding support for the study.

The paper is proposed to be submitted to *Vaccine*, or *Clinical Infectious Diseases* in August 2022 pending revisions recommended by the thesis supervisory committee.

[1] Cowling BJ, Lim WW, Perera RAPM, Fang VJ, Leung GM, Peiris JSM, et al. Influenza Hemagglutination-inhibition Antibody Titer as a Mediator of Vaccine-induced Protection for Influenza B 2019;68:1713–7. https://doi.org/10.1093/cid/ciy759.

CHAPTER FOUR: HEMAGGLUTINATION INHIBITION ANTIBODY MEDIATION OF RELATIVE VACCINE PROTECTION IN ADJUVANTED INFLUENZA VACCINATED CHILDREN

INTRODUCTION

Mediation analysis allows us to investigate causal relationships between predictor variables and outcomes [1]. In the case of seasonal influenza vaccination, vaccine efficacy (VE) is measured by the robustness of hemagglutination antibody (HAI) titers, which are assumed to correlate with protection against infection[2]. In this model, vaccination influences numerous immune mechanisms (including head-specific antibody titers, stalk-specific antibody titers, and T-cell mediated immunity), which in turn mediate the outcome of influenza infection. Previous work has found that HAI titers account for the majority (57%) of vaccine induced protection against influenza B [3], when comparing seasonal trivalent inactivated influenza vaccine (TIV) with placebo.

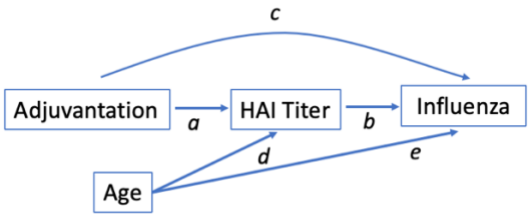
The adjuvanted seasonal influenza vaccine (aTIV) influences immune responses via numerous pathways, leading to enhanced protection[4–6]. Studies of the MF59 adjuvant have shown a direct effect on cytokine levels, indicating that it is able to activate immune cells and enhance antigen uptake [7,8]. Further studies have demonstrated that it induces immunogenicity by the induction of chemokines, increased recruitment of immune cells, enhanced differentiation of monocytes into dendritic cells, and facilitating dendritic cell migration into the lymph nodes, thus triggering the adaptive immune response [9,10].

Another study in children showed that the MF59 adjuvant elicited significantly higher seroconversion rates and geometric mean titers against all vaccine antigens, as compared to TIV vaccinated children [11]. Moreover, this study demonstrated that the persistence of HAI titers was significantly improved in adjuvant vaccinated children after six months, suggesting that the adjuvant may increase magnitutde and duration of protection. Consistent with these findings, another study observed higher HAI fold changes in aTIV vaccinated children, as compared to TIV vaccinated controls [12]. This study also observed higher HAI titers in aTIV vaccinees at six months post-vaccination, relative to controls. Uniquely, this study also found that adjuvant vaccinated children were primed to mount greater HAI titers than TIV-primed children when re-vaccinated in a subsequent season. Children who received adjuvanted vaccines two years apart, with no vaccination in the interim seasons, mounted 6.8-8.0-fold higher HAI titers to A/H3N2 and B influenza types, relative to the TIV vaccinated children. Titers against A/H1N1 were within one-fold change when comparing aTIV-primed and -vaccinated children with the reference TIV-TIV sequence.

As discussed above, the MF59 adjuvant may mediate vaccine protection against influenza through its ability to induce higher post-vaccination HAI titers. However, no study to our knowledge has determined the proportion of adjuvant vaccine protection against influenza which is mediated by HAI titers. Our study used a causal mediation analysis to quantify the proportion of relative vaccine protection attributable to increased post-vaccination HAI titers in adjuvanted vaccinees, as compared to non-adjuvanted vaccinees.

We propose the following pathway model:

Figure 1. *Mediation pathway model between vaccination, adjuvant and associated HAI titers, and influenza infection status. Adjuvanted influenza vaccine indirectly affects the likelihood of influenza infection, as it induces a rise in HAI titers (a), which correlate with reduced risk of flu (b). Adjuvantation may also act via other mechanisms to directly induce protection, such as enhancing cell-mediated immunity (c)[13]. Age has been shown to affect the magnitude of HAI titers which are induced in response to vaccination (d), and also the risk of influenza infection (e)[14,15].*



MATERIALS AND METHODS

Setting and Study Design

Our study was a cohort study, which made use of serum samples from the Adjuvanted Inactivated Vaccine Versus Inactivated Influenza Vaccine in Hutterite Children Trial (NCT02871206). In the original cluster randomized controlled trial, children aged 6 to 72 months were allocated to receive either quadrivalent inactivated influenza vaccine (QIV, Sanofi Pasteur: Fluzone®), or adjuvanted trivalent influenza vaccine (aTIV, Sequiris: Fluad Pediatric™). Adjuvant vaccinated children received either a 0.25 ml or 0.5 ml dose intramuscularly (for ages <36 months and ≥ 36 months, respectively), with the same dose

received four weeks after the first immunization. Quadrivalent vaccinated children received two 0.5 ml doses of the vaccine, administered four weeks apart. The parent study was conducted over three influenza seasons (2017-2019), and vaccines contained the recommended antigens for each season (Supplementary Table 1). Children were then followed for the duration of the influenza season for any signs or symptoms of respiratory infection. Children reporting two or more symptoms were sampled by nasopharyngeal swab for a multiplex respiratory pathogen panel and viral genotyping. Influenza infections were confirmed by reverse-transcriptase polymerase chain reaction (PCR).

Laboratory Testing

Our cohort included only children in whom there were data available on post-vaccination HAI titers. In the parent study, sera were collected from study vaccinated children at baseline and four weeks post-vaccination. Serum samples were tested by HAI assay against the four vaccine antigens recommended for each study year, per the standard protocol [16]. In brief, samples were plated in serial twofold dilutions from an initial dilution of 1:10, and incubated with erythrocytes and a stock titer of each vaccine strain virus (See Supplementary Table 1). HAI titers were determined to be the reciprocal of the last dilution at which hemagglutination was inhibited. Titers of <10 were imputed at 5 for the analysis.

