# Case Studies in Vaccine Evaluation

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## Table of Contents

## Abstract

Vaccines are estimated to prevent 2.5 million deaths each year. As with any public health intervention, vaccines progress through many stages of development and their evaluation must continue post-licensure. We have used case studies to highlight four key aspects of the lifecycle of vaccines: i) preclinical evaluation, ii) clinical development, iii) safety and iv) long-term effectiveness. *Preclinical evaluation* - We have demonstrated, for the first time, cellular immune responses to a candidate norovirus virus-like particle vaccine produced in *Nicotiana benthamiana* (Medicago Inc., Ste-Foy, QC). Both oral and intramuscular formulations of the vaccine elicited a proliferative response and IFN$\gamma$-producing cells in mice, and antibody-secreting cells in both mice and rabbits. *Clinical development* - In collaboration with MIT Canada Inc., an 80-subject clinical trial was performed to evaluate the use of the MedJet jet injection device to deliver seasonal influenza vaccines. Both cellular and humoral immune responses to a trivalent vaccine delivered by the MedJet were equivalent to those delivered by needle-and-syringe. Of particular interest, there were no significant differences in the frequency of responding memory $CD4^+$ T cells after re-stimulation or in the degree of poly-functionality of these cells in the healthy adult subjects. *Vaccine safety* – We performed a detailed immunologic investigation of peripheral nerve demyelination in a young woman that occurred in temporal association with human papillomavirus vaccination (Gardasil™). Working with peripheral blood mononuclear cells from the patient *in vitro*, unusually strong proliferative and cytokine responses were found to vaccine antigens, primarily driven by B cells. These data support, but do not prove, an association between the vaccine and peripheral nerve paralysis. *Long-term effectiveness* - To monitor the long-term persistence of antibodies after the rubella vaccination, we developed, optimized and validated a low-cost, serum-sparing ELISA to measure serum anti-rubella IgG titers. In collaboration with the Public Health Agency of Canada, this assay was used to assess rubella immunity in 1,752 pregnant women across Canada. This study (*Vaccine 2017*) highlighted socio-demographic inequalities in vaccine uptake and the need to address these issues in underserved populations. Together, these studies illustrate that the life-saving potential of vaccines relies on continuous evaluation of their safety and effectiveness throughout the vaccine lifecycle; both pre- and post- licensure.

## Abstrait

On estime que les vaccins permettent d'éviter 2,5 millions de décès chaque année. Comme pour toute intervention de santé publique, les vaccins passent par de nombreuses étapes de développement et leur évaluation doit continuer après l'approbation. Nous avons utilisé des études de cas pour mettre en évidence quatre aspects clés du cycle de vie des vaccins: i) évaluation préclinique, ii) développement clinique, iii) sécurité et iv) efficacité à long terme. *Évaluation préclinique* - Nous avons démontré, pour la première fois, des réponses immunitaires cellulaires à un candidat vaccin contre le Norovirus composé de particules pseudo-virales fabriquées à partir de plantes (Medicago Inc., Ste-Foy, QC). Les formulations orales et intramusculaires du vaccin ont induit une réponse proliférative et des cellules produisant l'IFNy dans le modèle murin, et des cellules sécrétant des anticorps chez les souris et les lapins. *Développement clinique* - En collaboration avec MIT Canada Inc., un essai clinique de 80 sujets a été réalisé pour évaluer l'utilisation de l'injecteur à jet MedJet pour l'administration de vaccins contre la grippe saisonnière. Les réponses immunitaires cellulaires et humorales à un vaccin trivalent administré par le MedJet étaient équivalentes à celles administrées par une aiguille et seringue. En particulier, il n'y avait pas de différence dans la fréquence de lymphocytes T responsives ou dans le degré de polyfonctionnalité de ces cellules. *Sécurité des vaccins* - Nous avons effectué une étude immunologique détaillée d'un cas de démyélinisation de nerfs périphériques chez une jeune femme qui s'est produit en association temporelle avec l'immunisation contre le virus du papillome humain (Gardasil$^{MC}$). En travaillant *in vitro* avec des cellules mononucléaires du sang périphérique, nous avons trouvé des réponses prolifératives et cytokines exceptionnellement fortes aux antigènes vaccinaux. Ces données appuient, mais ne prouvent pas, une association entre le vaccin et la paralysie nerveuse périphérique. *Efficacité à long terme* - Pour surveiller la persistance de l'immunité contre la rubéole, nous avons optimisé, validé et mis au point un test ELISA à faible coût afin de mesurer les titres sériques d'IgG anti-rubéole. En collaboration avec l'Agence de la santé publique du Canada, ce test a été utilisé pour évaluer l'immunité à la rubéole chez 1752 femmes enceintes au Canada (*Vaccine* 2017), ce qui a mis en évidence les inégalités socio-démographiques dans l'utilisation du vaccin et la nécessité de traiter ces problèmes dans les populations mal desservies. Ensemble, ces études montrent que l'impact des vaccins repose sur l'évaluation continuelle de leur innocuité et de leur efficacité tout au long du cycle de vie du vaccin; à la fois avant et après l'approbation.

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## Contribution of Authors

The specific contributions of the candidate to the manuscripts presented are as follows:

Chapter II

Animal studies were designed by Medicago Inc. and performed at ITR laboratories. *In vitro* experiments described in the manuscript were designed by J Shapiro and S Pillet and executed by J Shapiro. J Shapiro analyzed the data and wrote the manuscript. Editorial guidance was provided by S Pillet, BJ Ward and N Charland.

Chapter III

All authors were involved in study design and editing the manuscript. J Shapiro and B Hodgins performed the time and motion study with guidance from JA Pereira. J Shapiro and B Hodgins entered and cleaned the data. J Shapiro and HE Hendin performed the flow cytometry experiments. A Patel performed serologic testing. J Shapiro wrote the manuscript with significant editorial help from B Hodgins and BJ Ward.

Chapter IV

J Shapiro designed the studies with guidance from BJ Ward. J Schiller and G Panicker produced HPV VLPs. J Shapiro performed experiments, analyzed data and wrote the manuscript, with editorial contributions from BJ Ward.

Appendix I

Gilbert, N. L., Rotondo, J., **Shapiro, J**., Sherrard, L., Fraser, W.D. and Ward, B.J., Seroprevalence of rubella antibodies and determinants of susceptibility to rubella in a cohort of pregnant women in Canada, 2008–2011. *Vaccine* **35**, 3050-3055, doi:https://doi.org/10.1016/j.vaccine.2017.04.057 (2017).

The manuscript was reprinted from the journal Vaccine with permission from Elsevier. The study was designed by NL Gilbert and BJ Ward. ELISA optimization and validation were perfomrmed by J Shapiro. Samples were analyzed by J Shapiro, with help from Dr. Li Xing. Epidemiologic analyses was performed by NL Gilbert, J Rotondo and L Sherrard. Manuscript was written by NL Gilbert and B Ward, which editorial contributions from all of the authors including J Shapiro.

# CHAPTER I

## Literature Review

### 1.1 Quantifying the Impact of Vaccines

Over the past century, vaccines have had a tremendous impact on public health. In fact, of all health interventions, only the provision of safe drinking water has had a larger effect on human health than vaccines[1]. Vaccines are estimated to prevent 2.5 million deaths each year, and the WHO estimates that if all currently available vaccines are implemented globally, vaccines will prevent an additional two million deaths in children under five each year[2]. Vaccines have partially or completely controlled 14 major diseases[1], the incidence for all of which has been reduced by at least 90% in the United States[3]. In addition to saving lives, vaccines have tremendous economic effects. For example, it was estimated that the eradication of small pox generates 1.35 billion USD in savings annually[4].

Traditionally, vaccines have been evaluated based on their efficacy and their effectiveness. Efficacy relates the protection from disease that a vaccine provides to an immunized individual under optimal conditions[5]. Effectiveness, on the other hand, refers to the protection conferred by vaccination in a defined population, and includes both direct effects on the individual and indirect effects on the population[5]. Recently, experts in vaccinology from around the world have concluded that we must now move "beyond efficacy"[6] to estimate the "full public health impact"[7] of vaccines. These evaluations include investigating health economics and cost-effectiveness, long-term impacts, indirect effects, and also highlight certain parameters that have traditionally been ignored. For example, the need to incorporate geographic considerations into vaccine evaluations was exemplified by the recent implementation of the rotavirus vaccine. This vaccine was found to be very effective at preventing gastroenteritis in the developed world, but had lower efficacy in low and middle income countries[7]. Other critical parameters to consider were emphasized by the 2013-2015 Ebola epidemic, where the absence of an effective vaccine allowed the pandemic to spread and consequently have a disastrous and lasting effect on local healthcare systems[7].

The introduction of the pneumococcal conjugate vaccine is an excellent example of the need to look beyond efficacy to understand the full impact of vaccination. This vaccine was developed to prevent invasive pneumococcal disease, such as meningitis, in infants and it has proven to be efficacious[8]. In fact, in the United States a 94% reduction in disease caused by vaccine serotypes was observed in young children[9]. However, there have also been a multitude of indirect impacts. For *vaccinated individuals*, immunization prevents the long-term impacts of bacterial meningitis, which include lower educational achievement and reduced economic self-sufficiency in adult life[10]. In *vaccinated populations*, large reductions in disease have been observed in the unvaccinated as a result of herd immunity[2]. In a broader sense, this vaccine has also contributed to the fight against antibiotic resistance by preventing infection, and thus decreasing the need for antibiotic treatment[8].

Despite tremendous success, barriers remain to realizing the full public health potential of vaccines. First, new vaccines are needed to combat devastating infectious diseases such as malaria, HIV, TB and neglected tropical diseases[11]. In addition to improving health, hypothetical vaccines against a group of prevalent neglected tropical diseases have been branded as "antipoverty" vaccines due their ability to save populations from a poverty trap perpetuated by chronic disease[12]. Second, use of existing vaccines needs to be expanded and optimized for low and middle income countries, where children continue to die from preventable diseases[11]. One strategy to address this is the promotion of needle-free immunization delivery systems, which will increase acceptability and compliance, reduce occupational hazards for healthcare professionals and allow for rapid mass vaccination campaigns during pandemics[11]. Other strategies involve formulating vaccines that can elicit durable immunity from a single dose and that negate the need for cold-chain management[11]. Third, vaccine hesitancy is an ever-growing threat to the success of immunization programs[13]. Continuous demonstrations of the safety of both new and existing vaccines, and effective communication of these results, are crucial to maintaining confidence in vaccine programs[14].

To achieve all of the public health benefits described above, a vaccine, or a vaccine delivery system, must first be conceived, produced, thoroughly evaluated and licensed. The development process costs up to $1 billion and can take between 10-30 years[15,16], yet it is often unsuccessful. In fact most vaccine candidates fail in the preclinical stage, and less than 1 in 15 vaccines that are evaluated in a Phase II clinical trial are eventually licensed[15]. If a vaccine candidate is

licensed, continued research is required to monitor the effectiveness, impact and safety of the vaccine[17]. This review will focus on four key aspects of the vaccine development process: 1) Preclinical evaluation, 2) Clinical development, 3) Vaccine safety and 4) Long-term effectiveness.

## 1.2 Preclinical Evaluation

The purpose of preclinical, or nonclinical, testing is to determine if a vaccine can be moved from the laboratory to the clinic for human trials[18]. While the extent and specifics of this phase depend on the characteristics of the vaccine, there are two main criteria that any candidate must fulfill. First, the vaccine must be safe and non-toxic in a relevant animal model. Second, there must be demonstration of proof-of-concept and collection of initial immunogenicity data for the vaccine. In addition to the antigenic components of the vaccine, any adjuvants, additives, delivery devices and alternative delivery routes must also meet these two criteria[18]. The information gathered is then used to support the early phases of clinical evaluation[19]. As the number of new vaccine candidates under evaluation exploded in the early years of the 21st century, representatives from academia, industry and national regulatory agencies drafted the 'WHO guideline for nonclinical evaluation of vaccines' as a comprehensive resource to guide and standardize the first phase of vaccine evaluation[18,20]. This document has since become the gold standard for designing preclinical vaccine trials[15].

### 1.2.1 Toxicity & Safety

Vaccines have two main potential sources of toxicity: the intrinsic toxicity associated with the introduction of exogenous material, and toxicity caused by the ensuing immune response to the antigen or any of the vaccine's non-antigen componnents[21]. The goals of toxicity studies are therefore, to understand the potential toxic effects of a vaccine, predict toxicity in humans and decide if the candidate is safe enough to be administered to humans[18,20]. Both sexes of a single relevant animal model are typically used for these studies. The ideal model is an animal that is both susceptible to infection by the targeted pathogen and develops a measurable immune response to the vaccine – such a model is considered 'relevant'[20]. If the full criteria for relevance cannot be met, a second animal model may be needed[20]. The most commonly used animal models in vaccine toxicity studies are rodents (rats and more rarely mice) and rabbits[22].

Furthermore, if non-standard routes of administration are being considered, an animal that is relevant to the site of administration should be chosen[20].

Typically, vaccines are evaluated with repeat-dose toxicity studies[22]. The number of vaccine doses used and the administration schedule should exceed the dose that will be used in clinical trials on a mg/Kg basis to maximize both exposure to the vaccine and the elicited immune response[18]. Furthermore, all possible effort should be made to ensure that the materials used to produce the vaccine, the method of delivery and the route of administration mimic what will be used in human trials[20]. For example, if the vaccine will be delivered with an adjuvant, then in toxicity studies, the animals must be immunized with a combination of the vaccine and the adjuvant[22].

The parameters monitored after immunization, include mortality, body weight, food consumption, the local inflammatory response, serum chemistry and histopathology[18,22]. The type of vaccine dictates other parameters that must be observed. For example, the degree and stability of attenuation in live attenuated vaccines and the integration of DNA from DNA vaccines (which could lead to tumorigenesis or chromosomal instability) must be considered[20]. If the target product profile of the vaccine includes children or women of reproductive age, additional studies in juvenile animals and/or pregnant animals (ie: reproductive and developmental toxicity) may be required by regulatory authorities[23]. The results of these studies allow investigators to determine the starting doses in clinical trials and to identify any organs affected by toxicity that will be important to monitor in human studies[22]. Ultimately, toxicity studies inform the risk/benefit calculations that determine if a novel vaccine candidate can proceed on to clinical development.

### 1.2.2 Immunogenicity

Nonclinical testing provides investigators with an opportunity to demonstrate the proof-of-concept of a vaccine candidate by evaluating immunogenicity *in vitro* and in animal models[18]. *In vivo,* the immunogenicity of a vaccine is typically monitored prior to toxicity studies in order to establish the relevance of the chosen animal model(s)[18,22]. At this stage, vaccine efficacy is measured with immunoassays that address the most relevant endpoints[22]. Ideally, endpoints should be correlates of protection (CoP) that have been established in challenge studies[18]. Coupled with animal models that accurately represent human pathology, the existence of a

confirmed CoP can lead to predictions of vaccine efficacy that are quite accurate[24]. However, a truly predictive CoP is often lacking in the early stages of vaccine development[24].

Most vaccines rely on protection mediated by antibodies that block viruses and bacteria from infecting target organs[25]. Recently, increased emphasis has been placed on evaluating and improving the functionality of antibodies[25]. For example, antibodies that block the binding of norovirus to the cellular receptor were found to correlate with a lower risk of illness[26]. Therefore, in immunogenicity studies, humoral responses are often measured with multiplex assays to quantify the functionality of antigen-specific antibodies[22].

T cell responses, although often overlooked in vaccine development, can be required to induce antibodies and to elicit a durable memory response[27]. Mechanistically, $CD8^+$ T cells have the capacity to secrete anti-viral cytokines and are cytotoxic, meaning they can recognize and kill infected cells[27]. $CD4^+$ T cells produce cytokines and chemokines and support $CD8^+$ T cell and B cell responses[27]. Therefore, recent work has supported the investigation of T cell activity as CoPs in both clinical and preclinical work. For example, in influenza infection, pre-existing $CD4^+$ memory T cells are better correlates of immunity than antibody titers[28]. Furthermore, polyfunctional T cells (T cells that produce ≥2 cytokines) have been associated with protection and decreased progression of *Leishmania major*[29] and *Mycobacterium tuberculosis*[30], among other pathogens.

The multitude of tools for a comprehensive preclinical evaluation of a vaccine are well-illustrated by the nonclinical studies performed on the Sanofi Pasteur tetravalent, live-attenuated dengue vaccine[33]. First, the genetic and phenotypic stability of the viral vector vaccine was monitored through many passages. Next, human cell cultures were used to assess infectivity and the subsequent activation, maturation and secretion of cytokine and chemokines. DNA array profiling was also used to understand the genetic signatures of human monocyte-derived dendritic cells infected with vaccine strains, other attenuated strains and the wild type virus. Finally, non-human primates were chosen as an animal model, as they can be infected (although asymptomatically) with dengue. The level of viremia was used as a CoP to assess attenuation and protection from challenge. In addition, neutralizing antibodies and the induction of polyfunctional T cells were measured[31,32]. Ultimately this animal model was used to establish the administration schedule used in early clinical trials.

### 1.2.3 Injector Systems

In addition to evaluating a new vaccine, any new delivery system, such as pen or jet injectors, must also undergo preclinical development. This largely consists of performance testing to ensure that the injector is reliable, accurate, and does not interfere with the vaccine formulation[34]. Importantly, manufacturers must demonstrate that the injector can reliably deliver the correct dose of vaccine at the appropriate depth of injection[34]. Ideally, studies to demonstrate these parameters should be done in human skin from cadavers or surgery[35,36]. In addition any component of the device that comes into contact with the patient or the vaccine must be tested for bio-compatibility (ie irritants, leachables and extractables)[34]. Another critical performance characteristic of a novel delivery system that will be used for multiple administrations is prevention of cross-contamination[34].

### 1.2.4 Limitations

Despite the large effort and cost expended on the preclinical phase of vaccine development, it is important to consider that animal models often fail to predict either immunogenicity or efficacy (or both) in humans[18]. In fact, most vaccine candidates fail in either the preclinical stage or the early clinical stages of development[15]. The true capacity of a vaccine to reduce the burden of illness can only be measured in human clinical trials.

## 1.3 Pre-Licensure Clinical Development

Once a vaccine candidate has successfully met the standards for the preclinical phase, it can be tested in humans. The main goal of this phase of development is to collect the necessary evidence of vaccine efficacy and safety to support licensure of the product[37]. Because these trials rely on healthy human subjects, the study protocols have to be meticulously planned, approved by ethics committees and good clinical practice must be followed throughout[38]. Clinical trials also allow investigators to optimize dose regimens and schedules and to assess co-administration with other vaccines[37]. The pre-licensure development of a vaccine is divided into three stages: Phase I, phase II and phase III.

### 1.3.1 Phase I Clinical Trials

Phase I trials, also known as first-in-man studies, are the first time a vaccine is administered to human subjects. Typically, slowly-increasing doses of the vaccine are administered to small cohorts of healthy subjects under intense surveillance in order to detect adverse events and to generate preliminary data on the immune response[38]. Testing multiple doses allows investigators to establish a relationship between the dose of the vaccine and the measured responses[38]. While relatively straightforward, there are several issues that can complicate phase I trials. For example, live vaccines are sometimes shed or excreted, which may pose a risk to those in contact with clinical trial participants[38]. In this case, it may be necessary to perform the phase I trial under physical containment[38]. Another interesting complication of first-in-man trials arose during the 2013-2015 Ebola outbreak. When the WHO declared the outbreak a "public health emergency of international concern", a widespread effort began to accelerate development of vaccine candidates that had not been previously administered to human beings[39,40]. Accordingly, phase I trials of two Ebola vaccines were rapidly initiated in both Africa and Europe. The trials allowed authorities to rapidly select the appropriate vaccine dose for phase II/III field studies[40]. Accelerated clinical trials of this nature were unprecedented but highlight the requirement for the vaccine development process to adapt to novel technologies and public health emergencies.

Phase I studies can also be complicated by geography and politics. Many serious pathogens are most prevalent in low- and middle-income countries. Unfortunately, these countries often have weak regulatory structures and local authorities are hesitant to permit 'experimental' drugs or vaccines to be tested first in their populations[41,42]. Their hesitation is justified to some degree by the sad history of predatory or 'colonial' science: when drugs or vaccines are tested in low-resource countries for a variety of reasons, such as high disease prevalence or low cost to perform studies, but are then not made available in these same countries post-licensure[43].

### 1.3.2 Phase II Clinical Trials

In phase II trials, the vaccine moves from healthy adult subjects to participants who represent the target population of the vaccine[37]. In these placebo-controlled trials, hundreds of patients are typically included, primarily to establish the optimal dose of the vaccine and to assess adverse events[38]. To gain broader relevance, phase II trials are often performed in various geographical locations and can include progressively younger cohorts of subjects[38]. This "age de-escalation"

approach is used for vaccines that ultimately target young infants: initial safety and efficacy data are collected in adults or older children to minimize risks[37]. During this phase, the assays that will be used to measure the immune response in future trials are selected and validated, and the methods of vaccine production are finalized[38]. Another important aspect of this phase is the harmonization of the administration of the new vaccine with existing immunization schedules[38]. It is crucial to ensure that the new vaccine does not interfere with existing recommended vaccines and, conversely, that existing vaccines do not affect the safety or immunogenicity of the vaccine being introduced. These considerations are increasingly important as more vaccines become available that target people at key developmental phases and/or specific ages (ie infancy, adolescence, 65 year-olds)[38].

The second phase of clinical development can also include human challenge studies, in which healthy, consenting, vaccinated subjects are exposed to the wild-type infectious agent in a highly controlled setting[38,44]. This type of study can provide a preliminary demonstration of vaccine efficacy, allow for optimization of the vaccine formulation and identify correlates of protection, but is only ethical when the induced disease is either self-limiting or treatable[44]. When appropriate, phase II challenge studies can save time and money and prevent widespread exposure to an ineffective vaccine in further trials. This approach has been used in the development of vaccines to protect against many pathogens (eg: influenza, cholera and even malaria, among others), and has yielded important lessons in pathology, immunity and vaccine efficacy[44-46].