Statistical Analysis Plan

The primary outcome of our analysis was survival time to symptomatic influenza A infection, as confirmed by PCR. We hypothesized a mediation model (Figure 1), wherein adjuvanted influenza vaccination impacted HAI titers, as well as inducing cell-mediated immunity against influenza infection. The relative adjuvant vaccine-induced protection against infection was mediated by the rise in HAI titers generated by a robust response to the vaccine (the mediating, "indirect" effect). Since age has been shown to affect the magnitutde of HAI responses in children, as well as their likelihood of developing an influenza infection, we included this as a potential confounder in our models[14,15].

Estimation of Total Effect

To estimate the total effect of vaccination on protection, we constructed a Cox proportional hazards model, where the independent variables were the vaccine allocation (QIV or aTIV) and age, and the dependent variable was time of PCR-confirmed influenza infection. We included a term for the colony (the clustered variable) to estimate cluster-robust variance. The relative vaccine efficacy was calculated as 1- the hazard ratio (HR) X 100%. We tested whether there was an association between the adjuvant-induced rise in post-vaccination HAI titers and the hazard of influenza infection by fitting a Cox proportional hazards model, with age and vaccine allocation as the predictors, and a cluster-robust estimation term for colony. We tested the proportional hazards assumption using the "cox.zph()" function of the "survival" package in R, which uses the correlation between scaled Schoenfeld residuals and time-to-event to assess the independence between covariates and time. We assessed the proportional hazards and non-linearity

assumptions of the model using Schoenfeld and Martingale residuals plots. We determined whether there was an interaction between vaccine formulation and the post-vaccination titers by adding an interaction term to the model.

Estimation of Direct Effect: Relative Vaccine-Induced Protection

We estimated the direct effect of adjuvanted vaccination relative to quadrivalent vaccination on protection against influenza (the effect of the vaccine which does not act through the pathway of increased HAI titers) by first fitting a logistic regression model. This model included post-vaccination HAI and age as the independent variables, colony as the clustering variable, and vaccination with aTIV as the outcome. The regression coefficients of this model were used to generate odds ratios for each child, from which we derived a weighted score for each vaccinated participant (1-OR)[17]. Weights for children receiving the QIV vaccine were pre-specified at 1. We then fit a proportional hazards model for the time to influenza infection, adjusting for age, vaccine group, and colony, weighted according to the scores generated by the odds ratios. Direct effect was estimated from the hazard ratio of this weighted model.

Estimation of Indirect Effect: HAI- Mediated Relative Vaccine-Induced Protection

Indirect effect was calculated as the ratio between the total effect and the direct effect (steps described above). The proportion of effect which is attributed to the HAI titres following was estimated as [log(indirect effect HR)]/[log(total effect HR)].

We reported the estimates, 95% confidence intervals, and p-values from the models for total and direct effects. We conducted a sensitivity analysis in which we adjusted for pre-vaccination titers, to account for the possibility that vaccination would not have elicited a robust immune response due to a ceiling-effect for antibody titres in children with previously high titers. Models followed the same approach outlined above, with pre-vaccination titers for each participant included as a covariate. We included only samples with complete data on pre- and post-vaccination titers; due to study design, there were no observations with missing data. All analyses were done in the R environment, version 4.0.2, and an alpha of 0.05 was considered significant for all tests.

Sample Size

Given our available samples, we used all possible study vaccinated children with available HAI titer data (542 samples from 330 unique participants). Using the powerMediation for Cox proportional hazard models, the event rate of 4%, and assuming the log A/H3N2 titer as the mediator, we estimate that our sample would have 99.8% power to detect a relative risk of 2 in the coefficient of the mediator between vaccine groups. Under the same assumptions, and a relative risk of 1.5 in the coefficient of the mediator, we estimated our study power to be 81.4%. Vittinghoff et al. showed that for a Cox regression model with a mediating variable, the hazard ratio is equal to the baseline hazard plus the coefficients and values of all predictors, including the mediator ($\log(\lambda) = \log(\lambda 0) + b1xi + b2mi \ldots$)[18]. The null hypothesis is that in the Cox proportional hazards model which includes the baseline hazard and all relevant predictors and confounders, the

coefficient of the mediator-exposure relationship predictor (b2) will be equal to 0. The alternative hypothesis is that the coefficient of the mediator-exposure relationship is not equal to 0 [18].

Assuming simple random sampling methods, the sample size required under the above assumptions and a relative risk of 2 in the coefficient of the mediator, we determined that 205 subjects would be necessary to obtain 85% power to detect whether the mediation-exposure coefficient was not equal to zero. We calculated the design effect, or variance inflation factor using (1+((m-1) x ICC)), where m is the average cluster size and ICC is the intracluster correlation coefficient [19]. The overall design effect was calculated as 2.043, increasing the required sample size to 418. Using all available samples, we estimated that the power of our sample size would be 92.7% to detect a relative risk of 2 in the coefficient of the mediator in the hazard of influenza AH3 infections, after accounting for all other predictors [18].

RESULTS

Our cohort included 542 post-vaccination serum samples from 330 unique children across the three influenza seasons of the original study (Table 1). The mean age was 54.4 months (SD: 17.9) and did not differ significantly across vaccine groups. Among these children, there were 32 PCR-confirmed influenza infections: one A/H1N1 case, 22 cases of A/H3N2, and 9 B infections (untyped). Given the small number of cases in the available samples, and the significant differences between vaccine groups in post-

vaccination titers against A/H3N2, we focus on infections of this subtype. Of 22 A/H3N2 infections, two occurred in the aTIV group (0.8%), as compared to 20 in the QIV group (6.6%) (Chi-squared = 9.97, 95% CI: -0.09, -0.02, p = <0.001). Given the small number of events, and that 82% of cases occurred in season two, we pooled cases across all seasons.

The post-vaccination HAI titers against A/H3N2 are shown in Figures 2a, 2b, and Table 2. Of 542 observations, 239 were in aTIV recipients, and 303 in QIV recipients. The geometric mean HAI titers were 355.22 (SD: 3.49) in aTIV vaccinees, as compared to 202.05 (SD: 5.07) in QIV vaccinees (p<0.001). Pre-vaccination titers did not differ significantly (68.80, SD: 7.00 in aTIV vaccinees, vs 54.22, SD: 6.76 in QIV vaccinees, p = 0.15). Figure 3 shows the distribution of post-vaccination titers in children who went on to develop flu infections.

We estimated the hazard of influenza A/H3N2 infection in children who received the adjuvanted vaccine, as compared to QIV. We estimated the total effect HR to be 0.122 (95% CI: 0.026, 0.565), corresponding to a relative vaccine efficacy of 87.8% (95% CI: 43.6%, 97.4%). Under our proposed causal framework, we estimated that the direct effect, or the amount of vaccine protection which was not mediated by the increase in post-vaccination HAI titers, was 0.148 (95%CI: 0.03, 0.706). Indirect effect was estimated at 0.827. Using the ratio between the estimated log HRs for indirect and total effects, we estimated the proportion of relative vaccine effect mediated by the rise in

higher antibody titers in adjuvant vaccinated children as 9.02%. There was no interaction observed between the post-vaccination titers and the vaccine formulation (p=0.576).

We conducted a sensitivity analysis to account for pre-vaccination HAI titers, and the potential antibody ceiling effect, which might impact the magnitude of post-vaccination titer increases or fold-change (see Methods 2.4). In QIV vaccinated children, 21% of children had no change between pre- and post-vaccination titers. Similarly, 18% of aTIV vaccinees had identical titers between time points. In our sensitivity model including pre-vaccination titers for each child, the total effect was estimated at 0.1305 (95% CI: 0.032, 0.532). The direct effect was 0.129 (95% CI: 0.032, 0.519). The indirect effect was estimated at 1.01. Using the log of the hazard ratios from indirect and total effect estimates, we obtained a negative proportion (-0.00).

Shown in Figure 4a, the predicted hazard of infection in aTIV vaccinees relative to QIV vaccinees declined well before the accepted correlate of influenza protection HAI titer of 1:40. To estimate a relative risk reduction of 50%, we conducted a post-hoc analysis of the predicted risk scores at each titer dilution. We tested for a difference in the mean risk scores at each titer value by vaccine group using Welch's two-sample t-test, assuming unequal variance. Significant differences in the mean predicted risk scores were observed at every dilution ($p<0.05$ for all outcomes) (Table 3). Of note, we observed that relative to QIV vaccinees, children in the aTIV group were predicted to have a risk reduction of 50% or greater, regardless of the post-vaccination titer (Figure 4b).

DISCUSSION

Our study evaluated the proportion of relative vaccine protection which is conferred by in rise in post-vaccination HAI titers induced by adjuvanted influenza vaccines. The MF59 adjuvant has been shown to increase flu vaccine immunogenicity and efficacy [20]. Despite the observed changes in vaccine effectiveness, the mechanism by which MF59 acts to enhance protection remains unclear [13]. The HAI titer of <1:40 is the accepted correlate of protection against influenza, by which vaccine efficacy is assessed [21]. In our study, we observed that the relative additional vaccine protection in adjuvanted vaccinees was not largely driven by the greater HAI titers in this group. This finding offers interesting insight on both the utility of the HAI titer as a correlate of protection in children, and the adjuvant effect of MF59 on vaccine efficacy.

Our finding suggests that post-vaccination HAI titers mediate little of the relative protection of adjuvanted influenza vaccine, an estimated 9.0%. Despite non-significant mediation attributable to HAI responses, adjuvanted vaccinees had significantly improved vaccine efficacy against A/H3N2. This suggests that the MF59 adjuvant, while it does induce stronger antibody responses to vaccination, does not improve protection via this pathway. Studies in children have shown that seroprotection evoked by MF59-adjuvanted vaccines in children is superior to the immune responses following nonadjuvanted vaccines [9,11,12]. However, studies evaluating the HAI threshold of <1:40 in children have found that it was not consistently correlated with protection against influenza, with

new thresholds proposed of <1:110 [22,23]. Our study found that while immunogenicity was significantly enhanced in adjuvanted vaccinees, the relative protection of the adjuvanted vaccine was not conclusively mediated by the rise in HAI titers. This would suggest that MF59 adjuvant may confer improved relative vaccine efficacy via alternate pathways, such induction of innate and cell-mediated immune responses.

To our knowledge, our study is the first to use a causal mediation framework to assess relative vaccine protection. Cowling et al. used a similar framework to model the proportion of vaccine protection against influenza B that was mediated by HAI titers, as compared to unvaccinated children[3]. This study found that post-vaccination HAI titers mediated 57% of an overall vaccine efficacy of 68% against influenza B. Strengths of this work included the use of an unvaccinated comparator group, and its randomized controlled study design. In contrast, our study assessed the relative protection of adjuvanted versus non-adjuvanted influenza vaccines against influenza A/H3N2. Given that all study participants were vaccinated and mounted HAI titers, we would expect to see a more modest effect than might have been observed if we had compared with an unvaccinated control group. Our study also differs in that it investigates influenza A/H3N2, rather than B. Both antibody- and cell-mediated immune responses have been implicated in adaptive immunity against influenza A types [24–26]. It is possible that the effect of HAI titers on vaccine-induced protection may differ according to influenza type. Further work to compare the relative proportion of protection mediated by antibody titers

against influenza across types and subtypes would have valuable insight for novel vaccine development.

Most influenza A/H3N2 cases in our study occurred in season two. The mean age of study enrolled children during this season was 4.5 years, by which literature suggests children would have already experienced an exposure to influenza[27]. Studies have shown that early life exposures to influence imprint the host immune system, driving the strongest immune protection against those strains [14,28]. Antibody landscapes have shown that host immune histories can influence responses to subsequent exposures, either by natural infection or vaccination [29–31]. Effects can include blunting of the immune response and epitope-biased responses, wherein antibody titers are increased to the novel exposure but absolute titers to primary exposure strains remain highest due to back boosting[30,32]. For children in our cohort, this may mean that a proportion of HAI titers mounted in response to vaccination are memory responses to a previously encountered strain, rather than the vaccine antigen. In our cohort, 83.4% of children were at least three years of age. Therefore, it is very likely that primary A/H3N2 exposures in study subjects were to antigenically drifted strains of A/H3N2 in previous seasons, which had acquired mutations in antigenic site B [33,34]. These prior immune histories may have influenced the HAI responses to study vaccines, potentially limiting the quality and appropriateness of HAI titers and subsequent protection conferred.