### 1.3.3 Phase III Clinical Trials

Phase III trials are also referred to as pivotal trials because they provide the robust clinical evidence that is necessary to support the licensure of a new vaccine[37]. These studies typically include many thousands or tens of thousands of subjects[16], and either estimate the efficacy of a vaccine or rely on immunogenicity data to indicate whether the vaccine will be able to prevent disease[37]. The gold standard for phase III studies are large-scale, adequately powered, randomized, controlled, double-blind trials[38]. However, in some cases, other study designs may be necessary. For example, a cluster randomization trial allows the investigator to randomize larger units, such as a family or a village, as opposed to an individual[38]. The phase III trials initiated during the Ebola crisis are an excellent example of the necessity for cluster

randomization. In these trials, certain groups of individuals become clusters (or rings) because they were deemed at high risk of infection due to their social or geographical proximity to an index case[47,48]. Clusters were then randomized to either receive the experimental vaccine immediately or at a later time[35]. Clusters randomized to delayed vaccination acted as a control group for those vaccinated immediately. This somewhat controversial design combined vaccine evaluation and implementation, allowing investigators to estimate efficacy and effectiveness, while ensuring that all eligible participants were eventually vaccinated[35].

Regardless of study design, certain criteria must be met in phase III trials to support submission of a dossier for licensure (ie: a biologic licensure application (BLA))[49]. Importantly, the vaccine used during the trial must be manufactured under current Good Manufacturing Processes (cGMP) such that each step has been validated and will continue to be used post-licensure[20,37]. In addition, the primary outcome, as well as secondary and exploratory outcomes, must be precise, achievable and clearly defined before the trial begins[38]. Finally, the control or comparator preparation must be thoughtfully chosen. Many trials use a true placebo as a comparator because it cannot offer any protection from disease, will not cause any background safety issues and can be manufactured to look exactly like the experimental vaccine[38]. However, local ethics committees frequently object to the use of a true placebo – particularly in studies involving children – leading to the use of a comparator vaccine that provides some benefit to the 'placebo' group but is not widely available in the community, such as hepatitis A vaccine[38].

### 1.3.4 Assessing Immunogenicity and Efficacy in Clinical Trials

Assessment of immunogenicity throughout clinical development follows the principles discussed above for preclinical evaluations. Typically, immunogenicity is primarily characterized by assessing functional antibody responses in the serum but should also include other immune parameters relevant to the mechanism of action of the vaccine, such as T cell responses, mucosal responses, cross-reactivity, antibody-dependent cellular cytotoxicity (ADCC)[37,50]. During clinical trials, the immune response is measured from specimens collected pre-vaccination and at pre-determined timepoints post-immunization[37]. In double-blinded randomized controlled phase III trials, it is crucial that laboratory staff are blind to vaccination status. Data collected can serve to establish CoP for the pathogen in question, or to estimate vaccine efficacy.

### 1.3.5 Clinical Evaluation of Injector Systems

Novel vaccine delivery devices must also be evaluated in a clinical setting. Typically, injection devices are first tested with a mock vaccine. For example, a safety adaptor developed to increase reliability and reproducibility of intra-dermal (ID) injections was tested by delivering saline to healthy volunteers[51]. In this case, researchers measured parameters such as the diameter of ID wheals, amount of liquid on the surface of the skin post-vaccination, injection safety and pain to evaluate whether the adaptors could be used in the field. High-resolution ultrasound can also be used to establish the depth of injection[52]. Investigators must demonstrate that users can safely and effectively set up the device[34]. In the case of jet injectors, for example, users must be able to draw the proper dose, perform the injection and dispose of single-use material[34]. Following studies of safety and feasibility in healthy volunteers, injection devices can then be tested in non-inferiority trials with existing vaccines that have well-defined CoP, such as inactivated polio vaccine[53], the measles, mumps and rubella (MMR) vaccine[54], Bacillus Calmette–Guérin (BCG)[55] or influenza vaccines[56]. These field trials typically include the standard measures of immunogenicity for the vaccine being tested and a detailed safety analysis.

## 1.4 Evaluating Vaccine Safety

Assessment of vaccine safety must be included in all pre- and post-licensure studies[37,57]. Because prophylactic vaccines are given to healthy, and potentially vulnerable, individuals, the standards for vaccine safety are very high[57]. Thus, for both the public and regulatory agencies, the tolerance for risk is much lower than for a drug that is used as treatment[16]. National regulatory agencies provide guidelines for how vaccine safety should be monitored and reported[37]. Additionally, the WHO has published a manual detailing protocols for surveillance of adverse events following immunization (AEFI)[58].

AEFI can be classified into six broad categories[59]. First, the AEFI can be caused by the vaccine itself, such as redness at the site of injection or a fever in the days following immunization. Second, an adverse event can occur due an error in vaccine administration. Third, the symptoms experienced could be coincidental with vaccination. Fourth, an AEFI, such as syncope, can be a result of immunization anxiety. Fifth, the event could be related to a vaccine failure, such as a vaccine that was not kept in the cold chain. Finally, the cause of an AEFI can be unknown. All of these etiologies are important to consider when evaluating vaccine safety.

### 1.4.1 Safety Monitoring in Clinical Trials

Clinical trials are considered the gold standard for the evaluation of vaccine safety but even the largest trials (40,000-60,000 subjects) are 'small' compared to when a new vaccine is used in whole populations. As a result, safety data from clinical trials should only be generalized with caution[57]. Typically, after an experimental vaccine is administered, patients are monitored for 20-60 minutes for severe immediate reactions[37]. Solicited symptoms, such as pain, redness and swelling, are then collected for a minimum of 4-7 days following each dose. Unsolicited adverse events (AEs) are then collected for the entire period in between doses and for at least four weeks after the final dose[37]. Serious adverse events (SAE) should be collected for a minimum of six months following vaccination, or for a minimum of twelve months if the vaccine contains a novel adjuvant[37]. In addition to this standard protocol, specific safety issues identified in earlier phases of development should be monitored in phase III trials[38]. When an adverse event is reported, it must be characterized and assessed for severity and causality[37]. Only vaccines that meet the requirements of national regulatory agencies are considered for licensing.

### 1.4.2 Post-Licensure Safety Monitoring

Once a vaccine is licensed, continued monitoring of safety serves to identify rare AEs that occur at rates too low to be detected in clinical trials, and to identify safety issues that arise due to changes in manufacturing[57]. Post-licensure, AEs are monitored through both active and passive reporting. Active reporting relies on a continuous organized process to quantify, as completely as possible, the number of AEFIs in a given population[60]. Typically, this process revolves around one or more pre-specified AEFI of interest[60]. For example, due the association of intussusception with rotavirus vaccination, pre-licensure clinical trials of new vaccine candidates were powered to detect this rare SAE[61]. Despite this cautious approach, post-licensure studies to actively monitor the risk of this complication were initiated immediately upon introduction of new rotavirus vaccines into national immunization programs[60,61]. Active surveillance can include collection of data from sentinel sites, or formal epidemiological research such as cohort or case-control studies[60]. While these studies can be very effective, they require significant resources and expertise.

Alternatively, post-licensure vaccine safety can be monitored via passive reporting systems, in which any health care provider or patient can spontaneously report what they believe to be an

AEFI[58]. Passive surveillance is useful for the early detection of unknown SAEs that may signal a larger trend[58]. The Vaccine Adverse Event Reporting System (VAERS), which is co-administered by the U.S. Food and Drug Administration and Centers for Disease Control, receives and average of 30,000 reports annually[62]. Submitted AEFI are coded, classified and graded for severity, and monitored to detect vaccine safety signals[62]. Although clearly useful, passive reporting systems are often complicated by both under-reporting and over-reporting of AEFI, and reports of variable quality and completeness[62]. Furthermore, lack of an unvaccinated control groups makes it impossible to calculate the risk of a particular adverse event[62].

### 1.4.3 Assessing Causality

The WHO defines a causality assessment as the "systematic evaluation of the information obtained about an AEFI to determine the likelihood that the event may have been caused by the vaccine(s) received"[58]. Although such evaluations typically cannot establish a definite causal link between the vaccine and the AEFI, they can ascertain a degree of association[58]. Causality can be assessed at both the population and individual levels[58]. For populations, large scale epidemiological studies allow investigators to assess the absolute or relative risk of experiencing a particular AE after a particular immunization[63,64]. For individual cases, a statistical causal relationship is not sufficient to draw conclusions[63]. In 2013, the Global Advisory Committee for Vaccine Safety published a standardized protocol for collecting and interpreting data and assessing causality for a single AEFI[63].

Generally, a causality assessment relies on several basic principles[58]. First, it is essential that vaccination precede the adverse event. Second, the association should be compatible with existing knowledge of the biological process of the vaccine. Third, a relationship is more likely to be causal if there is a strong statistical association that is consistent among various populations. AEFI can be immune mediated, related to the biologic activity of the vaccine (ie: viral replication) or a reaction to the injection[64]. Of particular interest are immune-mediated AEFI, which can be associated with T cell effector functions, antibodies and autoantibodies, complement activation, hypersensitivity reactions and cytokines, among other components of the immune system[64]. Mechanistically, immune-mediated AEFI can be initiated by mechanisms such as molecular mimicry, antigen persistence, epitope spreading or bystander activation[64]. Together,

the process of assessing causality can serve as a catalyst for larger studies or to maintain confidence in vaccine programs by reassuring the public that vaccines are safe.

### 1.4.4 Vaccines and Autoimmune Diseases

Many publications have alleged an association between vaccines and autoimmune diseases[65]. In the majority of these cases, AEFI are reported as temporal associations and do not include a demonstration of causality[65]. This has become a larger issue in recent years as more vaccines targeting adolescents are introduced into immunization programs, because autoimmune diseases are often initially diagnosed in this age group[65]. Assessments of causality in this context are complicated by a lack of understanding of both autoimmune pathology and vaccine-mediated immunity. Evidence for such associations are therefore largely based on documented cases of infection acting as an environmental trigger for autoimmunity, such as the clear association between Guillain-Barre syndrome (GBS) and prior *Campylobacter* infection[65,66].

The link between autoimmunity and vaccines has only been confirmed in very few cases. Most notably, the relative risk of developing GBS within five weeks of receiving the 1976-77 vaccine for swine influenza was 7.6[67]. In addition, a small increase in the incidence of narcolepsy was found to be associated with the 2009 vaccine for pandemic swine flu in several Nordic countries[68]. While many other allegations have been made, demonstrating causality has proven difficult and large scale epidemiological studies and meta-analyses have found no increased risk compared to unvaccinated populations[69]. Given public concern about this issue and the many published cases of potential autoimmune-like AEFI, it is critical for the vaccine community to continue to collect data on this topic and to incorporate an assessment of autoimmune parameters into the preclinical and clinical stages of vaccine development[65].

## 1.5 Long Term Effectiveness

In addition to continuous safety monitoring, the effectiveness and impact of a licensed vaccine or a vaccination program should be evaluated over time[38]. Post-licensure, or phase IV trials, allow investigators to understand how a vaccine is working in a real-world context, assess the duration of vaccine-induced immunity (ie: the need for booster doses), and potentially expand use to new target populations[70]. These studies also provide information as to the effects

of herd immunity, how well the vaccine strain matches circulating strains and how microbial ecology is affected by the introduction of a vaccine[71].

One way to measure vaccine effectiveness is by quantifying the incidence or the number of deaths attributed to the vaccine-preventable disease[38]. However, it is well known that under-reporting leads to under-estimations of disease prevalence[72], and this method can mask substantial geographic and social heterogeneity[73]. This is particularly true for asymptomatic, mild or self-limiting infections that are not included in hospital records[74]. Seroepidemiology has therefore been used extensively over the past three decades as an alternative to measuring incidence of vaccine-preventable diseases, and has proven to be a valuable method for assessing the impact of vaccines post-licensure[75].

### 1.5.1 Seroepidemiology

Seroepidemiology is most often used to measure the prevalence of antigen-specific antibodies in sera collected from a cross-sectional cohort[72]. Quantifying levels of serum antibodies is a direct way to measure population immunity for a variety of infectious diseases[73]. This approach works best to detect immunity to pathogens that are antigenically stable and for which there is a clear serological marker of past infection or vaccination[73], such as measles, mumps, rubella, polio, hepatitis B and small pox[73,74]. Samples for serosurveillance studies can either be collected randomly from the population, or more commonly, are samples of convenience (ie residual sera from diagnostic tests)[72]. Once samples are collected, multiplex assays can be used to measure antibodies specific to multiple different pathogens[73]. For national or international programs, standardizing assays and cut-offs across study sites remains a challenge[72,76].

Data gathered from seroepidemiologic studies can be applied in many ways to better understand vaccine effectiveness and impact. Levels of seroprotection can indicate whether immunization targets have been reached[74]. This is most effective in situations where the vaccine induces a humoral response distinct from that induced by natural infection or when the wild-type pathogen is no longer circulating[74]. Studies performed in the same population at regular intervals can show changes in the number of susceptible people over time, and thus model waning immunity or predict potential outbreaks[72,75]. Importantly, serosurveillance allows public health authorities to identify sub-populations that are at increased risk of contracting a vaccine-

preventable disease[72]. For example, a study in Quebec found that immigrants and refugees had relatively high rates of susceptibility to measles, mumps and rubella, thus supporting the need for targeted supplementary vaccination programs[72,77]. Another example comes from yearly serosurveys in the years preceding and following the introduction of the measles vaccination program in the UK[78]. In 1994-95, a mass campaign to immunize all school-aged children was found to be successful in those 5-16 years of age, but susceptibility remained high in toddlers. This finding led to the implementation of a second dose of MMR at four years of age. Ultimately, seroepidemiology has proven to be an incredibly effective tool for monitoring the long-term impact of vaccines and informing vaccine policy. The proposed establishment of national and global serology banks for robust serosurveys thus has the potential to optimize vaccination strategies and contribute to eradication efforts[73].

## 1.6 Rationale & Research Objectives

This thesis is a series of distinct projects that address a range of barriers to achieving the full public health potential of vaccines. These projects involved four different vaccines, each at a different stage of the vaccine lifecycle. First, we had the opportunity to focus on one element of the preclinical evaluation of a novel norovirus vaccine candidate. Norovirus is a leading cause of acute gastroenteritis and food- or water-borne outbreaks globally and has a large impact on child health in low- and middle-income countries[79,80]. An effective vaccine would improve health in young children, the elderly, and travelers. A second project focused on the initial clinical evaluation of a novel jet injector for the delivery of seasonal influenza vaccine. Needle-free immunization has been heralded as a method to increase vaccine uptake and compliance, improve injection safety and allow for rapid mass immunization in public health emergencies[11]. Demonstration that a new delivery system is as good or better than existing systems in terms of safety and immunogenicity is crucial before such a device can be implemented on a large scale. A third project addressed the critical issue of vaccine safety by characterizing and assessing causality of an AEFI associated with Gardasil, a licensed human papilloma virus vaccine. The goal of this work was to demonstrate the value of detailed investigations of even an extremely rare SAE. The fourth project targeted the post-licensure phase of the vaccine lifecycle by developing, validating and implementing a low-cost, serum-sparing serological assay to measure the long-term effectiveness of the rubella vaccine in pregnant Canadian women. This project was

conceived to serve as a proof-of-concept in establishing inexpensive, multiplex, point-of-care tests to monitor vaccine-preventable disease serostatus in Canada. Collectively, the work described in the chapters below provides an overview of key issues in the lifecycle of a vaccine and contributes to our understanding of the vaccine development pathway.

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# CHAPTER II

## Preclinical Evaluation of the Immunogenicity of a Norovirus Virus-Like Particle Vaccine Candidate Produced in *Nicotiana benthamiana*

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## 2.1 Preface

When a new vaccine candidate is proposed, it must first undergo thorough evaluation in animal models. The objectives of this preclinical phase are to demonstrate the safety and immunogenicity of the vaccine, and to ultimately determine if the candidate can proceed to clinical development. While serological correlates of protection are historically the most commonly used to evaluate the efficacy of a vaccine, cellular markers of immunogenicity are increasingly recognized as important to consider. In particular, the ability of lymphocytes to proliferate, produce cytokines and secrete antigen-specific antibodies are relevant read-outs of vaccine immunogenicity, which can strongly impact efficacy. In this chapter, the immunogenicity of a plant-derived virus-like particle vaccine candidate to prevent norovirus infection is characterized in murine and rabbit models. These evaluations included 1) comparisons between the oral and intra-muscular routes of administration, 2) the use of adjuvants, 3) the addition of antacids to the oral formulation to avoid vaccine alteration during gastro-intestinal transit and facilitate mucosal delivery, and 4) preliminary dose-ranging studies. The work discussed here represents a small portion of a large effort to perform a comprehensive preclinical assessment of plant-derived norovirus virus-like particles as a vaccine candidate.

## 2.2 Abstract

Norovirus (NoV) is the most common cause of diarrheal episodes globally. Despite its prevalence, issues with *in vitro* cultivation systems, genetic variation and animal models have greatly hindered vaccine development. Plant-derived virus-like particles (VLPs) partly address these concerns because they are highly immunogenic, and their production can be rapid to accommodate emerging viral strains. Agroinfiltration of *Nicotiana benthamiana* has recently been exploited by Medicago Inc. to generate NoV VLPs (NoVLP) as a novel vaccine candidate. Here, we used the murine and rabbit models to evaluate the immunogenicity of NoVLP, and to compare vaccine doses, formulations and routes of administration (ie oral (PO) and intra-muscular (IM)). In mice, we demonstrated that the NoVLP elicits a robust proliferative response through both PO and IM immunization, and that IM delivery stimulates antibody- and IFNγ-secreting cells. In general, the vaccine formulation that elicited the greatest overall response was the high dose of VLP (10μg) administered IM with alhydrogel as an adjuvant. Oral immunization in mice led to an increase in NoVLP-specific memory B cells that was maintained for at least 42-days post-immunization. In the rabbit model, PO vaccination elicited IgA-secreting peripheral blood mononuclear cells. Taken together, these data suggest that both the PO and IM formulations of the plant-derived NoVLP are immunogenic in the two animal models studied. This work represents an important part of the on-going preclinical development of this novel vaccine candidate.

## 2.3 Introduction

Noroviruses (NoV), members of the *Caliciviridae* family, are the leading cause of acute gastroenteritis[1] and account for an estimated 200,000 deaths annually world-wide[2]. Infection with NoV causes self-resolving gastrointestinal symptoms in approximately one third of exposed immunocompetent adults[3]. However, in young children, the elderly and the immuno-compromised, infection can be more severe, chronic or even life-threatening[4]. Furthermore, malnutrition has been shown to contribute to pathogenesis, putting those in low-resource settings at increased risk of complications[5]. Global direct healthcare costs related to NoV are thought to exceed $4 billion, with an additional $60 billion of societal costs[6]. Because NoVs are

transmitted by the fecal-oral route[7], outbreaks often occur in crowded areas such as nursing homes, schools and cruise ships[8].

Despite the public health impact of NoV, there are currently no licenced vaccines or treatments. Vaccine development has been hindered by several key issues. Notably, the large genetic diversity within the NoV genus poses an important problem. A report of sequential infection of young children with two distinct NoV genotypes suggests that immune responses are not reliably cross-reactive[9]. This is critical to consider, as epidemiological data indicates that a novel variant of the dominant GII.4 genotype appears every 2-7 years due to genetic drift[7]. In addition, the duration of homotypic immunity after either natural infection or vaccination is not currently known. Human challenge studies in adults originally suggested that immunity lasts from 2-24 months[10,11]. However, a recent mathematical modeling exercise indicates that immunity can last from 4.1-8.7 years[12]. Both of these estimates represent a shorter duration of immunity than that elicited by many infections that are currently vaccine preventable[13]. Furthermore, there is considerable debate about appropriate correlates of protection with which to evaluate candidate vaccines. Several assays have been proposed and used in various challenge and vaccine trials, including salivary and fecal IgA, virus-specific memory IgG cells, serum hemagglutination inhibition antibodies and functional serum histo-blood groups antigen (HGBA)-blocking antibodies[14]. Of particular interest are HGBA-blocking antibodies, which can inhibit NoV VLPs from binding to pig gastric mucin *in vitro*, and have been suggested as a surrogate for neutralizing antibodies and thus as a correlate of protection[15]. However, recent reviews suggest that until more is known about NoV pathogenesis, it is important to consider multiple potential correlates of protection[7,14]. Vaccine development is also complicated by the fact that traditional animal models are not ideal for NoV due to the mucosal tropism and the species specificity of the virus. Notably, human noroviruses do not infect mice[4], and there are important differences between human and murine intestinal immunity[16]. For example, mice have lower levels of serum IgA than humans, and there are major differences in the subsets and functionality of B cells[16]. Finally, NoV has traditionally been very difficult to grow and therefore to study *in vitro*. A cultivation system that allows multiple strains of NoV to reproduce in a stem-cell derived, non-transformed human intestinal enteroid monolayer culture was only recently described but this system is not likely to be introduced widely in research laboratories and is

certainly not amenable to scale-up to commercial production[17]. These challenges not only complicate vaccine development but also encourage the investigation of novel vaccine platforms.

Virus-like particles (VLPs) are nanoparticles that mimic viral virions but do not contain any genetic material and are therefore non-infectious. VLPs elicit strong antigen-specific immune responses because they maintain the repetitive antigenic conformation of the wild-type virus[18]. Over two decades ago, the major capsid protein (VP1) of NoV was shown to self-assemble into VLPs[19]. Since then, several expression systems have been used to produce immunogenic NoV VLP vaccine candidates[20]. However, the duration of the immune response elicited by these vaccines is unknown[21], and early clinical trials with several vaccine candidates have failed to elicit immune responses that cross-react between genotypes[7]. Furthermore, the effectiveness of these vaccines in the populations most at risk of severe disease complications (young children, the elderly and the immunocompromised) has yet to be demonstrated[21].