There is a growing body of work on the effect of repeated vaccination on immunogenicity and efficacy of influenza vaccines[35–39]. A seminal modelling study by Smith et al. proposed the antigenic distance hypothesis, which predicts that vaccine efficacy is reduced when the vaccine antigen is closely similar to the prior season antigen, but dissimilar to the circulating strain[40]. In season two of our study, the vaccine antigen was unchanged from the previous season recommendation; however, phylogenetic analyses of A/H3N2 evolution showed rapid mutation and clade diversification from 2013-2018 [33,41,42]. Studies of the effect of these mutations on antibody escape have shown mixed results. Despite the antigenic drift, studies of antisera to the vaccine strain showed effective neutralization of the emergent variants[43,44]. Other work has shown that glycosylation changes in the evolved strains may be able to mask viral epitopes and promote immune evasion [34,45–47]. Skowronski et al. found that prior influenza vaccination had mixed effects across seasons, consistent with the antigenic distance hypothesis[42,48]. Prior seasonal vaccination was significantly associated with negative impact on current VE during the 2014-2015 season, in which vaccine antigens were homologous with the previous season's formulation but circulating strains had the greatest phylogenetic distance from the vaccine antigen[48]. In the 2017-2018 season, VE against A/H3N2 was found to be reduced in individuals who had been vaccinated in the prior season, relative to those without prior vaccination[42]. The lower VE was observed across the co-circulating clades of A/H3N2, including those with and without glycosylation changes linked to immune escape. This would suggest that both viral

evolution and changes in host immunity due to serial vaccination may contribute to poor vaccine effectiveness during season two of our study.

As a result of the potential interference by repeat vaccinations, and the acquisition of mutations in A/H3N2 which may have impeded efficient antibody binding during season two, HAI titers to the vaccine antigen may not have been sufficiently cross-protective. Therefore, it is challenging to ascertain the true proportion of relative vaccine protection which is mediated by the adjuvant-induced rise in HAI titers, had the vaccine been better matched to the seasonal circulating strain. Further study is warranted to investigate the causal mediation of relative adjuvanted vaccine protection by HAI titers during a matched influenza vaccine season. Our study has several other limitations, including the very small number of PCR-confirmed influenza cases, particularly in the adjuvanted group. It is possible that the low proportion of effect mediated by the rise in HAI titers in this group is underestimated due to the small sample size. Among confirmed influenza cases, HAI titers were not available for all children, further reducing our number of events and decreasing statistical power.

Our study provides unique insight on the potential mechanism of adjuvant-induced influenza vaccine protection. We found that while adjuvantation results in significant differences in post-vaccination titers, and confers significantly superior protection against influenza A/H3N2 infections, the increased HAI titers do not conclusively mediate this protection in adjuvanted vaccinees. We observed that relative to QIV vaccinees, the

hazard of A/H3N2 in adjuvanted children was reduced by 50% at titers > 1:20. Possible explanations for this include adjuvant-induced innate or cell-mediated immune responses. Moreover, influenza infections in our study were from a season of co-circulating H3N2 strains from divergent clades. Despite the differing epidemic strains, protection was significantly better in the adjuvanted group relative to non-adjuvanted vaccinees. This suggests that adjuvanted influenza vaccine may confer enhanced cross-protection against novel influenza antigens, potentially in seasons of vaccine mismatch. This would provide strategic data for the development of novel vaccine candidates against influenza A types, particularly A/H3N2, which shows consistently weaker vaccine effectiveness. Further research on the potential cross-protection conferred by adjuvanted influenza vaccine would be powerful for the design of next generation vaccines and adjuvant formulations.

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Table 1. Characteristics of children with paired serum samples included in the study cohort (*n* = 542 observations from 330 unique children)

Demographics	**All children** (n = 542)		**aTIV** (n= 239)		**QIV** (n= 303)	
	n	%	*n*	%	*n*	%
Age (months) (mean, SD)	54.4	17.9	54.8	18.0	54.11	17.8
Sex (Male = 1)	286	52.8	126	52.7	160	52.8
PCR-Confirmed Influenza	32	5.9	7	2.9	25	8.3
A type	23	4.2	3	1.3	20	6.6
A/H1N1	1	0.2	0	0.0	1	0.4
A/H3N2	22	4.1	2	0.8	20	6.6
B type	9	1.7	4	1.7	5	1.7

Table 2. Geometric mean pre- and post-vaccination HAI titers (mean, GSD) and significance of difference between vaccine groups (unadjusted)

	Pre-vaccination titers			***p***	**Post-vaccination titers**			***p***
Group	All	aTIV	QIV		All	aTIV	QIV	
Antigen								
A/ H1N1	56.21 (7.27)	80.93 (6.71)	42.16 (7.39)	<0.001	211.72 (5.53)	478.88 (3.21)	111.21 (6.03)	<0.001
A/ H3N2	60.23 (6.87)	68.80 (7.00)	54.22 (6.76)	0.155	259.12 (4.46)	355.22 (3.49)	202.05 (5.07)	<0.001
B/ Victoria	17.20 (4.41)	19.95 (4.49)	15.30 (4.31)	0.040	111.99 (4.56)	120.42 (4.54)	105.75 (4.58)	0.323
B/ Yamagata	25.85 (5.00)	26.73 (4.97)	25.17 (5.04)	0.667	148.37 (4.84)	122.89 (5.07)	172.15 (4.59)	0.014

Table 3. Mean predicted risk scores (SD) of post-vaccination titers, after adjustment for vaccine, age and colony

HAI Titer	***n* (%)**	**Predicted Risk Score** (mean, SD)			**95% CI**	**p**
		All Children	**aTIV**	**QIV**		
5	17 (3.1%)	1.97 (0.94)	0.37 (0.01)	2.47 (0.22)	1.96, 2.23	<0.001
10	8 (1.5%)	2.45 (0.19)	-	2.45 (0.19)	-	-
20	30 (5.5%)	2.18 (0.74)	0.37 (0.04)	2.45 (0.19)	2.00, 2.17	<0.001
40	35 (6.5%)	2.08 (0.96)	0.38 (0.03)	2.59 (0.21)	2.13, 2.30	<0.001
80	56 (10.3%)	1.38 (1.13)	0.38 (0.03)	2.62 (0.20)	2.16, 2.32	<0.001
160	64 (11.8%)	1.54 (1.15)	0.38 (0.03)	2.64 (0.22)	2.18, 2.34	<0.001
320	101 (18.6%)	1.68 (1.12)	0.39 (0.03)	2.63 (0.18)	2.19, 2.29	<0.001
640	84 (15.5%)	1.46 (1.11)	0.39 (0.03)	2.58 (0.18)	2.13, 2.25	<0.001
1280	147 (27.1%)	1.50 (1.14)	0.39 (0.03)	2.65 (0.19)	2.22, 2.31	<0.001

1. Differences in risk scores by vaccine group were tested in each stratum of post-vaccination titers using Welch's two sample t-test, assuming unequal variance.

Supplementary Table 1.
Vaccine antigen components used in the study vaccines and hemagglutination inhibition assays for vaccinated children

Year	A/H1N1	A/H3N2	B/Victoria	B/Yamagata
2016-2017	A/California/7/2009	A/Hong Kong/4801/2014	B/Brisbane/60/2008	B/Phuket/3073/2013
2017-2018	A/Michigan/45/2015	A/Hong Kong/4801/2014	B/Brisbane/60/2008	B/Phuket/3073/2013
2018-2019	A/Michigan/45/2015	A/Singapore/INFIMH-16-0019/2016	B/Colorado/06/2017	B/Phuket/3073/2013

Figures

Figure 1. *Mediation pathway model between vaccination, adjuvant and associated HAI titers, and influenza infection status. Adjuvanted influenza vaccine indirectly affects the likelihood of influenza infection, as it induces a rise in HAI titers (a), which correlate with reduced risk of flu (b). Adjuvantation may also act via other mechanisms to directly induce protection, such as enhancing cell-mediated immunity (c). Age has been shown to affect the magnitude of HAI titers which are induced in response to vaccination (d), and also the risk of influenza infection (e).*

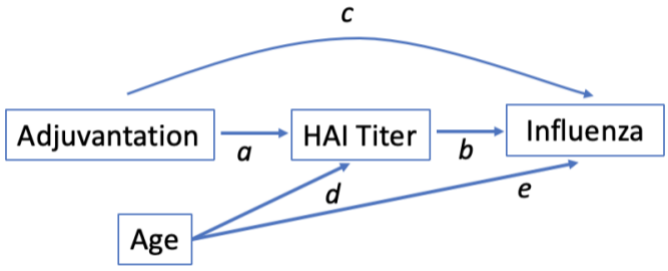


Figure 2a. *Distribution of post-vaccination HAI titers against the vaccine antigen for influenza A/H3N2. Titers are compared between quadrivalent vaccinees in black, and adjuvanted vaccinees in red. Horizontal lines indicate median titers for the group; vertical lines denote the interquartile range of titer values.*

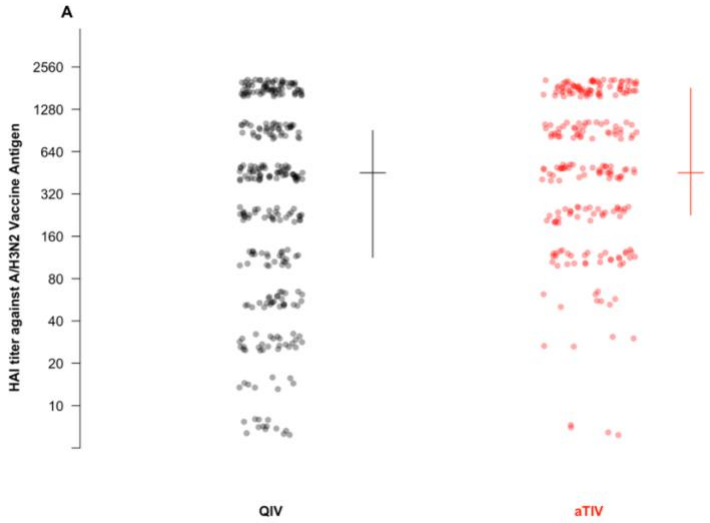


Figure 2b. *Histogram of post-vaccination HAI titers, by vaccine allocation. Quadrivalent vaccinees are presented in black; adjuvanted vaccinees in red. Bars represent the count of children with HAI titers in each range of titer dilutions, shown as the reciprocal of the titer value (ranging from <10 to 1280).*

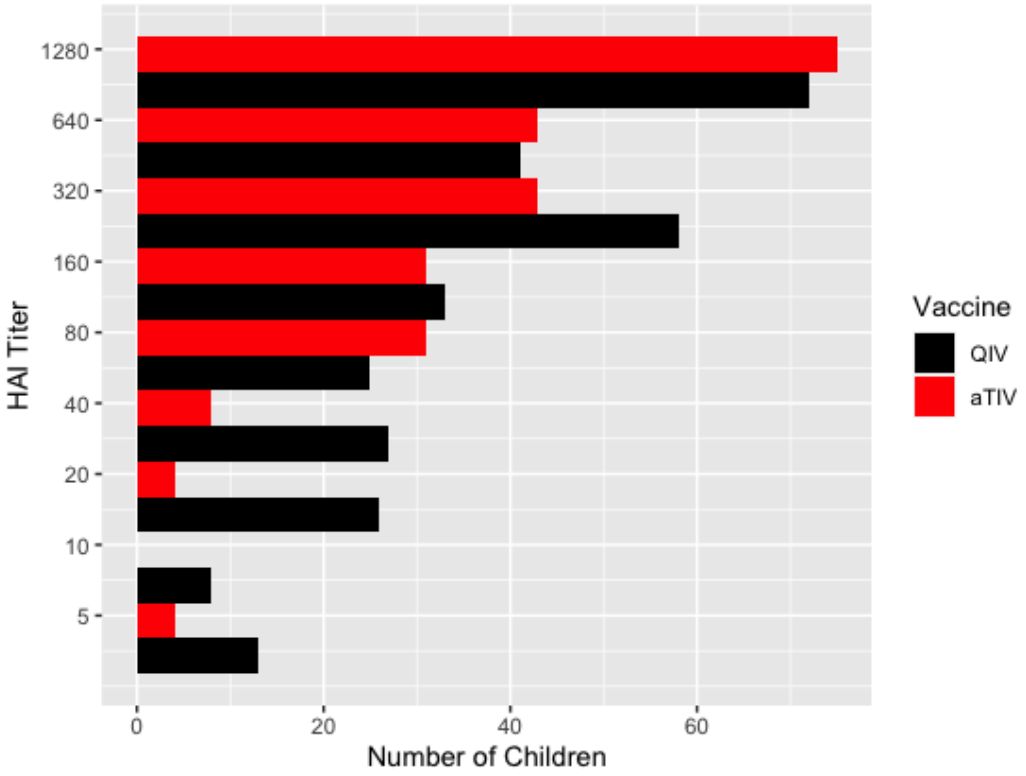


Figure 3. *Histogram of post-vaccination HAI titers in children who went on to develop influenza A/H3N2 infection, by vaccine allocation. Quadrivalent vaccinees are presented in black; adjuvanted vaccinees in red. Bars represent the count of children with HAI titers in each range of titer dilutions, shown as the reciprocal of the titer value (ranging from <10 to 1280).*