Plants are a promising and flexible platform for VLP production because they can safely produce large quantities of protein at low cost[22]. In particular, transient transfection of *Nicotiana benthamiana* using *Agrobacterium tumefasciens* has been shown to yield highly antigenic VLP vaccine candidates[22] and allows for rapid production of new vaccine strains to accommodate genetic drift[23,24]. Recently, this system has been used to produce a high yield of VLP bearing GII.4 NoV VP1 proteins (NoVLP) as a novel vaccine candidate. Here, we demonstrate for the first time that NoVLPs produced in *N. benthamiana* elicits a robust and functional immune response, as well as primary and memory antibody-secreting cells, when delivered either orally or intra-muscularly to mice. We also show that vaccination can elicit IgA-secreting cells in the rabbit model. Together with complementing data on the antigen-specific humoral and mucosal responses to the vaccine that will be reported elsewhere (S. Pillet, personal communication), this work suggests that NoVLP produced in plants are a promising vaccine candidate that merit further characterization and development.

## 2.4 Results

The experimental protocol for mice is summarized in Figure 1A. Briefly, 8-10-week-old female Balb/C mice were immunized three times at two-week intervals with NoVLP at doses of 0.1, 1 and 10μg for IM delivery and 200μg for PO delivery. Mice were sacrificed at three timepoints (D7, D21 and D42) to assess cellular responses. Alhydrogel (AH) was used as an

adjuvant for IM vaccination and an antacid (AC) composed of sodium bicarbonate was included for PO vaccinations. Oral immunizations were delivered by gavage in 0.6mL. Injections were delivered in the hind limb in 0.05mL. Animals that received saline administered by injection or gavage served as controls (referred to as 'placebo' groups throughout).

### 2.4.1 IM and PO NoVLP stimulate a lymphoproliferative response in the murine model

First, splenocyte proliferation after re-stimulation with the NoVLP was analyzed by bromodeoxyuridine (BrdU) incorporation (Figure 1B-D). **Seven days after the first dose of vaccine**, the IM+AH groups, but not the unadjuvanted IM groups, had greater proliferative responses than the placebo group (Figure 1B). The adjuvant consistently boosted this lymphoproliferative response, and the 0.1μg +AH vaccine elicited the greatest proliferation (mean stimulation index of 2.16). At this timepoint, there were no significant differences between the PO-VLP and PO-placebo groups, however this observation was likely confounded by inexplicably large baseline proliferation seen in the placebo PO groups. No significant differences in proliferation were found between the IM and PO vaccinated animals.

**At D21**, seven days after the second immunization, the proliferative response among vaccinated animals was generally larger than at D7 (Figure 1C). The middle and high dose IM vaccines ± AH tended to elicit larger responses than their respective placebo formulations, with the 10μg formulation reaching statistical significance (1.37 vs 0.37, $p = 0.019$). Consistent with D7, the adjuvated formulations elicited stronger responses than the unadjuvanted vaccines, except for at the highest dose of VLP. A dose-response was observed among the IM groups and the 10μg formulation yielded a significantly higher proliferative response than the 0.1μg dose (1.37 vs 0.39, $p = 0.0041$). For PO vaccinated animals, both groups had a strong response to the vaccine compared to placebo (without AC: 2.67 vs 0.15, $p = 0.0001$; with AC: 3.31 vs 0.67, $p = 0.0397$). It is interesting that, after two immunizations, the oral vaccine tended to elicit a stronger response than IM vaccination.

**By D42**, 14 days after the third immunization, the proliferative responses were unexpectedly lower than D21 (Figure 1D). Only the proliferative response to the 1μg +AH formulation was significantly stronger than placebo (1.54 vs 0.7, $p = 0.001$). The impact of the adjuvant was no longer consistent across doses, and the dose-response observed at D21 was no longer present. The two PO formulations were comparable and stimulated larger responses than the PO-placebo

groups, but these differences did not reach statistical significance. There were no notable differences between the IM and PO groups at this timepoint.

We next performed flow cytometric analysis of Ki67 expression as a marker of proliferation to gain a better understanding of the lymphocyte sub-populations that contributed to the proliferative responses observed. **At D21**, the response to the IM 10μg +AH was significantly different than the placebo and the other vaccinated groups. In this group, re-stimulation caused 0.89% of the $CD8^+$ T cell population to proliferate, compared to 0–0.16% in the other groups (Figure 1E). The frequency of proliferating $CD4^+$ T cells in this group was also higher than the placebo. Interestingly, despite the large BrdU response seen at D21, oral immunization with the VLP did not elicit a significant increase in proliferation compared to placebo among either B cells or T cell populations as measured by Ki67 expression. This anomalous result may be partially explained by the unexpectedly large frequency of $Ki67^+$ cells in the placebo-vaccinated group. **At D42,** the difference between the $CD8^+$ T cell response in the IM 10μg+AH group and IM placebo group was maintained, and both $CD4^+$ T cell and B cell responses were also observed in this group (Figure 1F). For all examined lymphocyte subsets, IM vaccination led to greater proliferation than PO vaccination.

Taken together, the BrdU and Ki67 data indicate that after *in vitro* re-stimulation, the NoVLP vaccine has the potential to elicit a splenocyte lymphoproliferative response when delivered to mice either intra-muscularly or orally.

### 2.4.2 NoVLP elicits antigen-specific INFγ-secreting splenocytes in the murine model

To better characterize the cellular response, we used ELISpots to measure the ability of splenocytes to produce IFNγ after *in vitro* re-stimulation with NoVLP. Stimulation indices (SI) were calculated by dividing the number of IFNγ-secreting cells in antigen-stimulated samples by unstimulated samples. **Seven days post-immunization**, the SI were 3.59 and 3.86 in the IM 10μg and IM 10μg +AH groups, respectively, and these were the only formulations that elicited larger responses than the placebo groups (Figure 2A). The adjuvant did not noticeably boost responses, and oral immunization did not increase the number of IFNγ-secreting cells compared to placebo. Interestingly, the IFNγ responses to the 10μg dose with and without AH were significantly larger than both PO groups (p values ranged from 0.0006 to 0.0069 for all comparisons).

**At D21**, animals that were vaccinated with 1 or 10μg of NoVLP + AH tended to have more IFNγ-secreting cells after re-stimulation than the placebo-vaccinated animals (Figure 2B). All of the adjuvanted IM groups had increased responses compared to D7 and to their respective unadjuvanted groups. Interestingly, there was an observable IFNγ response in the 200μg PO+AC group, although it did not reach statistical significance when compared to placebo. The SI in this group was, however, significantly larger than in the 200μg PO group (4.96 vs 0.28, p = 0.0035). When comparing the two routes of administration, the adjuvanted IM groups generally elicited stronger responses than the PO groups.

**By D42**, the 10μg and 10μg +AH groups had 8.37- and 8.04-fold increases in IFNγ-producing cells after re-stimulation, respectively, and these increases were significant compared to the adjuvanted placebo control (10μg: p =0.028; 10μg +AH: p = 0.0326) (Figure 2C). The lower IM doses did not elicit robust responses, and AH did not have a major impact on cytokine secretion. At this timepoint, the 200μg PO group had an 8.28-fold increase in IFNγ-producing cells over unstimulated samples. This was larger than the 200μg PO+AC group, and similar to the 10μg ± AH doses. When the three timepoints are considered together, these data suggest that vaccination with NoVLP, particularly with the largest dose IM + adjuvant, elicited a strong splenocyte IFNγ response for at least 42 days after immunization.

### 2.4.3 IM NoVLP elicits antigen-specific IgG-secreting cells in the murine model

Antibody-secreting cells (ASCs) were quantified by plating splenocytes directly after isolation to measure NoVLP-specific B cells that were producing IgG *in vivo*. **At D7**, there were relatively few ASC overall, and only animals that were vaccinated with the 10μg +AH dose had more ASC than the IM placebo (Figure 3A). There was no difference between the 200μg PO+AC group and the PO placebo group. When comparing doses and routes of administration, the 10μg +AH dose elicited the strongest response, with significantly more ASC than both the 1μg +AH and 200μg PO+AC doses (p = 0.0065 and p = 0.0003, respectively). **By D21**, there was an overall increase in the number of ASC in most groups, with the largest responses elicited by the 1μg+AH and 10μg +AH doses (Figure 3B). Despite considerable variation within each group, the IgG response stimulated by the 10μg+AH dose was significantly larger than the adjuvanted placebo formulation (131.25 vs 4.87 ASC/250,000 splenocytes, p = 0.0196). **At D42**,

the ASC response continued to grow in both the 10μg and 1μg IM groups with AH, reaching 197 and 298 ASC/250,000 splenocytes, respectively (Figure 3C). Throughout the study, the adjuvanted IM formulations elicited more ASC than the unadjuvanted formulations. Within the PO groups, the AC boosted the response compared to NoVLP alone, suggesting that this compound may have promoted delivery of the vaccine antigens to immunologically-relevant locations. At each timepoint, however, both the two higher dose formulations delivered IM yielded larger ASC responses than the PO formulations.

In addition to *in vivo* activated ASC, we also measured NoV-specific memory splenocytes on 42. To generate memory cells, we stimulated freshly isolated splenocytes with a cocktail of polyclonal activators (R848 and recombinant mouse IL-2) for 72 hours before using ELISpots to detect antigen-specific memory ASC. Interestingly, the 200μg PO formulation yielded the largest number of memory ASC (155/250,000 splenocytes) (Figure 3D). Although no comparisons reached statistical significance, the trends suggested that the 200μg PO vaccine yielded a stronger memory response than both the PO placebo and all of the IM-vaccinated groups.

Overall, the study in the murine model suggested that the NoVLP stimulates a robust immune response, whether it is delivered PO or IM. The adjuvated 10μg dose IM formulation was generally the most immunogenic, in terms of the proliferative response, IFNγ production and the capacity to secrete antibodies. However, significant lymphoproliferation and a strong memory ASC response was also induced by the 200μg PO vaccine formulations, suggesting that this route of administration may also be a viable candidate for further research. Although more complex, the PO route of administration is probably best evaluated in animal models that more closely mimic the human mucosal immune response.

### 2.4.4 Oral delivery of NoVLP to rabbits elicits a modest $CD4^+$ T cell proliferative response

Rabbits are a widely used model in human disease because their anatomy, physiology and phylogeny more closely resemble those of humans than the common rodents[25]. Rabbits are particularly relevant to this study, as they have been used to understand hepatobiliary transport of serum IgA and as models for enteric pathogens such as rotavirus, *Vibrio cholera, Salmonella,* and *Shigella*[26]. We therefore designed a series of experiments in rabbits to evaluate the

immunogenicity of orally-delivered NoVLP. Four-to-five-month-old New Zealand rabbits were immunized four times (orally or by gavage) at 14-day intervals and blood was collected at various timepoints to assess the immune responses (Figure 4A).

Once again, we began by measuring lymphoproliferation by BrdU incorporation after *ex vivo* re-stimulation with NoVLP. Unlike the murine model, there was no increased proliferation in vaccinated groups compared to placebo groups at any timepoint (Figure 4B). There were also no consistent differences between the two routes of oral administration. Next, we tried to use cell tracer (Invitrogen) dye dilution to identify and characterize proliferating cells by flow cytometry, but technical difficulties complicated the interpretation of these data at all timepoints. At D14, overall proliferation was modest, and trends indicated that administration of the vaccine by gavage yielded a larger response than both the placebo and oral administration (0.65% of total PBMC vs 0.26% and 0.04%, respectively) (Figure 4C). In the gavage group, the proliferative response was largely driven by $CD4^+$ T cells, although considerable variation was observed within this group (Figure 4D). Taken together, these data suggested that PO vaccination with NoVLP did not elicit a robust proliferative response in rabbits. As a result, other measures of PBMC functionality were pursued to try to better characterize the immune response to the NoV vaccine in this model.

### 2.4.5 Oral delivery of NoVLP to rabbits elicits a functional PBMC response that peaked 28 days post-immunization

We next used ELISpots to characterize the IgG, IgA and IFNγ producing potential of PBMCs from immunized rabbits 14, 28 and 56 days post-immunization. In both vaccinated groups (oral and gavage), the number of IgA-producing PBMC increased from D14 to D28 and then contracted by D56 (Figure 5A-C). The only difference between vaccinated and placebo groups was observed at the four-week timepoint, when oral vaccination elicited substantially more IgA-secreting cells than the placebo and gavage groups (1772 vs 424 and 390/200,000 PBMC, respectively) (Figure 5B). There was, however, considerable variation within the oral group. Overall, the rabbit IgG response was smaller than both the IgA response and the murine IgG response (Figure 5D-F). The kinetics followed a similar pattern to the IgA response, with an increase from D14 to D28, followed by a decrease at D56. The only observable (although not significant) difference between vaccinated and placebo groups was at four weeks post-

immunization, where gavage vaccination led to a spike in IgG-producing PBMC compared to placebo (20 vs 13 ASC/200K PBMC) (Figure 5E). In terms of IFNγ-secretion, a relatively modest response was seen throughout the study. On days 14 and 28, vaccination did not induce a different IFNγ response compared to placebo and this outcome was therefore not evaluated on D56 (Figure 5G-H). Taken together, these data showed that oral VLP vaccination can elicit a detectable NoV-specific IgA response in the rabbit model, suggesting that NoVLP can stimulate a mucosal immune response.

## 2.5 Discussion

The challenges associated with studying NoV *in vitro* and the large genetic diversity of the genus have greatly hindered vaccine development. Plant-derived VLPs could theoretically go a long way towards addressing both of these central concerns, as they do not require the capacity to grow virus and production can be rapidly adapted to emerging viral strains. Here, we demonstrate that NoVLP produced by agroinfiltration of *Nicotiana benthamiana* can elicit robust immune responses in both the murine and rabbit models, when delivered either orally or intra-muscularly.

Correlates of protection for NoV are not well understood. Unlike other vaccine-preventable diseases, serum antibody titers measured via ELISA have a short half-life and are not protective[27]. As an alternative, antibodies that block the binding of NoV to HBGAs have been reported to correlate with protection from gastroenteritis[28]. Additional assays performed by Medicago indicated that HBGA titers were significantly increased in mice and rabbits immunized with NoVLP delivered either IM or orally (S. Pillet, personal communication). However, in one clinical study with a different vaccine candidate, although vaccination induced detectable HBGA-blocking antibodies, these antibodies were not necessarily sufficient to protect vaccinees from infection or disease[14,15]. This finding suggests that other factors affect susceptibility to infection. One such factor may be pre-existing memory B cells. A recent challenge study found that this cell type is significantly correlated with protection from disease[29]. In this respect, the increase in memory ASC induced by oral delivery of NoVLP in our mouse study may prove to be a promising surrogate for vaccine efficacy.

Pre-existing IgA in the saliva has also been found to correlate with protection from disease in humans[29]. Furthermore, a recent study comparing the *in vitro* potency of IgA and IgG

serum antibodies suggested that IgA has a greater capacity to block NoV from binding HBGA, and may therefore be more protective[27]. Others have found that the level of IgA in nasal lavage fluids post-immunization strongly correlates with HGBA-blocking activity, indicating that IgA, not IgG, is the major functional antibody in NoV immunity[30]. While such serological responses were outside the scope of this study, we did observe a robust increase in IgA-secreting cells at D28 in the rabbit model. Consistent with our rabbit data, human challenge studies have also found a greater increase in IgA ASC than IgG ASC after infection[29]. Furthermore, the timeline of the immune response to the VLP vaccine is consistent with that of a viral challenge, with peak ASC numbers seven days post-challenge followed by a contraction at day 14[29]. In the mouse study, the day 21 timepoint was 7 days after the second dose, and the day 42 timepoint was 14 days after the third vaccine dose. Accordingly, both the proliferative and ASC response peaked at D21 and contracted by D42. These findings suggest that the immune response elicited by vaccination with plant-derived NoVLP follows a similar pattern to that observed after viral challenge.

In addition to proliferation and antibody secretion, we also measured the capacity of splenocytes or PBMC to produce IFNγ post-vaccination. Production of IFNγ in $CD4^+$ T cells has been observed after NoV challenge in humans and gnotobiotic pigs[31,32]. Furthermore, $CD8^+$ T cells from healthy volunteers who had previously been exposed to NoV secrete IFNγ after re-stimulation with a NoV peptide pool[33]. Mechanistically, work with murine NoV *in vivo* and cultured macrophages suggests that IFNγ can mediate the inhibition of viral replication[34]. In early clinical trials, a NoV VLP produced in insect cells led to a transient increase in IFNγ production post-vaccination[35]. It is therefore encouraging that, in mice at least, the NoVLP vaccine was able to elicit IFNγ-secreting cells.

As expected, the use of alhydrogel as an adjuvant generally boosted the immunogenicity of IM immunization throughout the study. The effects of the antacid on facilitating delivery of oral NoV vaccine formulations, however, remained unclear. The antacid used in the mouse study was sodium bicarbonate, which was chosen because it is the gastric acid neutralizer used with one of the oral cholera vaccines (Dukoral®) (Valneva's product leaflet). Comparisons between groups that received the antacid and those that did not yielded results that were inconsistent across timepoints and outcomes. Further studies, with larger groups and different antacids, will be required to fully understand the potential benefits of this strategy.

There were several limitations to this study. Groups vaccinated orally generally had much larger variability of outcomes than those vaccinated IM. Within orally vaccinated groups, the data were often bimodal, suggesting that some animals had responded well to the vaccine while others had not. This pattern could be due, at least in part, to challenges associated with administration such that animals in the same group received different vaccine doses. Variability could also be inherent to the oral delivery route. For example, evidence from both humans and animal models suggests that many factors, such as pre-exposure to antigen and microbiome composition, can contribute to the effectiveness (or lack of effectiveness) of oral vaccines[36]. Our animals were not screened for prior exposure to NoV and their microbiome composition was not controlled. In addition, the animal models that we used do not fully recapitulate the human condition. The mucosa of humans is very different from that of mice[37], and some oral vaccine candidates found to be effective in mice have failed in clinical trials[36]. To address some of the shortcomings associated with the mouse NoV model, we also preformed targeted studies in rabbits. Rabbits are widely used as a model to study infectious disease[38], and their anatomy and physiology more closely resembles humans than rodents[25]. In addition, studies are currently ongoing in the pig model. These animals, unlike mice and rabbits, develop clinical symptoms similar to those seen in humans when infected with a human NoV strain[39].

In this work, we demonstrated for the first time that plant-derived NoV VLPs can generate a robust immune response in mice and in rabbits. While IM administration generally elicited a stronger response in mice than the PO route, the large proliferative and memory ASC response elicited by oral vaccination suggest that this route of delivery should also be pursued. An interesting direction for future studies would be to evaluate multi-modality and prime-pull vaccination schedules that combine the IM and PO delivery routes. Further studies should also investigate the use of various mucosal adjuvants to promote the establishment of a protective immune response in the gut. Overall, these results confirm the immunogenicity of plant-derived NoVLP vaccines and represent an important step in the preclinical development of this vaccine candidate.

## 2.6 Methods

### 2.6.1 Vaccines & Immunizations

All animal work was performed at the International Toxicology Research (ITR) Laboratories (Baie-D'urfe, QC). Eight-to-ten-week-old BALB/c mice were immunized on days 0, 14 and 28 either IM with 0.1μg, 1μg or 10μg of NoV GII.4 Sydney 2012 strain VLP ± AH, or PO with 200μg of VLP ± AC (sodium bicarbonate). Placebo-vaccinated mice were immunized with formulation buffer ± AH or AC. Mice were sacrificed on days 7, 21 and 42, and spleens were harvested to analyze the immune response to the vaccine *in vitro*. Four-five-month-old New Zealand White rabbits were immunized on days 0, 14, 28 and 42 with 500μg NoV GII.4 Sydney 2012 strain in 2mL of sodium phosphate buffered saline. Rabbits were either immunized orally or via gavage. Placebo vaccinated animals received only formulation buffer.

### 2.6.2 Splenocyte Isolation

Spleens were collected and transported in complete RPMI (cRPMI) and stored on ice throughout processing. Spleens were pushed through a cell strainer using the back of a syringe plunger and then washed in HBSS. Ammonium-chloride-potassium buffer (ACK) was added to each sample, mixed and then immediately diluted in HBSS to stop the reaction. Cells were then washed three times in HBSS and re-suspended in cRPMI for further processing.

### 2.6.3 PBMC Isolation

PBMC were isolated from fresh whole blood using SepMate tubes (Stemcell, Vancouver, BC) as per the manufacturer's instructions, using density gradient medium (Ficoll, Wisent, St Bruno, QC). Cells were counted in cRPMI (D0 & D14) or in ACK (D28 & D56) and then re-suspended in cRPMI for further processing.

### 2.6.4 IgG, IgA and IFNγ ELISpots

ELISpots were performed as per manufacturer's instructions (Mabtech, Cincinnati, OH: Mouse IgG and IFNγ (D7), Rabbit IFNγ; BD, Franklin Lakes, NJ: mouse IFNγ D21 & 42). For IgG and IgA ELISpots, PVDF membrane plates (Millipore, Burlington, MA) were activated with ethanol and coated overnight with the 5μg/ml NoV-VLP. Cells were plated in duplicate at

250,000/well (mouse) or in triplicate at 200,000/well (rabbit). For detection of memory IgG, splenocytes were expanded poly-clonally for 72 hours with recombinant mouse IL-2 and R848 (Mabtech). Cells were washed to remove the stimulants and secreted IgG, and then plated on NoVLP-coated PVDF membrane plates. For IFNγ assays, cells were plated in duplicate at 250,000/well (mouse D7 & D21), 400,000/well (rabbit) or 500,000/well (mouse D42) and stimulated with NoV-VLP (2.5μg/ml) or phorbol 12-myristate-13-acetate (PMA) (1.56 μg/mL) and ionomycin (3.125 μg/mL) (Sigma, St. Louis, MO).