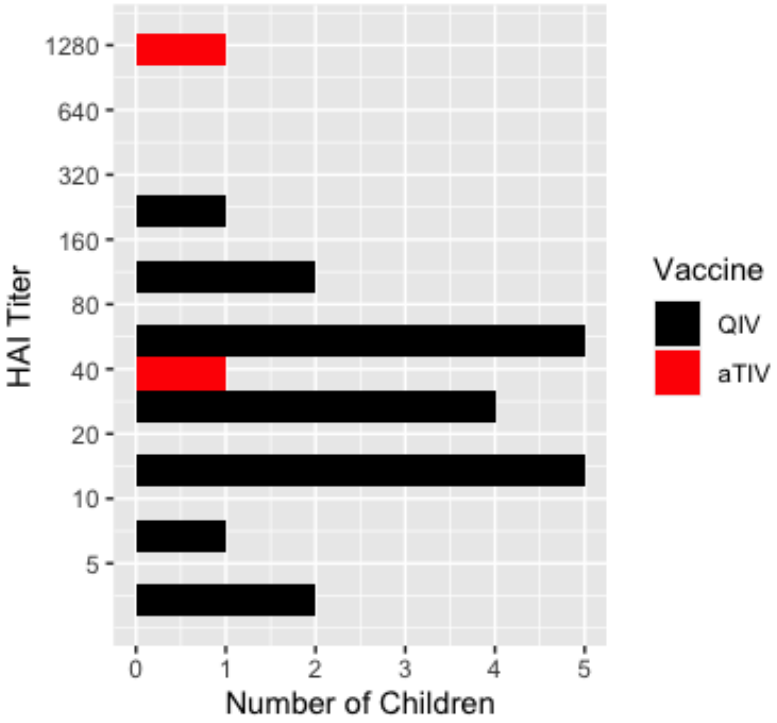


Figure 4a. *Correlation of HAI titer with protection against influenza A/H3N2 infection in a proportional hazards model. Assuming that the baseline risk is equal to one, the increase in post-vaccination HAI titer demonstrates relative risk reduction as HAI titers increase. Where the relative risk in quadrivalent vaccinees is one, this figure shows that adjuvanted vaccinees are at a reduced risk of influenza A/H3N2 infection at any value of HAI titer. An HAI titer of 40 is accepted to correlate with 50% protection against influenza; here we show that adjuvanted vaccinees are at 50% reduced risk at titers of less than 40.*

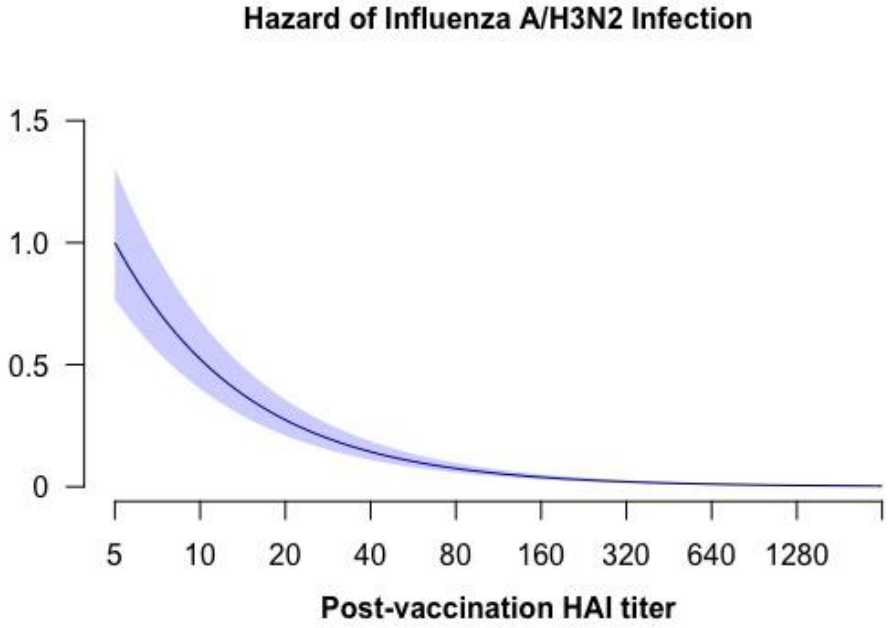
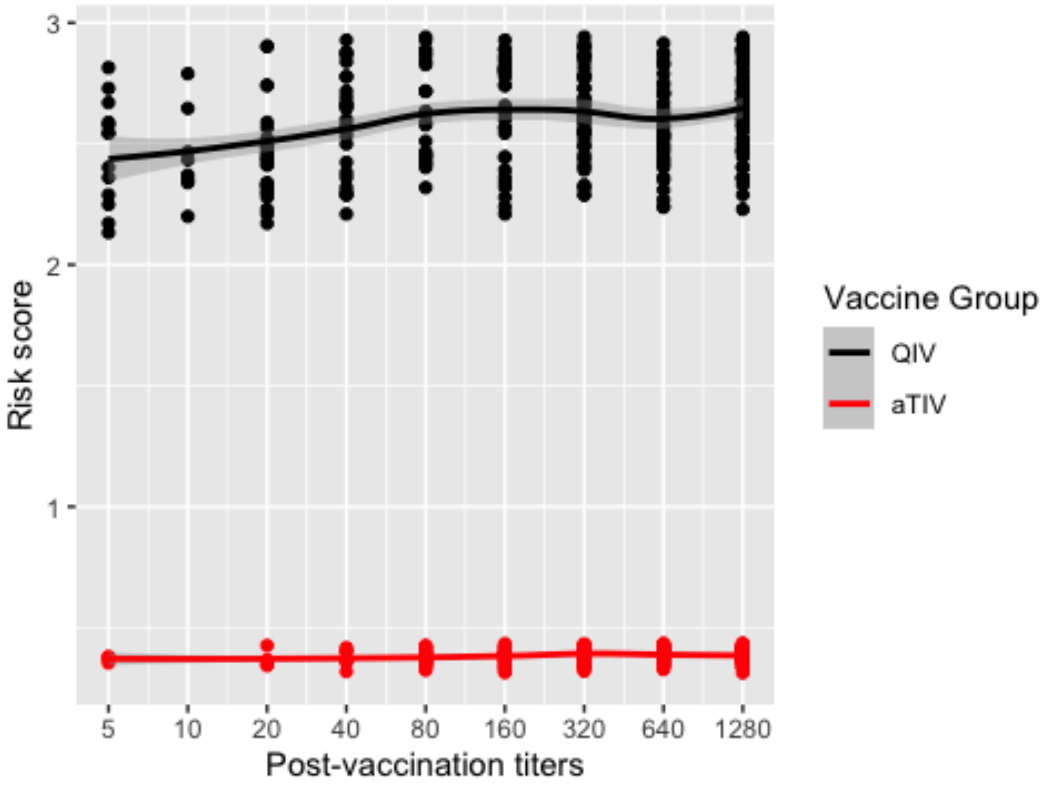