For all assays, cells incubated on ELISpot plates for 16 hours. Spots were detected as per the manufacturer's instructions for all mouse assays and for rabbit IFNγ ELISpots. For rabbit ASC assays, spots were detected with anti-rabbit IgA conjugated to HRP (1:2000, Abcam, Cambridge, UK) and tetramethylbenzidine (Mabtech), or anti-rabbit IgG conjugated to ALP (1:1000, Mabtech) and BCIP/NBT (Mabtech). All plates were read using a CTL reader and Immunospot software for automated spot counting. Quality control was performed on a well-by-well basis.

### 2.6.5 Cell Proliferation Assays

For cell proliferation assays, cells were plated in duplicate at 250,000/well (mouse) or 200,000/well (rabbit) in black flat-bottom plates. NoV-VLP or concanavalin A (ConA) (2.5μg/ml) were added as stimulants to respective wells for 72 hours. 18-24 hours prior to the end of the incubation, BrdU labelling reagent (Sigma, Oakville, ON) was added to each well. At 72 hours, stimulants were removed, and plates were processed as per manufacturer's instructions (Sigma). Plates were read using the "luminescence" function on the Tecan infinite 200Pro reader.

### 2.6.6 Flow Cytometry

One million mouse splenocytes were stimulated with either NoVLP (2.5 μg/ml) or ConA (1.25 μg/ml) for 48-72 hours. After stimulation, cells were stained with viability dye (efluor 780; 1:375, eBioscience, San Diego CA) and then stained for 30 minutes with a cocktail of extracellular markers including anti-CD3-AF 700 (Biolegend, San Diego,CA), anti-CD4-V500 (BD), anti-CD8a-BV 650 (BioLegend) and anti-CD19-PE-CF594 (BD). Cells were fixed for 30 minutes with FOXP3 fix/perm buffer (Invitrogen, Carlsbad, CA), washed in PBS and kept at 4°C

for a maximum of 72 hours. On the day of acquisition, cells were washed 3 times in perm wash (Invitrogen) and then stained with anti-Ki67-ef450 (eBioscience) for 30 minutes. Rabbit PBMCs were stained with Cell Tracer Far Red (Invitrogen) as per the manufacturer's instructions and then plated at 1 million/well for 72 hours. After the 72-hour incubation, the staining protocol described above was followed. The extracellular cocktail consisted of anti-CD4-FITC and anti-IgM-RPE, and the intracellular stain consisted of anti-CD3-IgM (all Bio-Rad, Hercules, CA). Cells were fixed overnight with Cytofix/Cytoperm plus (BD) and the intracellular stain was performed on the day of acquisition. For all experiments, 2-3 million events were acquired on the Fortessa X-20 (BD), and data were analyzed using FlowJo software (Treestar, Ashland).

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## 2.8 Figures

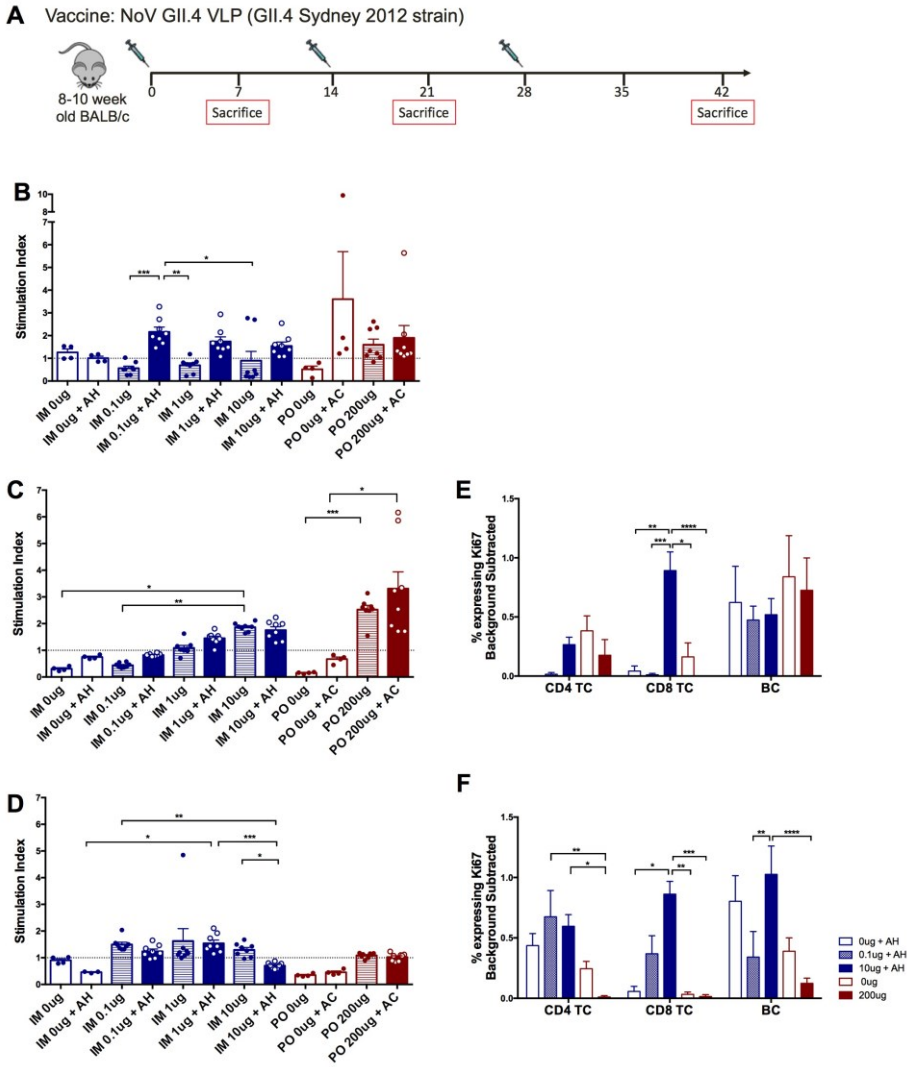


**Figure 1: IM and PO immunization with NoVLP generates a robust proliferative response in mouse splenocytes.** (**A**) Balb/C mice were immunized three times with the NoV GII.4 VLP and sacrificed at three timepoints to assess the immune response to the vaccine. **(B-D)**Splenocyte proliferation was measured by BrdU incorporation after 72 hours of *ex-vivo* re-stimulation. Stimulation indices represent a ratio between proliferation in stimulated and unstimulated cells. Spleens were harvested at day 7 (**B**), day 21 (**C**) and day 42 (**D**). **(E-F)** Flow cytometry was used to quantify the expression of Ki67 in splenocyte sub-populations after 48 hours of *ex-vivo* re-stimulation. The composition of the $Ki67^{+}$ population was analyzed at day 21 (**E**) and day 42 (**F**). Statistical significance was calculated using the Kruskal-Wallis test and Dunn's test to correct for multiple comparisons (**B-D**), or a two-way ANOVA and Tukey's test for multiple comparisons (**E-F**). Abbreviations: AH – alhydrogel; AC – antacid; TC – T cell; BC – B cell.

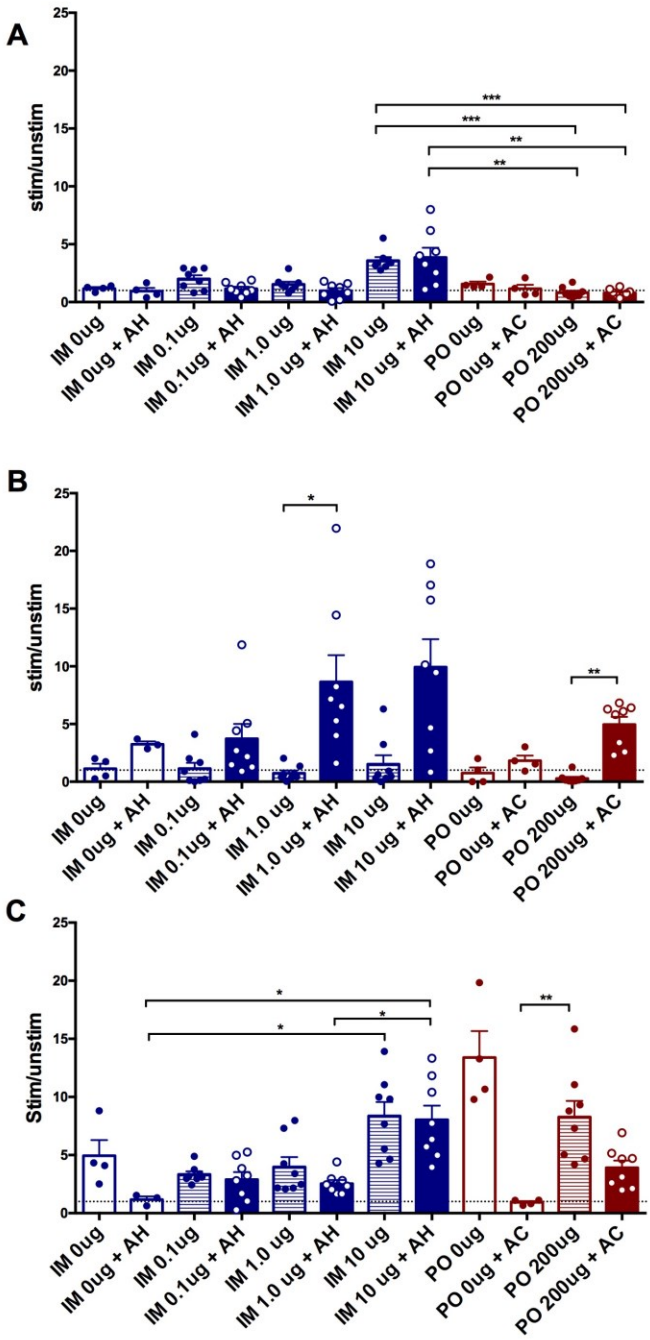


**Figure 2: IM immunization of mice with NoVLP generates an antigen-specific IFN$\gamma$ response.** IFN$\gamma$-secreting cells were identified using ELISpots. The results are reported as a ratio between the number of spots in VLP-stimulated and unstimulated wells. Splenocytes were harvested on day 7 (**A**), day 21 (**B**) and day 42 (**C**). Statistical significance was calculated using the Kruskal-Wallis test and Dunn's test to correct for multiple comparisons. Abbreviations: AH – alhydrogel; AC – antacid.

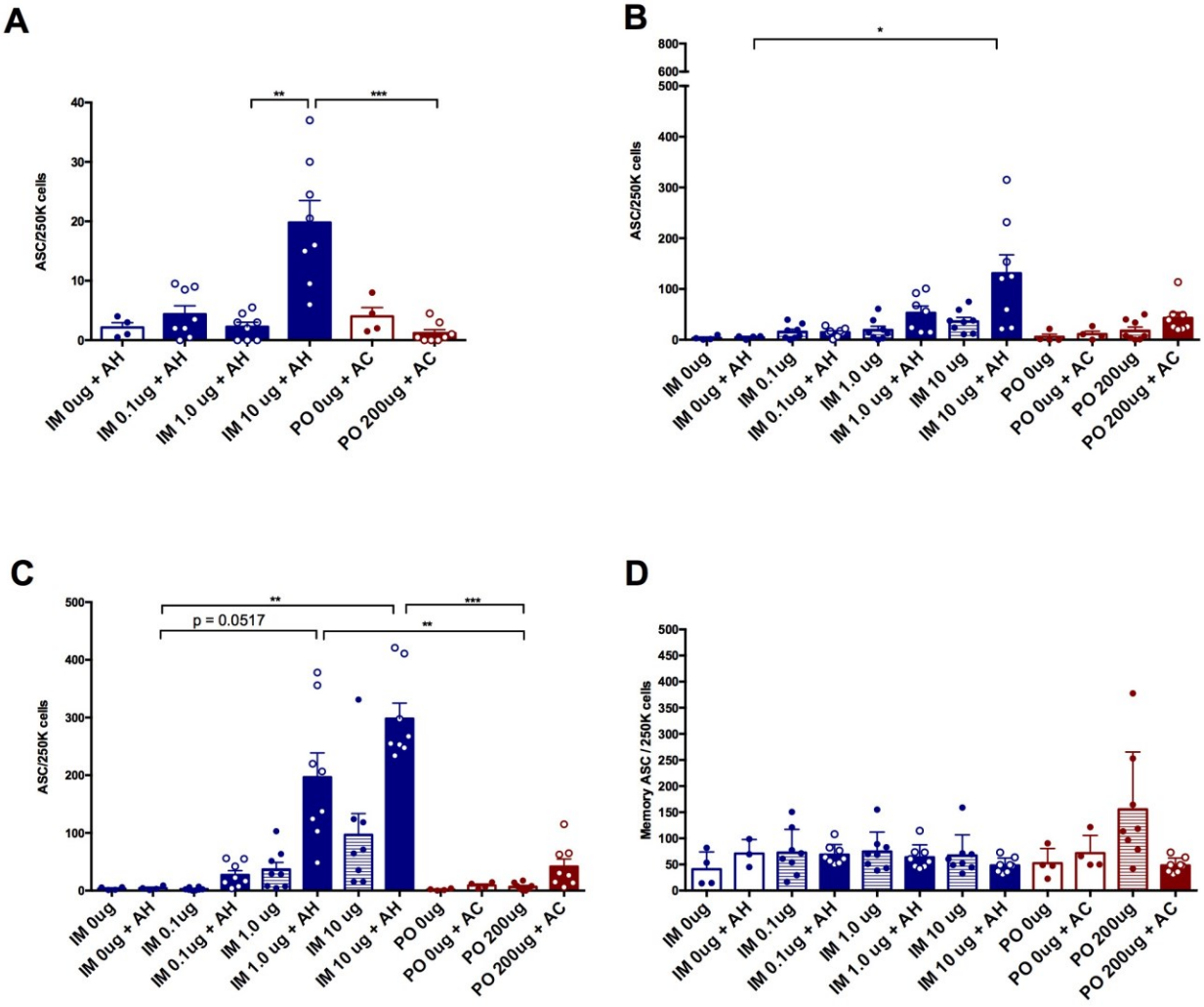


**Figure 3: Immunization with NoVLP results in primary and memory antigen-specific IgG-secreting cells in mice.** Antibody-secreting cells were identified by ELISpot, using the NoVLP as a capture antigen. Spleens were harvested, and fresh splenocytes were analyzed on day 7 (**A**), day 21 (**B**) and day 42 (**C**). To stimulate memory cells, splenocytes harvested on day 42 were expanded poly-clonally with a cocktail of polyclonal activators (R848 and recombinant IL-2) for 72 hours before ELISpot analysis (**D**). Statistical significance was calculated using the Kruskal-Wallis test Dunn's test to correct for multiple comparisons. Abbreviations: AH – alhydrogel; AC – antacid.

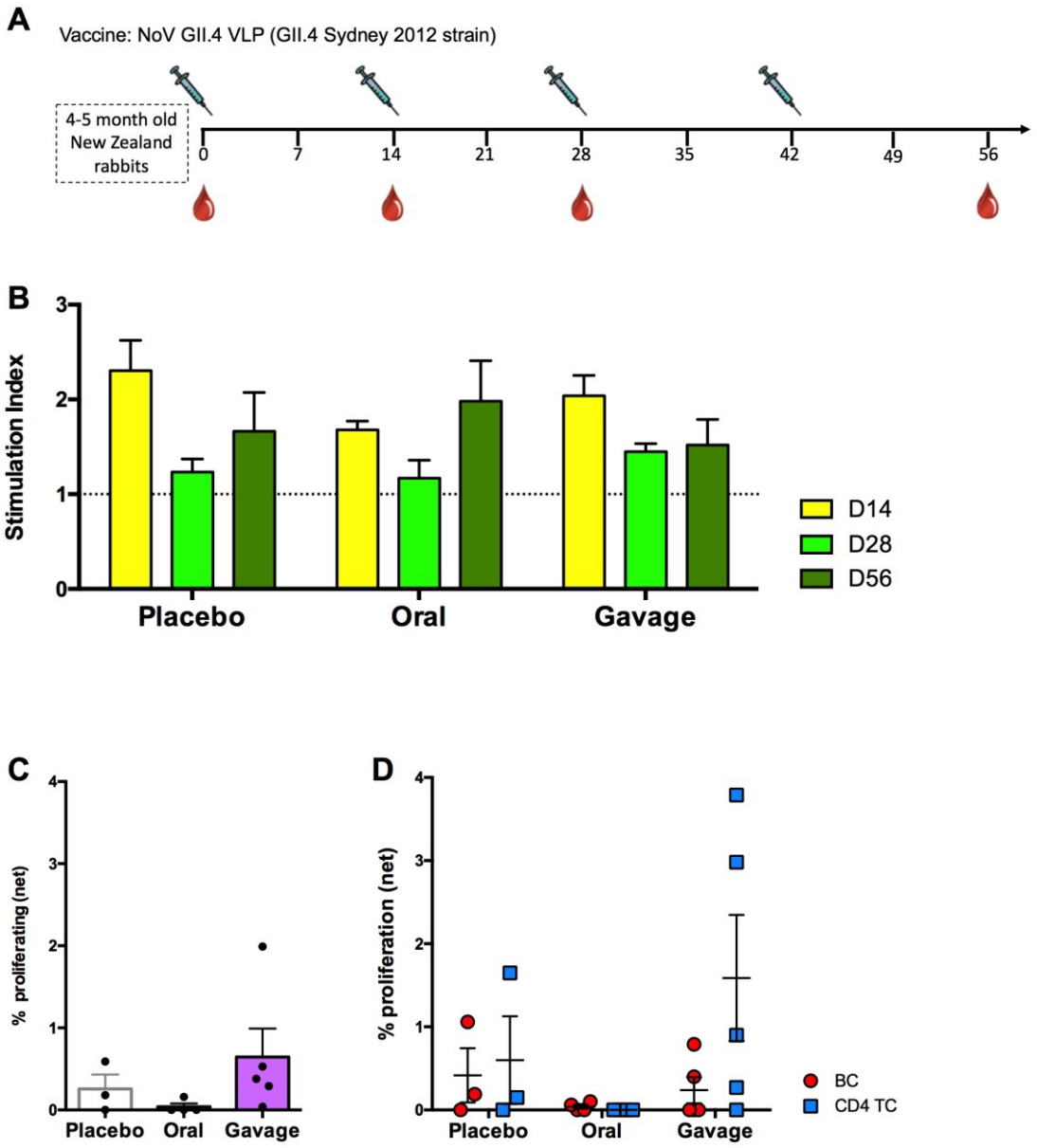


**Figure 4: Oral delivery of NoVLP to rabbits elicits a modest CD4$^+$ T cell proliferative response**. (**A**) 4-5-month-old New Zealand rabbits were immunized orally or via gavage on days 0 and 14, 28 and 42, and blood was collected at various time points to assess the immune response to the vaccine. (**B**) Splenocyte proliferation was measured by BrdU incorporation after 72 hours of *ex-vivo* re-stimulation. Stimulation indices represent a ratio between proliferation in stimulated and unstimulated cells. Flow cytometry was used to quantify cell tracer labelling on total PBMC (**C**) and on sub-populations (**D**). Values shown are background subtracted with unstimulated samples. Statistical significance was calculated using a two-way ANOVA and Tukey's test to correct for multiple comparisons.

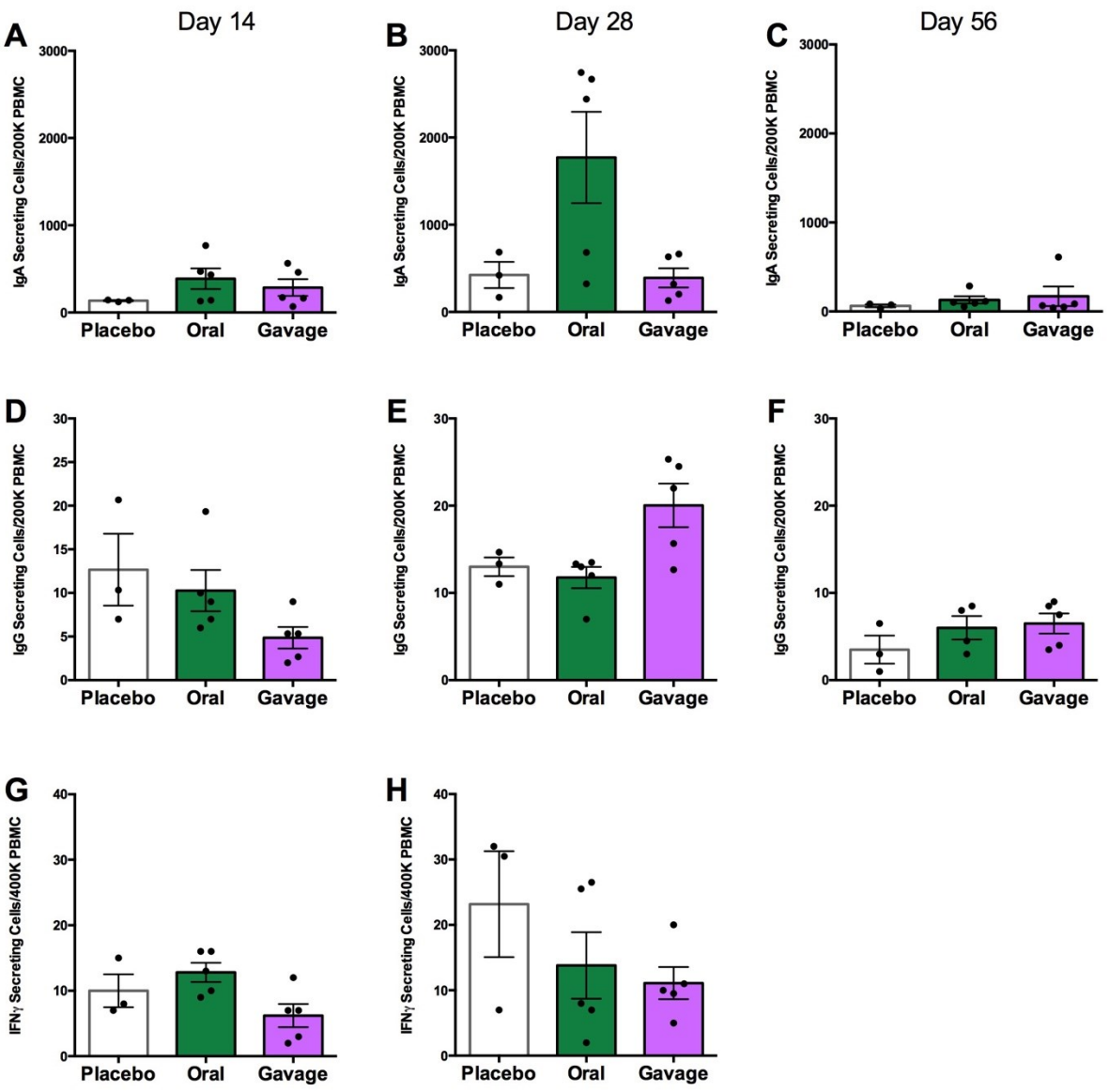


**Figure 5: Vaccination with NoVLP elicits IgA- and IgG-secreting PBMC in rabbits.** Functional PBMC were enumerated by ELISpot on days 14 (**A, D, G**), 28 (**B, E, H**) and 56 (**C, F**) post immunization. The antibody responses are shown as the number of IgA (**A-C**) or IgG (**D-F**) secreting cells per 250,000 PBMC. The IFNγ response is reported as the number of IFNγ-secreting cells/400k PBMC in VLP-stimulated wells (**G-H**). Statistical significance was calculated using the Kruskal-Wallis test and Dunn's test to correct for multiple comparisons.