Figure 4b. *Predicted risk of influenza A/H3N2 infection from a proportional hazards model. In this model, risk of infection is predicted from the values of covariates (age, vaccine group) and weighted by the post-vaccination titer. Relative to an equal risk of one, adjuvanted vaccinees (red) are less than half as likely to develop influenza A/H3N2, regardless of HAI titer.*



CHAPTER FIVE. CONCLUSIONS

The aim of this thesis was to investigate whether relationships between vaccine formulation and host factors were significantly associated with influenza vaccine-related outcomes. My research has focused on identifying correlates of infection severity, vaccine immunogenicity, and protection in children. Addressing these questions is important for several reasons. First, children are at greater risk for complications related to influenza, and reducing infections is necessary to prevent morbidity, epidemic outbreak, and associated burden on health care systems [1]. Secondly, immune responses to influenza exposure are highly multifaceted and shaped by host characteristics [2–4]. As such, identifying correlates of protection against influenza continues to remain challenging. Determinants of vaccine effectiveness are particularly valuable in children, in whom standard inactivated influenza vaccines are variably immunogenic [5,6]. We are unaware of any other study to date which examines adjuvant-mediated effectiveness outcomes in influenza vaccinated children. In this concluding chapter, I summarize the key findings from each manuscript chapter. I discuss the overall implications of the work and identify areas where further research work may be valuable.

SUMMARY OF RESEARCH AND IMPLICATIONS

In the second chapter, I investigated whether adjuvanted vaccine differed significantly from a nonadjuvanted formulation in its ability to attenuate the symptom severity in breakthrough influenza infections. We concluded that adjuvanted vaccination was associated with attenuated systemic and febrile symptom severity in breakthrough

influenza infections, particularly by A type infections. To our knowledge, this is the first study evaluating attenuated symptom severity of infection in adjuvant-vaccinated children, relative to a non-adjuvanted vaccine.

In influenza B infections, we observed that incidence rate ratios of composite severity outcomes were increased in adjuvanted children, relative to quadrivalent vaccinees. Given the vaccine lineage mismatch in aTIV, we may be able to make hypotheses about quadrivalent vaccine attenuation of symptoms, using aTIV children as essentially unvaccinated controls [7,8]. In this lens, quadrivalent vaccination may reduce total symptoms and course of disease, relative to unvaccinated controls, in influenza B infections. However, our study is limited by the small number of events, and work with larger sample sizes of B infections would be warranted to investigate this further.

Our work is uniquely valuable as it demonstrates the ability of adjuvanted vaccination to reduce symptom severity despite antigenic mismatch. Research on the development of a universally cross-protective influenza vaccine is ongoing, driven by the divergent phylogenies of influenza virus as a result of antigenic shift and drift [9,10]. Further studies have shown that adjuvanted vaccines induce a distinct antibody profile, with enhanced magnitude, longevity, affinity and breadth of antibody responses[11–16]. It may be hypothesized that these broader enhancements of antibody responses may influence protection when encountering antigenically distinct influenza viruses. While protection may be hindered by the highly strain-specific HAI antibodies, the greater

cross-reactivity may afford some protection against clinical presentation of disease[17,18]. Given the possible mechanisms by which adjuvanted vaccination may generate more broadly protective responses, studies are warranted which examine the vaccine protection against both symptomatic infection, and attenuation of the severity and course of disease.

Our findings speak to the potential of adjuvants to mitigate influenza severity in the face of novel antigenic evolution, which has meaningful implications for future immunization responses to emergent influenza viruses with pandemic potential. Further work to characterize adjuvant-specific attenuation of disease would be valuable for informing immunization recommendations, particularly in populations at high risk of influenza-related complications.

In the third chapter, I investigated whether reactogenicity to immunization was predictive of vaccine immunogenicity, as measured by HAI titers mounted four weeks post-vaccination. We found that increases in reactogenicity scores were associated with significant reductions in immunogenicity, particularly against A/H1N1 and B/Victoria vaccine antigens, and that there was a significant moderation effect of adjuvanted vaccination. Increasing local reactions were the only positive correlation we identified, which was independent of vaccine effect moderation. To our knowledge, this is the first study evaluating reactogenicity as a predictor of immunogenicity, accounting for the moderating effect of adjuvantation.

Our findings challenge the widely held belief that reactions to vaccination are indicative of a stronger immune response [19,20]. While we observed that the coefficients of reactogenicity predictors in adjuvanted vaccinees were significantly increased relative to QIV controls, reactions were consistently associated with diminished immunogenicity. This might suggest that while adjuvantation is able to organize innate immune responses to coordinate the adaptive response, systemic inflammatory responses in the absence of adjuvantation may indicate immune interference. This may fit with literature characterising the pathology of cytokine storms [21–24].

The interaction effects we observed between systemic reactogenicity and vaccine group may that the inflammatory cascade may be a correlate of adjuvanticity. In adjuvanted vaccinees, this could present as increased antibody titers post-vaccination; however, other measurements of increased cellular immune measures would support this hypothesis. Studies have shown that MF59 adjuvant facilitates rapid recruitment of immune cells primarily through the induction of chemokines, which are upregulated following adjuvant exposure[14,25–28]. Greater induction of chemokines, which are known potentiators of inflammatory signalling, may lead to both increased innate immune activity and greater inflammatory reactions in the host[21,29–35].