## CHAPTER III

# Needle-free delivery of influenza vaccine using the Med-Jet® H4 is efficient and elicits the same humoral and cellular responses as standard IM injection: A randomized trial.

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## 3.1 Preface

After successful preclinical development, a vaccine, or an injection system, must be thoroughly tested in human subjects. During the clinical stage, data on the safety and efficacy of the product are collected first in healthy volunteers (phase I), then in increasing numbers of the target population (phase II), and finally in large phase III randomized controlled efficacy or immunogenicity trials. In the case of a vaccine delivery system, the device is first tested with a mock vaccine in healthy volunteers, and then in field trials with a well-characterized, commercial vaccine. The goal of these studies is to demonstrate that the device is safe and elicits an immune response that is comparable (or superior) to traditional delivery methods. The following chapter describes an 80-person randomized trial evaluating the use of a jet-injector, the Med-Jet H4, for the administration of a seasonal influenza vaccine.

## 3.2 Abstract

**Background:** Needle-free vaccine delivery systems have many potential advantages including increased vaccine compliance and decreased risk of needlestick injuries and syringe reuse. The Med-Jet® H4 is a gas-powered, auto-disabling disposable syringe jet injector. The Med-Jet family of products are currently being used in dermatology, podiatry, pain management and veterinary practices. The objectives of this study were to assess patient attitudes, time-efficiency, safety and immunogenicity of the seasonal influenza vaccine delivered by Med-Jet H4 compared to the traditional needle-and-syringe.

**Methods:** A total of 80 patients were randomized 2:1:1 to receive a commercial trivalent vaccine by Med-Jet H4 or by needle injection from a single-dose or multi-dose vial. Patient attitudes were assessed pre-randomization and post-immunization. Safety data were collected for 21 days post-immunization. Efficiency of administration was measured through a time-and-motion study during vaccinations. Humoral and cellular responses were assessed on Day 0 and Day 21.

**Results:** Overall, the participants readily accepted Med-Jet vaccination despite greater frequency of transient local reactions (eg: redness, swelling) immediately following immunization. Vaccine administration took slightly longer with the Med-Jet, but this difference decreased over time. Geometric mean hemagglutination inhibition titers, seroconversion and seroprotection rates in the Med-Jet and needle groups were equivalent for all influenza strains in the vaccine. Microneutralization responses were also essentially identical. There were also no significant differences between the groups in the frequency of functional $CD4^+$ T cells, memory subset distribution or poly-functionality.

**Conclusions:** These data suggest that the Med-Jet H4 is an acceptable means of delivering seasonal influenza vaccine. The system was attractive to the subjects, rapidly learned by skilled vaccine nurses and elicited both humoral and cellular responses that were indistinguishable from those elicited with intra-muscular needle injection. To our knowledge, this study is the first to assess cellular responses elicited following jet injection of an influenza vaccine.

(ClinTrials.gov NCT03150537)

## 3.3 Introduction

Needle-free immunization systems have been suggested as a strategy to increase compliance with immunization recommendations, deliver vaccines rapidly in emergencies (eg: pandemic influenza) and mitigate the public health impact of needlestick injuries and syringe reuse[1]. The development of safe jet injectors has been promoted by public health authorities including the Center for Disease Control and the WHO as well as major non-governmental organizations such as PATH and the Bill and Melinda Gates foundation[2]. Initially developed in the 1860s, jet injectors typically deliver vaccines to targeted tissues through a high-pressure liquid stream[2]. Most modern jet injectors use disposable cassettes to ensure safe delivery[2]. Medical International Technologies (MIT Canada) Inc. has developed a series of jet-injection devices based on low-pressure, gas-powered delivery of an ultra-fine stream (0.11 mm: 6 times smaller than a 30 G needle) of vaccine that minimizes patient discomfort and leakage. Unlike spring-powered jet injectors, the MIT devices use $CO_2$ or compressed air as a power source, permitting more consistent and accurate injections for the lifetime of the device. The newest model, the Med-Jet® H4 (Med-Jet), uses disposable cartridges equipped with a piston tip that breaks upon injection, effectively preventing reuse and thus mitigating the risk of cross-contamination. This approach also affords the possibility of manufacturing pre-filled cartridges to maximize efficiency of vaccine delivery.

In the present study, patients were randomized to receive the seasonal influenza vaccine by Med-Jet or by needle-and-syringe from either single- or multi-dose vials. The seasonal influenza vaccine was chosen for this study because it is typically offered in a 'mass vaccination' context. An observer-based time-and-motion study was used to compare the efficiency of these delivery methods, and patient attitudes and beliefs were surveyed pre- and post-immunization. Humoral and cellular immune responses to vaccine delivered by Med-Jet or needle & syringe were assessed.

## 3.4 Methods

### 3.4.1 Study Design, Participants and Vaccine

This trial was approved by the McGill University Health Center (MUHC) Research Ethics Committee and was registered at ClinTrials.gov (NCT03150537: May 4, 2017). The 80

subjects planned for the study were recruited and immunized at the McGill Vaccine Study Centre (Montreal, QC) between May 12-18, 2017 and randomized 2:1:1 to be vaccinated by Med-Jet H4 injection or by intramuscular needle & syringe (NS) injection from either a single-dose (NS-SD) or a multi-dose vial (NS-MD). Random numbers were generated using 'R' version R-3.3.3 for Mac OS X. Randomization was performed by the principle investigator and implemented by the vaccine center. The sample size calculations were patterned after a similar study performed using the Pharmajet jet injector to deliver influenza vaccine[3]. In our study, the most interesting comparisons were between the Medjet and any NS group. We calculated that a sample size of 40 volunteers/group would provide 0.90 power at alpha 0.05 (two-sided) to detect >15% differences in the HI seroconversion rates for H1N1 A/California/07/2009 and H3N2A/Hong Kong/4801/2014 and >20% for B/Brisbane/60/2008 at day 21 after vaccination.

To participate in the study, patients had to be healthy, 18-49 years of age and have a body mass index between 18-32 kg/m$^2$. Patients who had received an influenza vaccine during the 2016-2017 season were excluded. In all groups, the vaccine used was a standard commercial trivalent split-virion product (FluZone™: Sanofi Pasteur, NY) supplied either in single-dose or multi-dose vials that contained 15 $\mu$g hemagglutinin (HA) for each of the WHO-recommended strains for the 2016-17 season (ie: H1N1 A/California/07/2009, H3N2 A/Hong Kong/4801/2014 and B/Brisbane/60/2008 viruses). Serum was collected from each patient prior to vaccination and 21 days (d21) post-immunization for serologic measures. Whole EDTA-anticoagulated blood was collected from approximately half of the subjects in each group to evaluate the cellular immune response. All immunologic assays were performed by operators blinded to group assignment. A brief questionnaire about attitudes and beliefs regarding needles and needle-free immunization was completed by all subjects pre- and post-immunization. Safety data (local and systemic adverse events) were collected in person (30 minutes), daily for 4 days (by diary) and between d5 to d21 (by diary and report at the d21 blood draw).

### 3.4.2 Vaccine Delivery

The MIT jet injectors are licensed by Health Canada, and are commercially available in the European Union, as well as many other countries. The injectors are designed and manufactured in Montreal and are ISO 13485 and ISO 9001 compliant. Nurses were trained to use the Med-Jet and were given an opportunity to practice administering mock vaccine (sterile

saline) before the study began. Because pre-filled cartridges are not yet available, sterile cartridges were filled with an adapter attached to a multi-dose vial and screwed into the device immediately prior to use (Figure 1A). The device is primed by pulling back gently on the trigger and the nozzle of the device is then pressed firmly onto the patient's skin over the deltoid muscle. The vaccine is delivered by pulling back on the trigger. After vaccination, the cartridge is released from the device for disposal.

### 3.4.3 Time-and-Motion Study

A time-and-motion (TM) study was carried out by two trained, independent observers during all injections. The tasks involved in vaccination were divided into three categories: preparing for vaccine administration, vaccine administration and post-vaccination duties. The visual and verbal cues associated with these steps are detailed in Supplemental table 1. Each observer recorded task-times in seconds using an electronic data collection form that included time-stamp functionality.

### 3.4.4 Serology

The hemagglutination inhibition (HAI) assays and microneutralization (MN) assays were performed as previously described[4] using H1N1 A/California/07/2009-like, and B/Brisbane/60/2008-like viral stocks grown in eggs and H3N2 A/Hong Kong/4801/2014-like grown in MDCK cells (National Microbiology Laboratory, Winnipeg, MN). Testing for non-specific hemagglutinating activity was performed prior to the HAI assay. Briefly, receptor destroying enzyme (RDE)-treated sera were diluted 1:4 with PBS and 0.5% turkey erythrocytes. If hemagglutination occurred, sera underwent hemadsorption that was repeated until non-specific hemagglutination activity was no longer observed.

### 3.4.5 PBMC Isolation and Flow Cytometry

Peripheral blood mononuclear cells (PBMC) were isolated within 6 hours of blood collection by differential density centrifugation and cryo-preserved as previously described[5]. Thawed PBMC were plated at 1 million cells per well and either left unstimulated or stimulated with 1.5 μg/mL total HA (0.5μg/mL HA per strain) of each targeted virus for 18 hours. Single-dose Influvac vaccine (Mylan, Maidenhead, UK) was used as the source of viral antigens. After

14 hours of stimulation with HA antigen, golgi stop and golgi plug (BD, San Jose, CA) were added to each well according to manufacturer's instruction and positive control wells were stimulated with phorbol 12-myristate-13-acetate (PMA) (1.56 μg/mL) and ionomycin (3.125 μg/mL) (Sigma, St. Louis, MO). Cells were stained extracellularly with aqua live/dead viability dye (Invitrogen, Carlsbad, CA), anti-CD4 (clone SK3), anti-CD8 (clone RPA-T8) and anti-CD14 (clone M5E2) (all from BD), and anti-CD27 (clone 0323), anti-CD45RA (clone HI100) and anti-CCR7 (clone G043H7) (all from Biolegend, San Diego, CA). Cells were then fixed and stained intracellularly with anti-CD3 (clone SP34-2), anti-IFNγ (clone B27), and anti-TNα (clone 6401.1111) (all from BD), and anti-IL-2 (clone MQ1-17H12) (Biolegend). Samples were acquired on the BD Fortessa X-20. Data were analyzed on FlowJo (TreeStar, version 10.0.8r1) and SPICE (http://exon.niaid.nih.gov/spice) software. The gating strategy used is detailed in Supplemental Figure 3. HA antigen-stimulated samples for which <100,000 events were acquired were excluded from further analysis. For analysis that included both d0 and d21, six samples from the Med-Jet group and four samples from the NS group were excluded on this basis. Final analysis was performed on twelve subjects from the Med-Jet group and seven subjects from the NS group.

### 3.4.6 Statistical Analysis

Statistical analysis was performed on Graphpad Prism (GraphPad Software, version 6.0c, La Jolla, CA) and Stata 10 (Statacorp, College Station, TX). Note that, for immunologic parameters, the two NS groups were combined for analyses.

## 3.5 Results

### 3.5.1 Recruitment and Vaccine Safety

80 participants were recruited to the study and randomized to one of three intervention groups (Table 1). No patients were lost to follow-up between D0 and D21. Body mass index and demographic characteristics were similar between groups, with an even distribution of males and females, a mean age of 30 (±8.96), and the majority of participants identifying as Caucasian/White (Table 1). Both methods of vaccine administration were found to be safe, with no serious adverse events reported in the 21 days post-immunization. Participants in the Med-Jet

group experienced greater swelling and redness but not pain within 30 minutes of vaccination (Table 2). By the evening of d0, similar rates of local and systemic reactions were reported by all participants, and local reactions were generally resolved by d4 post-immunization in all groups.

### 3.5.2 Patient Attitudes

Participants answered a brief questionnaire pre-randomization and post-immunization exploring their attitudes towards needles and impressions of the Med-Jet. There were no significant differences between mean responses pre- and post- immunization (Figure 1B). The majority of participants were enthusiastic about the idea of needle-free immunization, were interested in the Med-Jet and thought that it would work just as well as a NS. Most participants hoped to be in the Med-Jet group and thought that the Med-Jet would hurt less than a needle. Post-immunization, those in the Med-Jet group paradoxically reported that jet injection hurt less than their memory of past needle injections, despite having reported higher levels of pain during the Med-Jet injection ($p = 0.0102$). Pain at the injection site was consistent with the slightly higher levels of local redness and swelling immediately following immunization in the Med-Jet group (Table 2). At d21 post-immunization, 60% of the Med-Jet group indicated they would prefer to receive vaccinations by jet injection in the future.

### 3.5.3 Time-and-Motion

The time-motion study revealed that the nurses were not as agile delivering the vaccine with the Med-Jet as with a traditional NS. The largest discrepancy between techniques was during vaccine preparation (Figure 1C), when the mean time to prepare for administration was significantly longer for the Med-Jet (84.39s [95% CI: 75.60, 93.18]) than for either NS-SD (7.85s [95% CI: 7.06, 8.64]) or NS-MD (43.16s [95% CI: 34.30, 52.03], both $p < 0.001$) (Figure 1C). However, when we eliminated the time to load the vaccine into the cartridge in order to simulate workflow with a pre-filled cartridge, vaccine preparation time decreased to 30s (95% CI: 26.77, 33.23) which was significantly shorter than the mean time for NS-MD ($p = 0.0011$) (Figure 1C). Similar to the first stage, the time to administer vaccine was significantly shorter for NS-SD (10.35s [95% CI: 9.61, 11.09]) and NS-MD (10.08s [95% CI: 8.55, 11.61]) than for Med-Jet (SD and MD) (22.24s [95% CI: 20.49, 23.99], both $p <0.001$) (Figure 1C). However, the time to perform post-vaccination duties was not significantly longer with the Med-Jet (15.61s

[95% CI: 12.96, 18.26]) than the NS-SD (13.28s [95% CI: 10.20, 16.36], p = 0.29) or NS-MD (14.95s [95% CI: 11.00, 18.91], p = 0.77) (Figure 1C). Despite the longer initial vaccination time, the time for each task generally decreased over the five days of vaccinations, indicating a rapid learning curve for nurses adopting this new technology (Supplemental Figure 1).

### 3.5.4 Serology

The HI titers (GMT) for the three virus strains increased significantly between d0 and d21 in both the NS and Med-Jet groups and there were no significant differences between the groups for any outcome including the mean GMTs, seroprotection rates (SPR) and seroconversion rates (SCR) (Figure 2A-C). Microneutralization titres also rose significantly between d0 and 221 in both groups for all three viruses and, again, there were no statistically significant differences between the NS and Med-Jet groups (Supplemental Figure 1)

### 3.5.5 Cell-Mediated Immunity

Both the number and functionality of $CD4^+$ T cells contribute to protection against viral infections such as influenza[6-8]. We therefore assessed detailed $CD4^+$ T cell responses after *ex-vivo* re-stimulation with HA antigens in a subset of patients. Functional $CD4^+$ cells were defined as expressing any cytokine (IFNγ, TNFα or IL-2) after re-stimulation. The increased frequency of functional $CD4^+$ cells between d0 and d21 was equivalent in both groups (Figure 3A). Memory cells among the functional $CD4^+$ cell sub-populations were classified as naïve ($CD45RA^+$ $CCR7^+$ $CD27^+$), central memory (CM: $CD45RA^-$ $CCR7^+$ $CD27^+$), transitional memory (TM: $CD45RA^-$ $CCR7^-$ $CD27^+$), effector memory (EM: $CD45RA^-$ $CCR7^-$ $CD27^-$) or effector memory $CD45RA^+$ (TEMRA: $CD45RA^+$ $CCR7^-$ $CD27^{+/-}$)[9]. At d21, the Med-Jet group had slightly higher proportions of $CD45RA^-$ memory $CD4^+$ T cell sub-populations (CM, EM and TM) compared to the NS, but none of these differences reached statistical significance (Figure 3B). When adjusted for pre-vaccination values (d21-d0), there were also no significant differences in $CD4^+$ T cell poly-functionality (ie: expression of ≥2 cytokines) between the NS and Med-Jet groups (Figure 3C). However, slightly higher triple-positive ($IFN\gamma^+$ $TNF\alpha^+$ $IL\text{-}2^+$) $CD4^+$ T cells were seen in the NS group and the Med-Jet group had slightly larger single-positive ($IFN\gamma^+$ or $TNF\alpha^+$) sub-populations. At d21 post-vaccination, there was a modest increase from

d0 in the proportion of influenza-specific poly-functional $CD4^+$ T cells in both NS and Med-Jet groups, but none of the differences reached statistical significance (Figure 3D). A similar analysis was repeated in the $CD8^+$ T cell population but no significant differences were found between the NS and Med-Jet groups (data not shown).

## 3.6 Discussion

Needle & syringe-based vaccine delivery has a long history of success but also has several serious shortcomings. Once quite rare (or denied), needle-phobia is rapidly becoming a barrier to achieving high vaccine coverage. A recent Canadian survey suggests that 24% of adults and 63% of children fear needles. In this study, needle-phobia was the primary reason for non-compliance with immunization recommendations for 7% of parents and 8% of children[10]. Furthermore, the routine use of needles in vaccination programs poses important risks for patients and healthcare workers. The WHO estimates that 3 million occupation-associated needle-stick injuries occur each year[11], accounting for 37%-39% of hepatitis B and C infections among healthcare workers[12]. In many low- and middle-income settings, the risk of needle-stick injuries extends well beyond the healthcare setting due to inadequate disposal practices[13]. Disposing of sharp waste can be quite costly. Although recent data on the cost of sharps waste is not available, a 1990's estimate suggested $0.55-$1.10(US)/Kg for a total cost of ~$450M/year in the USA[13]. At a micro scale, the McGill Vaccine Study Centre pays ~$400 CDN/year to responsibly dispose of ~40 small sharps containers (D. McCormack, personal communication). A disposable, needle-free, non-reusable jet injector system that uses a pre-filled vaccine cartridge could address all of these concerns.

Jet injector systems have been a theoretical possibility for many years. Early prototypes were plagued by contamination issues with some disastrous consequences including major outbreaks of iatrogenic hepatitis B[14-16]. More recently, several strategies have been introduced to address 'splash-back' and cross-contamination including the use of single-use cartridges. Because immune responses to influenza vaccines are relatively well-characterized, and because jet injectors would be useful during an infectious disease emergency such as an influenza pandemic, several such systems have been investigated for the delivery of influenza vaccines over the last 20 years[3,17,18]. The findings of our study are consistent with these previous trials in

both the equivalence of the humoral responses induced and the frequency of transient local reactions such as swelling and redness immediately following immunization. The benign nature of these local reactions is suggested by the fact that, despite their frequency, most of the subjects in the Med-Jet arm of our study still favoured Med-Jet over needle delivery in the post-immunization questionnaire.

Unlike other needle-free injection systems, MIT's injectors work at relatively low-pressure to deliver a medication or vaccine in an ultra-fine (0.11 mm) stream of liquid, and use a magnet instead of a spring to control the initial pressure of injection. Since springs typically weaken with time, the magnet results in more consistent and accurate delivery that does not need to be reset after each injection. The Med-Jet injectors are powered by compressed air and can be adjusted to permit dermal, subcutaneous and intramuscular injection. Another innovation of the Med-Jet system is the piston tip that breaks off in the disposable cartridge after the vaccine has been injected. This design ensures that each cartridge can only be used once, dramatically reducing cross-contamination risk. The Med-Jet family of products are currently being used in dermatology[19], cosmetics, podiatry, pain management[20] and veterinary practices in countries including Canada, Japan, South Africa, South Korea.

Although other studies have reported on humoral responses to jet injection of influenza vaccines[3,17,18], we believe that this is the first study to assess whether or not jet injection can also elicit a cell-mediated immune response. Currently available influenza vaccines largely rely on the generation of neutralizing antibodies to mediate protection[21]. However, frequent mutations in the surface glycoproteins have frustrated the efforts of vaccine developers to elicit long-lasting and cross-protective immunity to seasonal influenza[21]. In contrast to the relatively short-lived protective efficacy of neutralizing antibodies, both animal and human data suggest that memory $CD4^+$ T cells can contribute to durable protection against heterosubtypic strains[21-23], and thus correlate with protection. For example, in a recent human influenza challenge study, pre-existing memory $CD4^+$ T cells were found to be a better correlate of protection than antibody titres[6]. A growing body of evidence suggests that multiple factors, including intradermal (ID) delivery can influence the generation of cell-mediated immunity in response to influenza immunization[24]. Since vaccine antigen is distributed both ID and IM following the low-pressure Med-Jet injection, we hoped that vaccination using this device would elicit better cellular responses than deep IM injection. Using CD45RA, CCR7 and CD27 to delineate five sub-populations of

memory $CD4^+$ T cells, we found that NS and Med-Jet administration elicited modest but comparable $CD4^+$ and $CD8^+$ cellular responses in terms of both memory phenotypes and functional status.

Another innovation of this study was the inclusion of a time-motion element that permitted each stage of the 'act' of vaccination to be broken-down into discrete segments. Although we did observe differences in time between the two delivery methods, we expect that this can be at least partially attributed to the nurses having been very experienced with NS delivery and having very limited time for training with the Med-Jet prior to the study. Our results over the five study days demonstrate that, even during this short period, nurses were able to adapt to using the Med-Jet with increased efficiency (Supplemental Figure 3). If the cartridge-filling step is removed (ie: were a pre-filled cartridge to become available), administration of the vaccine was at least as fast with the Med-Jet as NS delivery from a multi-dose vial (Figure 1C). Furthermore, the time-motion study did not consider the time needed to dispose of 'sharps' waste. With experience and pre-filled cartridges, it is likely that the speed of Med-Jet vaccination would be comparable to or even faster than single-dose syringes.

This study has several obvious limitations. The first is its relatively limited size with only 40 subjects in each of the Med-Jet or needle & syringe arms. Despite its small size, the humoral and cellular immune responses to vaccination were remarkably consistent between the two injection techniques. A second concern is the fact that the study was carried out at the McGill Vaccine Study Centre with highly-skilled vaccination nurses. This setting initially put the Med-Jet at a slight disadvantage in terms of the speed of administration although the learning curve for Med-Jet injection was very rapid. However, it is not at all certain that other healthcare professionals who administer vaccines would adapt as rapidly to this novel delivery system.

In conclusion, this study demonstrates that the Med-Jet delivery system performs very well in terms of patient attitudes, safety and the immune response elicited by a commercial influenza vaccine. These findings suggest that use of the Med-Jet for seasonal influenza campaigns might increase vaccine up-take while decreasing needle-stick injuries and the transmission of blood-borne diseases.

**Conflicts of Interest:**

JS, BH, HH, AP, JAP and BJW have nothing to disclose. KM is president and CEO of MIT Canada. MM and CM are both employees of MIT Canada

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## 3.8 Tables

**Table 1: Demographic Characteristics of Study Groups**

| Demographics | Needle (SD) | Needle (MD) | MedJet H4 | Total |
|---|---|---|---|---|
| **Participants** n | 19 | 21 | 40 | 80 |
| Male | 9 | 8 | 23 | 40 |
| Female | 10 | 13 | 17 | 40 |
| **Age** | 30.5 ± 9.4 | 27.2 ± 6.9 | 31.0 ± 9.4 | 30.0 ± 8.96 |
| **Race/Culture** n | | | | |
| Caucasian/White | 10 | 17 | 27 | 54 |
| African/Black | 0 | 0 | 3 | 3 |
| Hispanic | 5 | 3 | 10 | 18 |
| Asian | 2 | 0 | 0 | 2 |
| Native American | 1 | 0 | 0 | 1 |
| Other/No answer | 1 | 1 | 0 | 2 |
| **Weight (Kg)** | 73.2 ± 12.6 | 70.3 ± 14.2 | 76 ± 12.7 | 73 ± 13 |
| **BMI** | 25.4 ± 3.7 | 24.4 ± 3.4 | 25.9 ± 3.2 | 25.4 ± 3.4 |

| Adverse Effect | Needle | MedJet H4 | Significance |
|---|---|---|---|
| **Redness** mm (% ≥ 10mn | | | |
| Immediate (30 min) | 1.52 (2.5%) | 41.2 (85%) | **** |
| Day 0 (evening) | 14.9 (35%) | 8.73 (35%) | |
| Day 1 | 6.18 (32.5%) | 15.2 (45%) | |
| Day 2 | 9.8 (30%) | 13.8 (35%) | |
| Day 3 | 6.0 (17.5%) | 8.0 (20%) | |
| Day 4 | 2.6 (10%) | 1.70 (5%) | |
| **Swelling** mm (% ≥ 10mn | | | |
| Immediate (30 min) | 0.56 (2.5%) | 14.2 (75%) | **** |
| Day 0 (evening) | 6.33 (25%) | 5.53 (27.5%) | |
| Day 1 | 6.18 (27.5%) | 8.98 (30%) | |
| Day 2 | 4.10 (20%) | 7.15 (25%) | |
| Day 3 | 1.76 (12.5%) | 3.43 (12.5%) | |
| Day 4 | 0.21 (10%) | 0.73 (2.5%) | |
| **Pain** score 1-5 (% ≥ scor | | | |
| Immediate (30 min) | 0.09 (0%) | 0.22 (10%) | |
| Day 0 (evening) | 0.53 (7.5%) | 0.55 (0%) | |
| Day 1 | 0.53 (7.5%) | 0.50 (7.5%) | |
| Day 2 | 0.23 (2.5%) | 0.23 (0%) | |
| Day 3 | 0.13 (2.5%) | 0.10 (0%) | |
| Day 4 | 0.08 (2.5%) | 0.08 (0%) | |
| **Itching** score 1-5 | | | |
| Immediate (30 min) | 0.03 | 0.08 | |
| Day 0 (evening) | 0.15 | 0.03 | |
| Day 1 | 0.08 | 0.03 | |
| Day 2 | 0.13 | 0.05 | |
| Day 3 | 0.15 | 0.03 | |
| Day 4 | 0.10 | 0.03 | |
| **Systemic (headache, muscles aches, tiredness, nausea)** n (%) | | | |
| Immediate (30 min) | 1 (2.5%) | 7 (17.5%) | |
| Day 0 (evening) | 10 (25%) | 11 (27.5%) | |
| Day 1 | 11 (27.5%) | 12 (30%) | |
| Day 2 | 5 (12.5%) | 6 (15%) | |
| Day 3 | 2 (5%) | 3 (7.5%) | |
| Day 4 | 0 (0%) | 4 (10%) | |
| **Symptoms After Day 4** n (%) | | | |
| Local | 5 (12.5%) | 7 (17.5%) | |
| Systemic | 3 (7.5%) | 3 (7.5%) | |

Local reactions: Two-tailed P-values calculated with Mann-Whitney test (unpaired, non-parametric)
Systemic and D4+: Two-tailed p-values calculated with Fischer's exact test

**Table 2: Adverse Events**

## 3.9 Figures

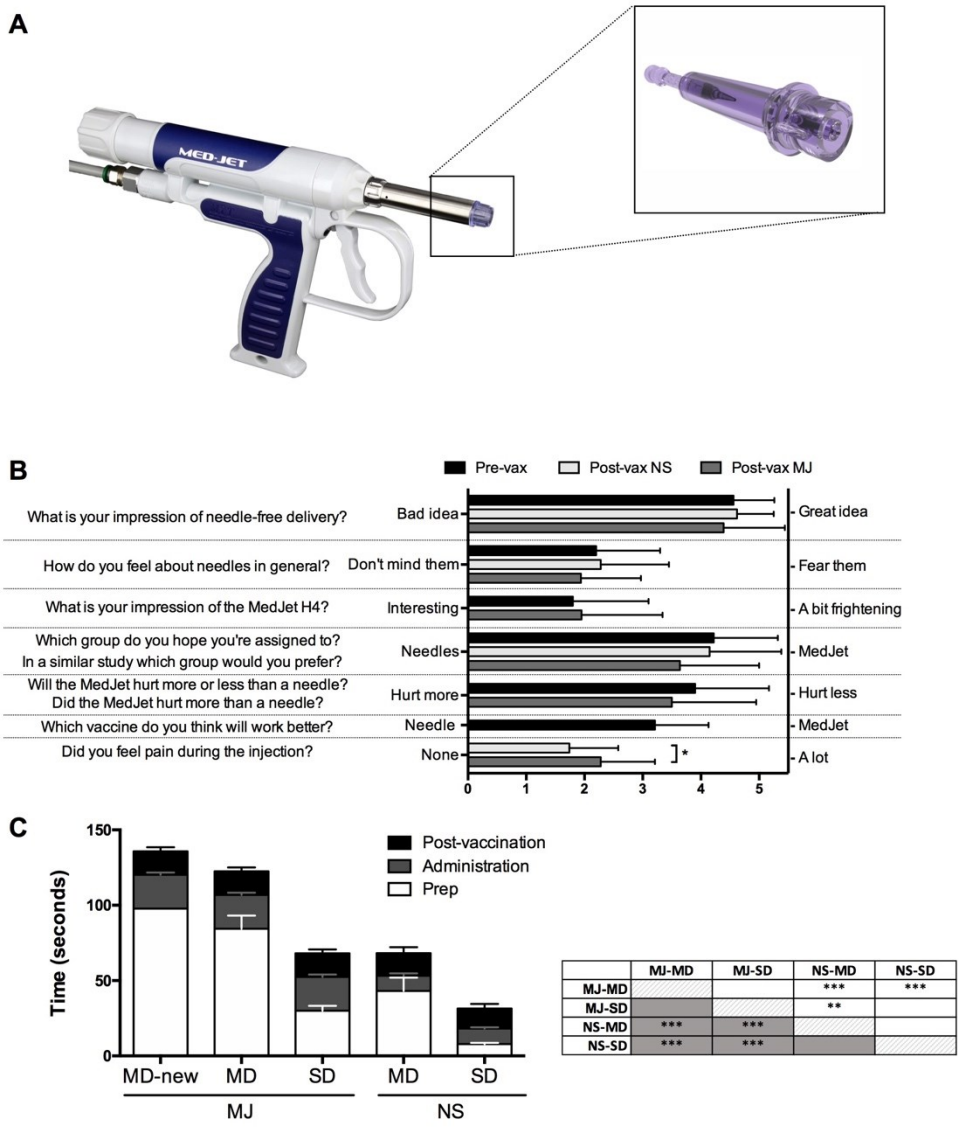



**Figure 1: Attitudes, beliefs and time efficiency of Med-Jet vaccine delivery. A**) Med-Jet H4 device used in the study. Enlargement shows cartridge that is loaded with vaccine from a MD vial. **B**) Patient attitudes and beliefs about needle-free injection, as determined by a pre-randomization and post-immunization questionnaire. Answers were reported on a 5-point likert scale, and error bars represent the SD. Significance was calculated with a Mann-Whitney test. **C**) TM data for each stage of vaccine administration, with 95% CI intervals. MD-new represents time for the first dose from MD vial, when vial adapter had to be attached. The table indicates significance for preparation and administration phases, based on the two-tailed p-values

calculated with t-tests. There were no statistically significant differences in the post-vaccine phase.

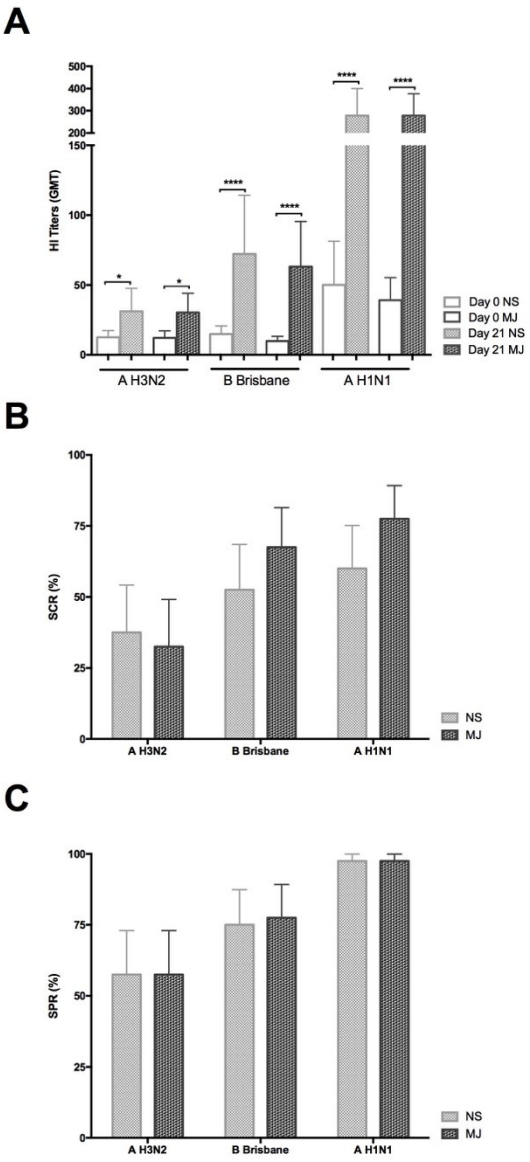



**Figure 2: Sera HI response to Influenza vaccine.** Serum HI antibody responses to the three viruses in the TIV: A H3N2 A/Hong Kong/4801/2014, B/Brisbane/60/2008 and A H1N1/A/California/07/2009 at day 0 and day 21 post-vaccination administered with either NS or Med-Jet. **A**) Geometric mean titer (GMT), **B**) percent of seroprotection rate (SPR) and **C**) percent of seroconversion rate (SCR). N=40 for both groups and error bars depict 95% CI. Turkey tests for multiple comparisons and Fisher's Exact T-tests were used to calculate two-tailed p-values.

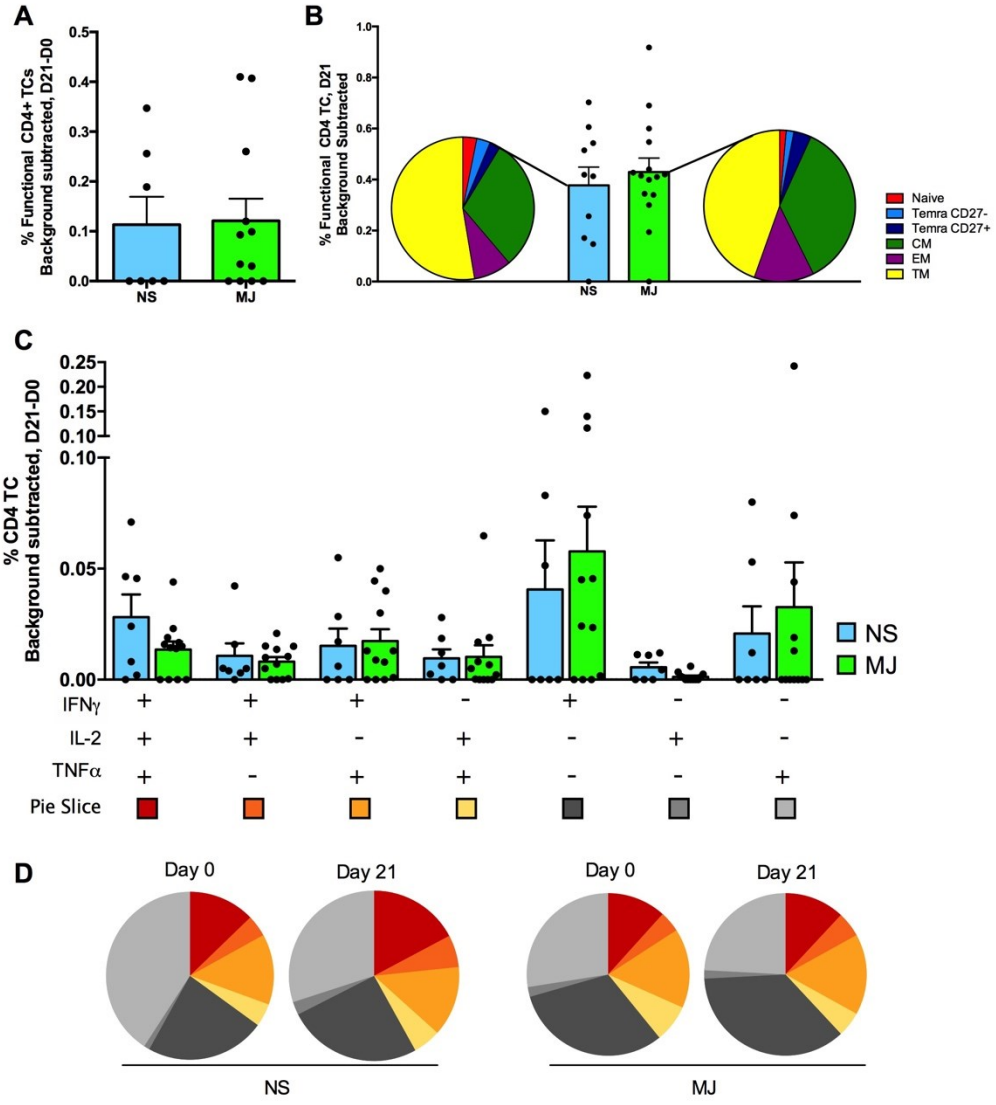
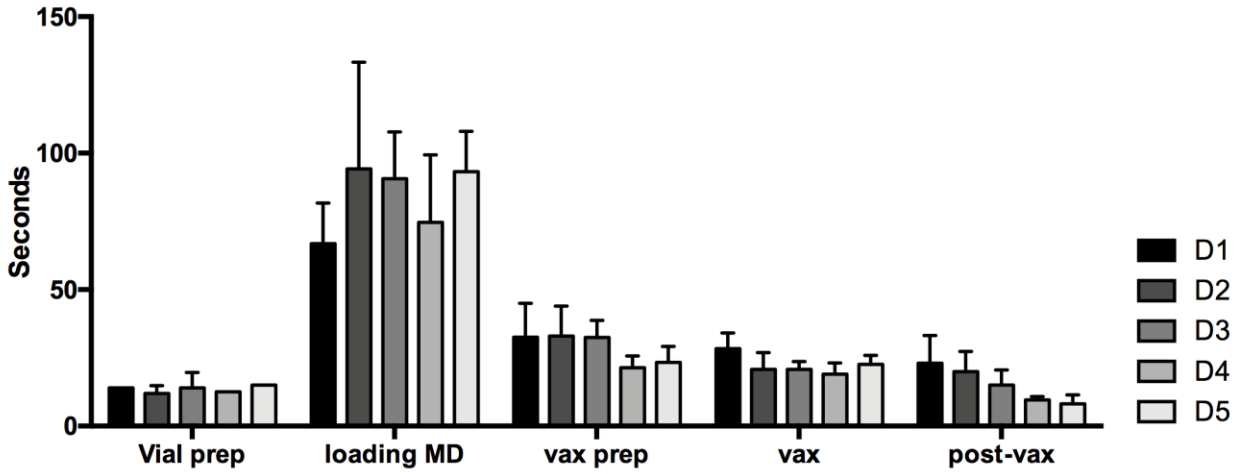



**Figure 3: Cell-mediated immune response to influenza vaccine delivered by NS or Med-Jet.** PBMCs from D0 and D21 were re-stimulated with trivalent influenza vaccine and stained for memory phenotypes and cytokine production. In bar graphs, the mean + SEM and individual results (dots) are represented, n=7 for NS group and n=12 for Med-Jet group. (**A**) Increase in functional $CD4^+$ TCs (expressing IFNγ, TNFα or IL-2) from D0 to D21. (**B**) Memory phenotype of functional $CD4^+$ TCs on D21. (**C**) Qualitative analysis of $CD4^+$ TCs, based on the expression of IFNγ, TNα and IL-2. (**D**) Relative distribution of 7 cytokine-secreting subsets among functional $CD4^+$ TCs. Based on two-tailed p-values calculated with Mann-Whitney and multiple T-tests, no statistically significant differences were found between the NS and Med-Jet groups.