Of further interest, the coefficient of respiratory reactogenicity was significantly increased in the aTIV group when predicting A/H3N2 immunogenicity. Despite the

antigenic evolution in A/H3N2 over the course of the study, increasing respiratory reactions in adjuvanted children were associated with significant improvements in A/H3N2 immunogenicity, relative to QIV controls. [36,37]. It is possible that respiratory reactions were the result of inflammatory mediators arising from the rapid proliferation of memory B and T cells, boosted from A/H3N2 exposures in prior seasons [38–41]. Were this the case, it would suggest that reactogenicity presents differently in adjuvanted induction of adaptive immune responses, based on whether they are de novo or recalling immunological memory. Further work to characterize reactions after novel exposures in comparison to antibody back-boosting, and the relationship to vaccine immunogenicity, could be valuable in forecasting vaccine effectiveness and identifying individuals at greater risk of vaccine failure. Moreover, identification of correlates of adjuvanticity in children would have noteworthy value for developing next-generation vaccines for this high-risk group, where correlates of protection remain uncertain.

In the fourth chapter, I used a causal mediation framework to investigate relationships between vaccine formulation, post-vaccination HAI titers, and the hazard of influenza infection. I quantified the proportion of relative vaccine protection against influenza A/H3N2 in adjuvanted vaccinees which is mediated by the rise in HAI titers. We found that adjuvanted vaccination conferred a relative vaccine protection of >87%. Of this, an estimated 9% was mediated by the increased post-vaccination HAI titers in the adjuvanted group, as compared to nonadjuvanted vaccinees. We further examined the correlation between predicted hazard of influenza A/H3N2 infection and post-vaccination

titers. We found that in adjuvant-vaccinated children, the hazard of infection declined at much lower titers than the accepted correlate of protection, an HAI of 1:40. To our knowledge, this is the first study which estimated the proportion of relative vaccine protection in adjuvanted vaccinees which is attributable to the increased post-vaccination antibody responses.

Our findings suggest that the MF59 adjuvant confers superior protection against influenza A/H3N2; however, in a season of vaccine mismatch, this protection is not largely mediated by the antibody titers. This may be unsurprising, in light of the known deficiencies in vaccine immunogenicity and effectiveness against A/H3N2 [5,36,42]. However, the difference in protection outcomes, despite limited mediation by antibody responses, raises further questions about the utility of HAI responses as a correlate of protection in children. It may be that adjuvant protection, or perhaps that protection against heterologous variant strains of influenza is driven by cell-mediated immunity. Further studies investigating the relative contribution of humoural and cellular immune responses to adjuvant protection would be novel, and have especially unique value in children.

Moreover, the finding that adjuvanted vaccination confers superior protection irrespective of the accepted HAI correlate warrants further research. While antibodies measured in each season were specific to the vaccine antigen, it is possible that older children with prior seasonal vaccination or exposure histories may preferentially mount memory

responses to those early life exposures [43–47]. Our study did not measure antibodies mounted to heterologous strains of influenza; however, it may be that antibodies induced by adjuvantation are of greater breadth and cross-reactivity. Coupled with the enhanced immunological memory responses to previously encountered strains which may have been more strongly back-boosted in adjuvanted children, this may explain some of the mechanism of enhanced protection. Further work to evaluate the proportion of vaccine protection which is mediated by de novo or anamnestic immune responses would be particularly ground-breaking.

LIMITATIONS

The studies discussed throughout this thesis were secondary analyses of a rigorously designed cluster-randomized controlled trial. This had many advantages including a large sample size, randomization and blinding of vaccine interventions, detailed and standardized prospective follow-up of outcomes, and supporting laboratory methods of serological testing and viral genotyping. However, there are limitations to the studies which must be considered when interpreting our findings. Standardized reporting of symptoms and reactions are subject to misclassification errors by self-reporting. Self-reporting is especially challenging in very young children, who may have more limited communication. This may have resulted in better quality data collected from older children, possibly biasing estimates in younger participants. Moreover, a minimum of two symptoms were required to be referred to testing for influenza infection; as such, we must acknowledge that our findings are only meaningful in evaluating protection against

symptomatic influenza infections. We were not able to make any inferences about the possible vaccine effectiveness or vaccine-attenuated symptom severity in asymptomatic influenza.

Our study used HAI antibody titers as the measurement of adaptive immune responses, and only examined HAI titers against the antigens contained within the vaccines. We acknowledge that the immune response to influenza vaccination is complex, and that HAI titers provide information relevant to humoural immunity. While additional serological data would have been valuable to provide a more fulsome picture of the immune response, this was not possible due to cost limitations and limited sera from participant samples. Evaluating innate or cell-mediated immune activity would allow for a more complete investigation of several hypotheses put forth throughout this thesis on the innate-adaptive cross-talk induced by adjuvanted vaccination.

Methodologically, our studies did not adjust for multiple testing. It is possible, due to the number of models and analyses undertaken, that some significant findings are spurious and the result of type 1 error. We are also limited by the number of influenza events, which are very modest, particularly in the adjuvanted vaccine group. This increases the degree of uncertainty around the estimates comparing influenza protection between vaccine groups. Our study is also influenced by several clustering variables, including colony, participant, and season. These clustered variables increase the similarity between grouped observations, leading to under-rejection of the null hypothesis and biased

estimates if not accounted for. Where appropriate, we adjusted for clustering using robust standard errors and random intercepts in our models. However, it is possible that some findings may not have proven significant due to power limitations for hypothesis testing in our sample.

CONCLUSION

The results of our studies suggest that adjuvanted influenza vaccination may moderate key activities of innate and adaptive immunity in children, leading to enhanced protection and reduced severity of subsequent influenza infections. Research characterizing the possible interplay between innate, humoural and cell-mediated immune responses induced by adjuvanted vaccination should be explored. The studies discussed here have contributed to our understanding of adjuvanted vaccine effectiveness in children. We have used several statistical approaches to address the aims of these studies, using the best possible methodology and benefiting from the rigour of the parent study design. Through this thesis, we have identified several novel research directions to expand the understanding of adjuvant-mediated protection and influenza outcomes. These include greater study of adjuvanted vaccine effectiveness against antigenically distinct strains, and exploring the relative contribution of various immune cell subsets to adjuvanted vaccine protection. Greater understanding of the network of these relationships and their causal contribution to vaccine protection would contribute to the fields of immunology, vaccinology, and epidemiology.

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