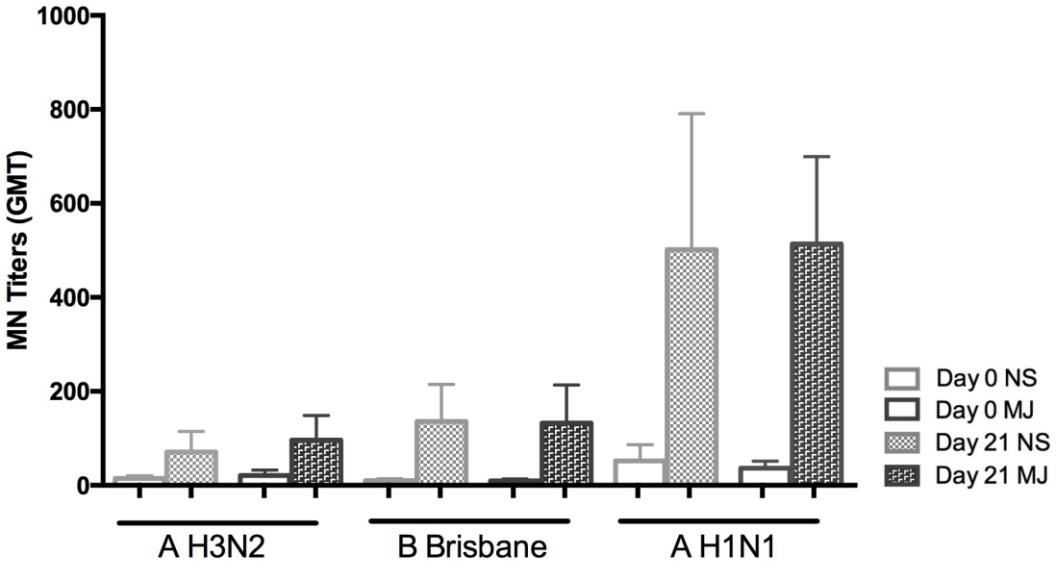
## 3.10 Supplementary Material

**Supplemental Table 1: Tasks involved in vaccine administration**

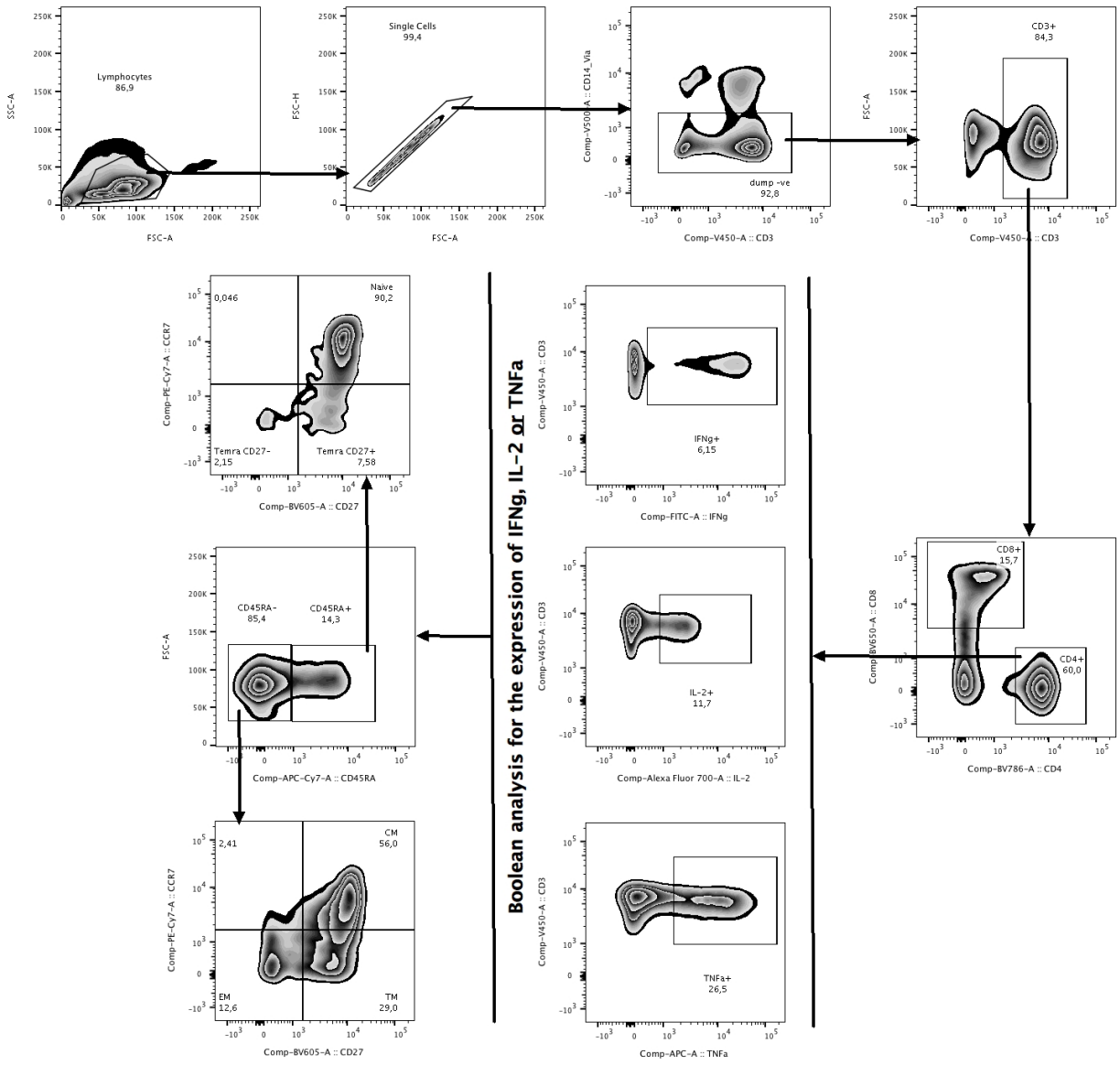
| | MedJet H4 | | Needle-and-syringe | |
|---|---|---|---|---|
| | **Multi-dose** | **Single-dose** | **Multi-dose** | **Single-dose** |
| **Stage 1**: Preparing for vaccine administration | Open vial adapter package and attach adapter to vaccine vial (for first dose in multi-dose vial), remove cartridge from packaging, attach to adapter, load vaccine, remove cartridge from vial and attach to MedJet injector | Remove cartridge from packaging and attach to MedJet injector | Remove syringe and needle from packaging, attach needle to syringe, load vaccine and replace needle | Remove needle from packaging and attach to syringe |
| **Stage 2**: Vaccine administration | Prepare arm and inject dose. | Prepare arm and inject dose. | Prepare arm, remove needle shield and inject dose. | Prepare arm, remove needle shield and inject dose. |
| **Stage 2**: Post-vaccination duties | Clean/treat arm (as needed), remove cartridge from injector and dispose of it along with packaging | Clean/treat arm (as needed), remove cartridge from injector and dispose of it along with packaging | Clean/treat arm (as needed), dispose of syringe and packaging | Clean/treat arm (as needed), dispose of syringe and packaging |




**Supplemental Figure 1: TM by day of vaccine administration.** Daily mean of time to complete tasks involved in vaccine preparation, administration and disposal. "Vial prep" refers to attaching vial adaptor to new MD vaccine vial. "Loading MD" indicates the time to load the vaccine into the single-use cartridge. "Vax prep" entails loading the cartridge into the Med-Jet device. Error bars represent 95% CIs.




**Supplemental Figure 2: Microneutralization titers from day 0 and day 21 after trivalent influenza vaccine (TIV).** Titers were measured for the following three viruses: H3N2 A/Hong Kong/4801/2014, B/Brisbane/60/2008 and A H1N1/A/California/07/2009 for both the NS and Med-Jet. No significant differences were observed between the two groups (n = 40 for both groups).




**Supplemental Figure 3: Flow cytometry Gating Strategy.** Doublet and CD14-positive/viability-negative lymphocytes were excluded from analysis. $CD3^+$ cells were separated into $CD8^+$ and $CD4^+$ populations. $IFN\gamma^+$, $TNF\alpha^+$ and $IL\text{-}2^+$ populations were delineated, and boolean analysis was used to analyze the expression of functional cells (producing IFNγ, TNFα or IL-2). The functional $CD4^+$ TC population was then divided into 6 memory phenotypes based on CD45A, CCR7 and CD27.

## CHAPTER IV

# Immunologic investigation of a possible vaccine-associated adverse event: Bilateral phrenic nerve paralysis in a young woman after human papilloma virus immunization

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## 4.1 Preface

Clinical trials for vaccines intended for widespread or even universal use typically include fewer than 100,000 closely-monitored subjects. As a result, adverse events that may occur at frequencies below 1:10,000 are unlikely to be identified pre-licensure. Detecting, reporting and investigating serious adverse events (SAE) post-licensure are therefore all crucial aspects of any vaccination program. A better understanding of SAEs can help to maintain confidence in vaccination programs, lead to the development of safer vaccines and identify populations at risk of developing a serious side-effect after immunization. An area of particular interest is the potential for vaccine-induced autoimmune disease. As more vaccines are being developed to prevent or treat a growing range of illnesses, it is important to maintain active surveillance to ensure the safety of the vaccines that are currently being administered on a large scale.

## 4.2 Abstract

Over a 6-month period in 2009, a healthy 19-year-old woman (PK) received three doses of Gardasil$^{TM}$, a quadrivalent vaccine used to prevent human papilloma virus (HPV) infection. This vaccine contains virus-like particles (VLP) of the L1 proteins of HPV genotypes 6, 11, 16 and 18 and a proprietary aluminum phosphate adjuvant. PK experienced mild dyspnea and orthopnea 2-3 weeks after the first dose which worsened with each subsequent dose. She was eventually diagnosed with bilateral phrenic nerve paralysis. The rarity of this condition and its temporal association with HPV vaccination (ie: challenge-rechallenge-rechallenge) raised the possibility that one of more components of the vaccine had triggered demyelination of the phrenic nerves. Working with serum and peripheral blood mononuclear cells collected between 2 and 9 years after HPV vaccination, we compared PK's humoral and cellular responses to HPV, and other vaccine antigens, with those of eight healthy, age- and sex-matched HPV-vaccinated control subjects. Even 9 years after vaccination, PK had a strong B cell-driven proliferative response to the vaccine and its component VLPs (up to 4.5-fold greater than controls) as well as a broad-based increase in HPV-specific T cell cytokine production. Although PK's B cells were found to proliferate spontaneously and produced abundant pro-inflammatory cytokines in response to HPV-VLP (eg: IL-2$^{+}$: 0.16±0.07% vs 0.018±0.04% in controls: $p<0.005$), her anti-HPV antibody titres were only 50-65% of the control levels. Her humoral and cellular responses to other vaccine antigens (eg: mumps virus, tetanus toxoid) were either normal or slightly elevated. HLA haplotyping revealed a rare HLA*C allele as well as other alleles associated with autoimmune pathologies, consistent with her family history of autoimmune illnesses. We have not yet been able to conclusively link these unusual responses to HPV antigens and peripheral nerve damage. These data therefore support, but do not prove, an association between the HPV vaccinations and the development of the bilateral phrenic nerve palsy.

## 4.3 Introduction

In April of 2009, a healthy and athletic 19-year-old woman with a family history of autoimmune disease, referred to as PK, received the first dose of the HPV vaccine (Gardasil™: Merck, USA). In the month following immunization, she began to experience fatigue, tightness of the fingers and hands and a malar rash. These symptoms largely resolved through April but

rapidly returned when the second dose of HPV vaccine was given in June, including worsening of her malar rash and more severe fatigue. New symptoms also developed, including sharp chest pains at rest and dyspnea upon moderate exertion. Although some of these symptoms persisted at low levels, PK felt much better by the end of July and had returned to most of her normal daily activities. In September, she received the third dose of HPV vaccine and, in the following week, her exhaustion returned with dyspnea upon mild exertion and she began to experience weak and heavy limbs. In the subsequent 3-4 months, her symptoms steadily worsened and orthopnea and tachycardia developed. In October 2009, she was hospitalized and in December she was diagnosed with asthma, complete paralysis of the left diaphragm and partial paralysis of the right diaphragm due to bilateral phrenic nerve involvement. By June 2010, there was no improvement in right nerve function and slight improvement in left nerve function. In the years since diagnosis, PK has made a nearly full recovery, but has never returned to the level of physical she maintained prior to the event.

Two highly unusual features of this case are noteworthy. First - paralysis of one or both phrenic nerves is most commonly associated with traumatic injury, such as major thoracic surgery[1]. Spontaneous phrenic nerve paralysis is an exceedingly unusual event, with a small number of case reports associated with serious neurological disease[2], infections (pneumonia, herpes zoster infection, dengue)[1,3] and immunomodulatory therapy[4,5]. Second - PK's history is strongly suggestive of challenge-rechallenge (and even re-re-challenge) with the HPV vaccine. Her symptoms appeared more quickly after each dose (ie several weeks after the first dose but within days of the third dose) and they became more severe with each vaccination. This pattern is considered to be highly relevant in assessing causality of vaccine-associated severe adverse events (SAE)[6]. We could find no other published cases of phrenic nerve paralysis associated with HPV vaccine in the literature and the only case in the Vaccine Adverse Event Reporting System (VAERS) is PK herself.[7]

Gardasil is a qaudrivalent virus-like particle (VLP) vaccine that targets HPV6, 11, 16 and 18. Globally, 270 million doses of this vaccine have been distributed over the past decade[8]. Each dose contains four different VLPs composed of the self-assembling L1 major capsid protein of one of the four targeted viral strains[9]. Gardasil also contains a proprietary amorphous aluminum hydroxyphosphate sulfate (AAHS) adjuvant that is physically and functionally distinct from other aluminum-based adjuvants[10]. Multiple clinical trials have demonstrated that Gardasil is 90-

100% effective in preventing pre-cancerous cervical lesions and genital warts caused by the targeted viruses in women aged 15-26 and causes no statistically significant increase in short- or medium-term adverse events compared to placebo[11].

Despite the broad consensus that HPV vaccines are very safe, like other vaccines given to adolescents and young adults, the age group when many autoimmune conditions first manifest[12], there are numerous reports of autoimmune-type illnesses/events following HPV immunization[13]. For example, one small case series describes six healthy young women who developed symptoms of postural tachycardia syndrome (POTS) after receiving Gardasil and, in two of these cases, the symptoms worsened with subsequent doses (i.e. challenge-rechallenge)[14]. A search of the VAERS database revealed 29 reports of POTS temporally-associated with HPV vaccination that fully met diagnostic criteria[15]. In another report, four patients developed neuromyelitis optica after immunization[16]. In this study, peptide sequences found in both the vaccine antigens and human aquaporin 4 raised the possibility that shared T cell epitopes may have led to immunologic cross reactivity[16]. Another small case series describes six patients who developed systemic lupus erythematosus (SLE) in temporal association with HPV vaccination[17]. In addition, there are multiple case reports of central nervous system demyelinating disease after HPV immunization, including cases of acute disseminating encephalomyelitis[18-21]. In several of these reports, the subjects either had a family history of autoimmune disease or had symptoms before vaccination that were sub-clinical[17,18,22,23], and in none of these cases has a causal relationship with HPV vaccination been proven. Large epidemiologic studies have found no association between HPV vaccines and autoimmune diseases[24-29].

Although the triggers for most autoimmune conditions are unknown, it is clear that both genetic and environmental factors can contribute[30]. Large-scale sequencing efforts have revealed a steadily enlarging number of associations between specific human leukoctye antigen (HLA) alleles and one or more autoimmune conditions[31]. Infections may be among the most important environmental triggers of autoimmunity; most often attributed to molecular mimicry between microbial and self-antigens[32]. Although remarkably rare given the massive exposure of populations to vaccines, there are nonetheless a small number of autoimmune events that can be attributed to vaccination with some confidence: among them post-infectious encephalomyelitis after measles or MMR vaccines[33,34], Guillain-Barre Syndrome (GBS) after seasonal influenza vaccine[35], and possibly narcolepsy after the 2009-10 pandemic adjuvanted influenza vaccine[36].

Regardless of mechanisms that trigger autoimmunity, these conditions are typically characterised by abnormal lymphocyte activation and proliferation[37]. Classic immunology proposes that Th1-type responses, characterized by the expression of IL-2, IFNγ, TNF and lymphotoxin are the primary drivers of cell-mediated inflammation in auotimmunity[38]. More recently, Th2-type responses, traditionally considered to be anti-inflammatory, have also been implicated in immunopathology, particularly in antibody-mediated autoimmune diseases[38,39]. Furthermore, there is increasing evidence that an absence of regulatory activity mediated by either T or B cells can also push an otherwise innocuous response towards autoimmunity[40-42].

The challenge-rechallenge and even re-re-challenge pattern of PK's symptoms raised the obvious question of whether or not a causal link might exist between one of more components of the HPV vaccine and demyelination of PK's phrenic nerves. Preliminary studies demonstrating that PK's peripheral blood mononuclear cells (PBMC) proliferated in response to *ex vivo* stimulation with Gardasil to a far greater extent than other vaccinated individuals led to a series of experiments of increasing complexity to more fully assess her unusual response. Almost a decade after vaccination, PK's T cell ($CD4^+$ and $CD8^+$) and B cell responses (ie: proliferation, cytokine production) to Gardasil and the individual VLPs were between 2 and 20-fold higher than controls. Her B cell responses were particularly intriguing since her anti-HPV antibody titres were only 50-65% of the controls. These abnormal immune cell responses to HPV antigens persisted for almost a decade after exposure to Gardasil, although assays that were repeated with samples taken 2 and 9 years after vaccination suggested a waning of the intensity of response. These *in vitro* observations were consistent with her slow but almost complete recovery of phrenic nerve function during this period.

## 4.4 Results

### 4.4.1 PK has strong lymphoproliferative responses to Gardasil and its components

In 2011, approximately 2 years after HPV vaccination, PK's PBMC were found to proliferate actively in response to *ex vivo* Gardasil stimulation with stimulation indices (SI) 2- to 9-fold greater than healthy age- and sex-matched vaccinated controls (data not shown). PK's lymphoproliferative response to *ex vivo* Gardasil re-stimulation was still >2-fold higher than appropriate controls in 2017 (Figure 1A). There were no significant differences between the

lymphoproliferative response of PK's PBMC to PHA stimulation compared to controls either in 2011 or 2017 (data not shown, Figure 1A), suggesting an antigen-specific effect. However, the Gardasil data were obviously confounded by the presence of the proprietary aluminum-based adjuvant. When PBMC from either 2011 or 2017 were stimulated with the individual VLPs at an equivalent total L1 antigen dose, the antigen-specificity of PK's lymphoproliferative response was confirmed and a hierarchy of response for the individual VLPs was strongly suggested: VLP 11 >> VLP 16 = VLP 6 > VLP18 (Figure 1B).

### 4.4.2 Shared T cell epitopes between L1 sequences and human peripheral nerve proteins

Bioinformatic analyses focused on the L1 sequences of two of the main drivers of the proliferative response (ie: VLP 11 and VLP 16) revealed three peptides with sequence homology to human proteins involved in the peripheral nervous system (Suppl. Table 1). When these peptides were synthesized and used to stimulate PBMC *in vitro,* PK's responses were not significantly different from the vaccinated controls (Figure 1C). Although these observations do not completely eliminate the possibility of a molecular mimicry mechanism in PK's case, they steered the focus of the investigation away from T cells to some extent.

### 4.4.3 PK's proliferative response is driven by antigen-specific B cells

This reorientation in focus was reinforced when the Ki67 flow cytometric data showed that PK's strong lymphoproliferative response to HPV antigens was driven primarily by B cells. After stimulation with the four VLPs combined, 7.13% of PK's B cells expressed Ki67 compared to a mean of 2.1% for the vaccinated controls ($p = 0.0008$) (Figure 1D). The difference between PK and the vaccinated controls was also significantly different after stimulation with each of the 4 monovalent VLPs (VLP 6 $p = 0.004$, VLP 11 $p = 0.039$, VLP 16 $p = 0.003$ and VLP 18 $p = 0.007$) (Figure 1D). In contrast, there were no significant differences between PK and the control subjects in $CD4^+$ T proliferation in response to Gardasil, the individual or combined VLPs (Figure 1F). Although simulation with the four VLPs led to a slightly greater proliferative response of PK's $CD8^+$ T cells ($p = 0.038$) (Figure 1G) when compared to the controls, there was no consistent pattern of CD8 proliferation to the different vaccine antigens.

### 4.4.4 PK has enhanced cytokine production in response to HPV antigens

In 2011 (~2 years after HPV vaccination), ex vivo stimulation of PKs PBMC with Gardasil resulted in ≥10-fold increases in the release of IL-2, TNFα, TNFβ, IL-6, IL-5 and IL-13 into the culture supernatant (versus 3-5-fold in the control PBMC cultures), and a >50-fold increase in IFNγ (versus to <20-fold in controls) (Figure 2A). Compared to controls, these increases reached statistical significance for both Th1-type cytokines IL-2 (22.4 vs 5.1, $p = 0.001$), IFNγ (57.2 vs 15.6, $p = 0.03$) and TNFβ (16.8 vs 2.8, $p = 0.01$) and Th2-type cytokines IL-5 (20.5 vs 3.5, $p = 0.0012$) and IL-13 (17.1 vs 3.2, $p = 0.015$). A similar pattern of broad cytokine production was seen in the supernatants from PK's antigen-stimulated PBMC collected in 2017 but responses were generally lower than those of the 2011 samples (Figure 2A). Again, these *in vitro* observations were complicated by the presence of the adjuvant in the Gardasil used to stimulate the PBMC. When PK's PBMC isolated in 2017 were stimulated with individual VLPs, two overall patterns of response were observed. The cytokine production profile elicited from PK's PBMC by VLP18 was overall very similar to the control samples (Figure 2E). PK's response to VLP6 stimulation was more vigorous, with 2- to 4-fold higher levels of IL-2, IL-13 ($p<0.005$) and IL-6 (Figure 2B), and the response to VLP16 was dominated by 9-fold higher production of IL-6 (61-fold versus 7-fold over unstimulated PBMC in PK and controls respectively) with slightly increased levels of IL-5 ($p<0.02$), IFNγ and TNFα (Figure 2D). The cytokine profile of PK's PBMC to VLP11 was the most consistently different from the control samples with 2- to 8-fold higher levels of Th1 (IL-2, TNFα, TNFβ: $p<0.02$), Th2 (IL-5: $p<.03$, IL-13: $p<0.03$) and Th17 cytokines (IL-6, IL17). Only some of these differences between PK's responses reached statistical significance compared to the control subjects, in large part due to limited power by imbalanced group size (Figure 2). It is noteworthy that none of the individual VLPs elicited the strong Th1 response (IL-2 or IFNγ) seen after Gardasil stimulation and none of the conditions induced a strong IL-10 (regulatory) response in the PBMC isolated from either PK of the controls (data not shown).

### 4.4.5 PK has inflammatory cytokine responses, but not strong proliferative responses, to non-Gardasil antigens

The lymphoproliferative response of PK's PBMC to a mitogen (PHA) and to other vaccine antigens (tetanus toxoid (TT)) were slightly higher than the control samples but these differences did not reach significance (Figure 3A, B). Stimulation with TT elicited the release of a broad range of cytokines by PK's PBMC that generally exceeded production by the control PBMC (all except IFNγ and TNFα) but none of the differences for individual cytokines reached statistical significance (Figure 3C). PK's PBMC also responded with strong cytokine release following stimulation with mumps antigen; particularly the Th1 cytokines IFNγ (32-fold vs 6-fold increase over unstimulated PBMC; $p<0.02$), TNFα (7.2-fold vs 3.8-fold increase; $p<0.03$), TNFβ and IL-6 (Figure 3D). When PK's PBMC were stimulated non-specifically with PMA/ionomycin and analyzed by intracellular cytokine staining and flow cytometry, there were no differences from control samples in cytokine production by B cells or $CD4^+$ T cells, but more of PK's $CD8^+$ T cells produced TNFα than the controls (59.22% vs 30.53%, $p < 0.02$) (Figure 3E).

### 4.4.6 PK has a weak antibody response to HPV antigens despite B cell activation

Despite the strong proliferative response of PK's B cells to *ex vivo* stimulation with either Gardasil or the individual HPV VLPs, PK's serum IgG titers measured by ELISA were 20-40% lower than the controls for Gardasil, the four VLPs combined and for all of the individual VLPs, except VLP16 (Figure 4A). In contrast, PK's capacity to produce antibodies to other vaccine antigens (eg: rubella, measles) appeared to be comparable to the control subjects (Figure 4A). The lower anti-HPV titres were not attributable to waning immunity since PK's titers were stable from 2011 to 2018 (Figure 4A). PK's IgG electrophoresis was essentially normal except for a slightly elevated level of IgG2 (Suppl. Table 2). A closer examination of PK's PBMC revealed that B cells made up a lower proportion of her circulating lymphocytes than the controls (3.63 vs 5.98 % of $CD14^-$ PBMC: $p=0.055$) (Figure 4B) but that many of these B cells were proliferating spontaneously (5.5±1.4 vs 2.4±0.36% in controls; $p<0.006$) (Figure 4C). Furthermore, intracellular cytokine staining demonstrated striking cytokine production by PK's B cells upon *ex vivo* stimulation of PBMC cultures with Gardasil, the 4 VLPs together or the individual VLPs (Figures 3D-F). For example, more of PK's B cells produced IL-2 than the vaccinated controls in response to both Gardasil (0.21% vs 0.015%, $p = 0.00004$) and the 4 VLPs combined (0.16% vs

0.0175, p = 0.005) (Figure 4D). Compared to control samples, TNFα expression was significantly increased in PK's B cells in response to VLP 11 (0.13% vs 0.03%, p = 0.009) and VLP 16 (0.11% vs 0.02%, p = 0.01) (Figure 4E) and IL-6 production was higher in response to VLP18 (1.0% vs 0.30%, p = 0.035) (Figure 4F).

### 4.4.7 PK's HLA haplotype is associated with autoimmunity

As noted above, PK's family history included individuals with idiopathic thrombocytopenia, Raynauds, rheumatoid arthritis (RA) and GBS, and PK herself had experienced Raynauds, mild asthma and hives prior to receiving Gardasil vaccination. Analysis of PK's HLA haplotype revealed that several of her alleles are associated with a variety of autoimmune conditions, including HLA*DRB1:07:01, which is associated with autoimmune hepatitis type 2[43], Crohn's disease[31,44,45], primary biliary cirrhosis[46], GBS[47], vitiligo[48,49] and dermatomyositis[50] (Suppl. Table 3). One of PK's HLA*C alleles, 17:01:01:02, is considered to be very rare as it is not defined in the "Common and Well Documented Allele Catalogue" and there are no matches in the allele frequency database[51-53].

## 4.5 DISCUSSION

It is very likely that the large majority of serious adverse events associated with vaccination have multifactorial causation with contributions from both the genetic makeup of the host and environmental triggers, including infections and antigen exposures[30]. In most cases, once the event occurs, it is extremely difficult, if not impossible, to determine whether an abnormal immune response to a specific antigen contributed to the initiation of the event or arose as a result of the event: the 'chicken versus egg' conundrum. In PK's case, we found several peptide sequences that are shared by the L1 proteins of HPV 11/16 and human peripheral nerve components. It is therefore possible that any unusual pattern of immune reaction to the vaccine antigens is the ***result*** of her phrenic nerve damage rather than evidence of the vaccine ***causing*** the damage. However, the rarity of the event itself (bilateral phrenic nerve palsy) and the pattern of challenge, re-challenge and even re-re-challenge strongly suggest that HPV vaccination at least contributed to the development of the nerve damage.

It seems likely that PK's genetic heritage put her at higher risk for this kind of event, since her family history included individuals with a range of autoimmune conditions. The GBS that

occurred in her maternal grandmother may be of particular significance because it involves demyelination of peripheral nerves and has a clear association with both infection (eg: Campylobacter jejuni, influenza) and influenza vaccination[54]. Prior to developing the phrenic nerve palsy, PK herself had experienced autoimmunity in the form mild Raynaud's syndrome. Beyond the family history, the finding that several of PK's HLA alleles are strongly associated with a number of autoimmune conditions certainly supports the supposition of a genetic risk. On the other hand, the rarity of one of PK's HLA*C alleles may provide a plausible explanation for the fact that no other similar cases of phrenic nerve palsy have been reported in association with HPV vaccination, despite its use in hundreds of millions of young women. Future experiments are planned to examine the possible relationship between the HLA*C allele carried by PK (17:01:01:02) and aberrant B cell responses to HPV antigens.

In this work, we have demonstrated that, almost a decade after vaccination, PK had an unusually strong *ex vivo* response to Gardasil and to several of the individual VLP components of the vaccine. Although there was some evidence of T cell hyper-responsiveness (ie: broad-based T cell cytokine production and modest T cell proliferation), the most striking differences between PK and the age- and sex-matched vaccinated controls were seen in B cells. PK had a slightly lower frequency of B cells in peripheral circulation compared to the control subjects, but a greater proportion of these cells were proliferating spontaneously (5.5 ± 1.4 versus 2.4 ± 0.36%), their proliferative response to HPV-antigens was 3- to 4-fold greater than controls, and intracellular production of IL-2, TNFα and IL-6 that was 3- to 14-fold higher than seen in the control cells. These observations are particularly interesting since PK's antibody response to HPV antigens was generally lower than that of the control subjects. Although PK had a slightly elevated serum concentration of IgG2, and IgG subclass imbalances have been reported in a number of autoimmune conditions (ie autoimmune pancreatitis, hypothyroidism, primary biliary cirrhosis and irritable bowel syndrome)[55,56], we have not yet assessed whether or not PK has autoantibodies that react directly with peripheral nerve tissues. However, in addition to the secretion of autoantibodies, B cells have the potential to contribute to autoimmunity through antigen presentation and the secretion of cytokines[57]. Indeed, antigen-presenting B cells have been implicated in models of multiple sclerosis (MS) and RA[58,59] and cytokine dysregulation in B cells has also been observed in several autoimmune diseases. For example, the ratio of TNFα- to IL-10-producing B cells is higher in SLE patients compared to healthy individuals[60]. In

murine experimental autoimmune encephalitis (EAE), animals with a B cell-specific inability to produce IL-6 are resistant to disease but EAE is exacerbated when B cells cannot express regulatory cytokines such as IL-10 or IL-35[61,62]. Indeed, the murine EAE model suggests that there may be a reciprocal relationship between pro-inflammatory cytokine production by B cells and their ability to develop into antibody-secreting cells (ASC)[61].

Even though the most striking difference between PK and the healthy controls were the B cell responses, both spontaneous and VLP-induced $CD8^+$ T cell proliferation were generally greater in PK than in healthy controls. Furthermore, stimulation of PK's PBMC with HPV-antigens elicited a broad-based cytokine response that was much more vigorous than was seen in PBMC cultures from the control subjects. Several of the cytokines over-produced by PK's PBMC in response to the HPV vaccine antigens have certainly been implicated in autoimmunity. Most notably, Th1-type cytokines such as IFNγ are associated with pathology in type 1 diabetes, MS, RA and other autoimmune diseases[38], while the Th2 cytokines IL-4, IL-5 and IL-13 have been implicated B-cell mediated autoimmune diseases such as SLE[38]. Furthermore, MS patients were found to have significantly more $CD4^+$ and $CD8^+$ T cells that produced IL-13, and this frequency increased substantially during a relapse phase, although the causes and effects of this increase remain unclear[63]. A relative lack of regulatory T cells and/or regulatory cytokines such as IL-10 and IL-35 have also been implicated in the development of autoimmunity in both animal models and humans[40-42]. Future experiments with PK's T and B cells will examine an expanded range of cytokines and chemokines.

There are many limitations to this study. From the outset, our investigations were unnecessarily complicated and delayed by difficulties accessing appropriate reagents. Neither the proprietary adjuvant nor the individual VLPs were made available by the manufacturer of Gardasil. Although we eventually found alternate sources for the VLPs, we were not able to assess the response of PK's PBMC to Merck's propriety aluminum adjuvant (AAHS) in isolation. Unlike other adjuvants, AAHS is not charged at neutral pH, possibly increasing its capacity to bind antigen and then release it after injection[10]. Although AAHS is thought to preferentially promote humoral responses, it can also elicit IFNγ production by human T cells[10]. In our experiments with Gardasil and the VLPs, it was clear that the adjuvant (or some other component of the whole vaccine) had a strong effect on PBMC, decreasing cell viability by both classical apoptotic and non-apoptotic mechanisms at higher concentrations (Suppl. Figure 2A-E).

Obviously, the work described herein relates to a single case of phrenic nerve paralysis. To our knowledge, there are no other documented reports of this type of SAE temporally associated with HPV immunization. Furthermore, the PBMC used in this study were obtained between 2 and 9 years after the 'events' and only tiny amounts of the VLP reagents were available to us in 2011. However, in the limited experiments that were repeated at both time-points (eg: preliminary lyphoproliferation data, cytokine profiles (Figure 2)) there is a strong suggestion of a waning effect consistent with PK's slow clinical improvement.

The evidence that we have accumulated to date do not permit us to establish a clear causal link between the series of HPV vaccinations PK received and the contemporaneous development of bilateral phrenic nerve palsy. Unfortunately, we did not have any serum from PK prior to vaccination so we do not know if there had been any priming to HPV antigens through wild-type exposure(s). We can never know if PK would have developed bilateral phrenic nerve palsy spontaneously, and there is good reason to believe that PK was at risk for the development of autoimmunity based on her family history. However, even nine years after vaccination, PK's response to HPV antigens was strikingly abnormal while responses to other vaccine antigens (eg: tetanus toxoid, mumps) were vigorous but within the normal range. Although this work may eventually have implications for PK's close relatives, it does not address the safety of Gardasil in the general population. HPV vaccination is a pivotal health intervention to prevent genital warts, cervical cancer and other associated cancers, and many large epidemiological studies have found no association between HPV vaccination and autoimmune disease[24-29]. Therefore, this work has no bearing on vaccination recommendations made by governmental and non-governmental authorities. Ultimately, this project underscores the importance of monitoring post-licensure vaccine safety and the need for detailed immunologic evaluation of unusual vaccine-associated adverse events.

## 4.6 METHODS

### 4.6.1 PBMC isolation & cryopreservation

PBMCs were isolated from whole blood by differential density centrifugation using SepMate tubes (Stemcell, Vancouver, BC) according to the manufacturer's instructions. Before processing, serum was collected from a subset of samples and stored at -20°C. For

cryopreservation, cells were re-suspended at $5x10^6$ cells/mL in FBS + 10% DMSO (Sigma Aldrich, Oakville, ON) and stored in liquid nitrogen until use. After thawing, trypan blue was used to ensure viability was >80% and PBMC were allowed to rest for a minimum of 2 hours at 37°C before further processing. PK donated blood samples in 2011 and in 2017/2018. Unless otherwise indicated, the samples from 2017/2018 were used for analysis.

### 4.6.2 Cell Proliferation Assays

A chemiluminescent bromodeoxyuridine (BrdU) cell proliferation assay (CPA) kit (Roche, Manheim, Germany) was used in 2017/2018, following the manufacturer's instructions. Thawed PBMC were plated at 200,000 cells/well and stimulated with 6 ug/mL of Gardasil or 1.5ug/mL of the four VLPs for 5 days or with phytohaemagglutinin (PHA) for 3 days. For peptide CPAs, 5 ug/mL of each peptide was used to stimulate cells for 72 hours. Twenty-four hours prior to the end of stimulation, BrdU labelling solution was added to each well. Plates were read on the Infinite M200 plate reader (Tecan). Limited early experiments conducted in 2011 used $H^3$-thymidine incorporation to assess the lymphoproliferative response to *ex vivo* Gardasil stimulation for 72 hours (2 or 4 ug/mL). Results are reported either as stimulation indices (SI = mean of antigen-stimulated wells/mean of control wells) or as fold-difference compared to age- and sex-matched healthy vaccinated controls.

### 4.6.3 Flow Cytometry

Proliferation was also measured via nuclear staining of Ki67. Cells were plated in triplicate at 200,000 cells/well and stimulated for 72 hours with 2ug/ml total antigen of Gardasil, the 4 VLPs combined, the individual VLPs or PHA. Triplicates were pooled and then stained for viability (efluor 780; 1:375, eBioscience, San Diego CA) and then a panel of extracellular markers (anti-CD3-V500 (clone UCHT1; 1:40, BD Horizon, San Diego CA), anti-CD4-efluor 450 (RPA-T4;1:80, eBioscience), anti-CD8-Brilliant Violet 605 (RPA-T8; 1:40, Biolegend), anti-CD19-Brilliant Violet 650 (HIB19; 1:80, Biolegend, San Diego CA) and anti-CD14-BUV 395 (MØP9; 1:40, BD Horizon)). Cells were then fixed with Foxp3/Transcription Factor Permeabilization/ Fixation buffer (eBioscience) and stained with anti-Ki67–FITC (20Raj1; 1:20, ebioscience).

To assess the apoptotic state of PBMC after *ex vivo* exposure to Gardasil and the VLPs, cells were stimulated with antigen for three days and stained with viability dye as described above.

Cells were then stained with Annexin V and 7-AAD as per manufacturer's instructions (PE Annexin V Apoptosis Detection Kit I, BD Pharmingen).

Cytokine production was assessed with intracellular staining. PBMC were plated at 600,000/well and stimulated with 5ug/ml total antigen for 18 hours. After 14 hours of stimulation, 1 μL of brefeldin A (Ebioscience) was added to each well, and phorbol 12-myristate-13-acetate (PMA) (1.56 μg/mL) and ionomycin (3.125 μg/mL) (Sigma, St. Louis, MO) were added to positive control samples. Cells were stained with the extracellular panel detailed above, fixed and stained with the following intracellular panel: anti-IL-2-Alexa fluor 700 (MQI17H12; 1:200, Biolegend), anti-IL-6-PE CF54 (MQ2-13A5; 1:40, BD Horizon) and anti-TNFα-Brilliant Violet 711 (Mab11; 1:20, Biolegend).

For all experiments, compensation was performed according to the manufacturer's instructions using one-comp beads (ebioscience) and/or single stains, and a minimum of 100,000 events were acquired on the LSR Fortessa (BDbioscience). Data were analyzed using FlowJo software (Treestar, Ashland). The gating strategy is described in supplemental figure 1.

### 4.6.4 Cytokine & Antibody ELISAs

To determine cytokine concentrations in supernatant from antigen-stimulated PBMC, a Q-Plex Array Chemilluminescent kit, the Q-view Imager Pro and Q-view software were used following the manufacturer's instructions (Quansys Bioscience, Logan, UT). The cytokines/ chemokines assessed were: IL-1$\alpha$, IL-1$\beta$, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, Il-15, IL-17, IL-23, IFN$\gamma$, TNF-$\alpha$ and TNF-$\beta$. For each cytokine, a stimulation index was calculated by dividing concentrations in the stimulated wells by those in the unstimulated control wells. These data are presented as log-scale radar plots with 10-fold changes highlighted in bold.

ELISAs were performed to measure levels of antigen-specific IgG in serum according to standard procedure. Plates were coated with 2.7ug/ml of antigen[64], or with serially diluted IgG from human serum (Sigma-Aldrich) to create a standard curve. Heat inactivated serum samples were diluted 1:50. Antigen-specific IgG was detected with a mouse anti-IgG antibody conjugated to horseradish peroxidase (Fisher Scientific, Ottawa, ON) and tetramethylbenzidine (Millipore, Burlington, MA).

### 4.6.5 Bioinformatic Analysis

Sequence similarities between the four HPV L1 proteins were identified using the UniProt sequence alignment tool[65], and differences were identified using the Reinforced Merging for Unique Sequences tool (REMUS) (http://biotools.cs.ntou.edu.tw/Remus.asp)[66]. The Basic Local Alignment Search Tool was used to identify human proteins with sequence homology to VLP peptides[67], and then the Panther Classification System of Gene Ontology (http://geneontology.org/) was used to identify human proteins involved in biological processes of interest[68,69]. Peptides were synthesized by GenScript (Piscataway, NJ).

### 4.6.6 HLA Typing

HLA class I typing was performed as previously described[70] using reagents from GenDX (Netherlands) and GenDx SBTengine Software (Genome Diagnostics, Netherlands). HLA class II typing was also performed as previously described[71]. Briefly, sequencing libraries were generated following manufacturer's instructions (HLA 96/11 kit: Omixon, Hungary). Libraries were then sequenced in the MiSeq platform (Illumina) using MiSeq Reagent Kit v2, 500 cycle (Illumina). Finally, the paired-end reads (2x250 bp) generated were analyzed for HLA typing using HLA Twin software v 2.1.4 (Omixon) with default settings.

### 4.6.7 Blinding and Statistical analysis

All key experiments were conducted or repeated by operators blind to sample origin. Statistical analysis was performed on Graphpad Prism (GraphPad Software, version 6.0c, La Jolla, CA). All error bars represent the standard error of the mean. Significance was calculated with multiple t-tests.

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## 4.9 Figures

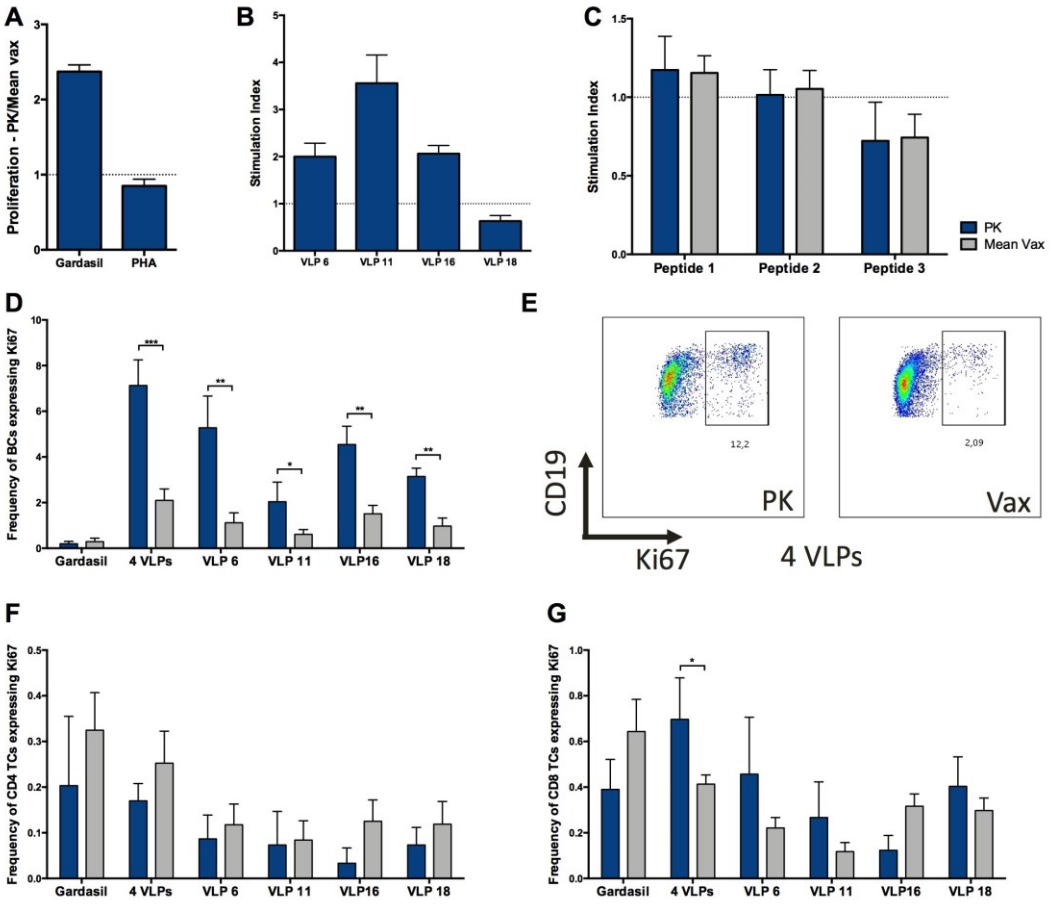



**Figure 1: PKs B cells undergo abnormal proliferation in response to Gardasil vaccine antigens. (A - C)** PBMC proliferation was measured by BrdU incorporation. Stimulation indices (SI) are a ratio between proliferation in stimulated and unstimulated cells. (**A**) The ratio of PKs SI to the mean vax (n=4) SI from two independent experiments. (**B**) PK's mean SIs after VLP stimulation, from 2 independent experiments with PBMC from either 2011 or 2017. **C)** Proliferation after 72-hour stimulation with peptides. Values for PK are the mean from 4 individual experiments, and mean vax is data from 10 control subjects. **(D)** Frequency of $Ki67^+$ B cells after 72-hour stimulation with Gardasil and vaccine antigens, as measured by flow cytometry. All values are background subtracted with unstimulated samples. Values for PK are from 3 independent experiments, and mean vax is the compiled data from 8 control subjects. **(E)** Representative flow cytometry plots from PK and a vaccinated control after 4VLP-stimulation. Events shown are live single cells that are $CD14^-/CD3^-/CD19^+$. **(F-G)** Same as **D**, but with $CD4^+$ TCs and $CD8^+$ TCs, respectively. Significance: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ and **** = $p < 0.0001$.

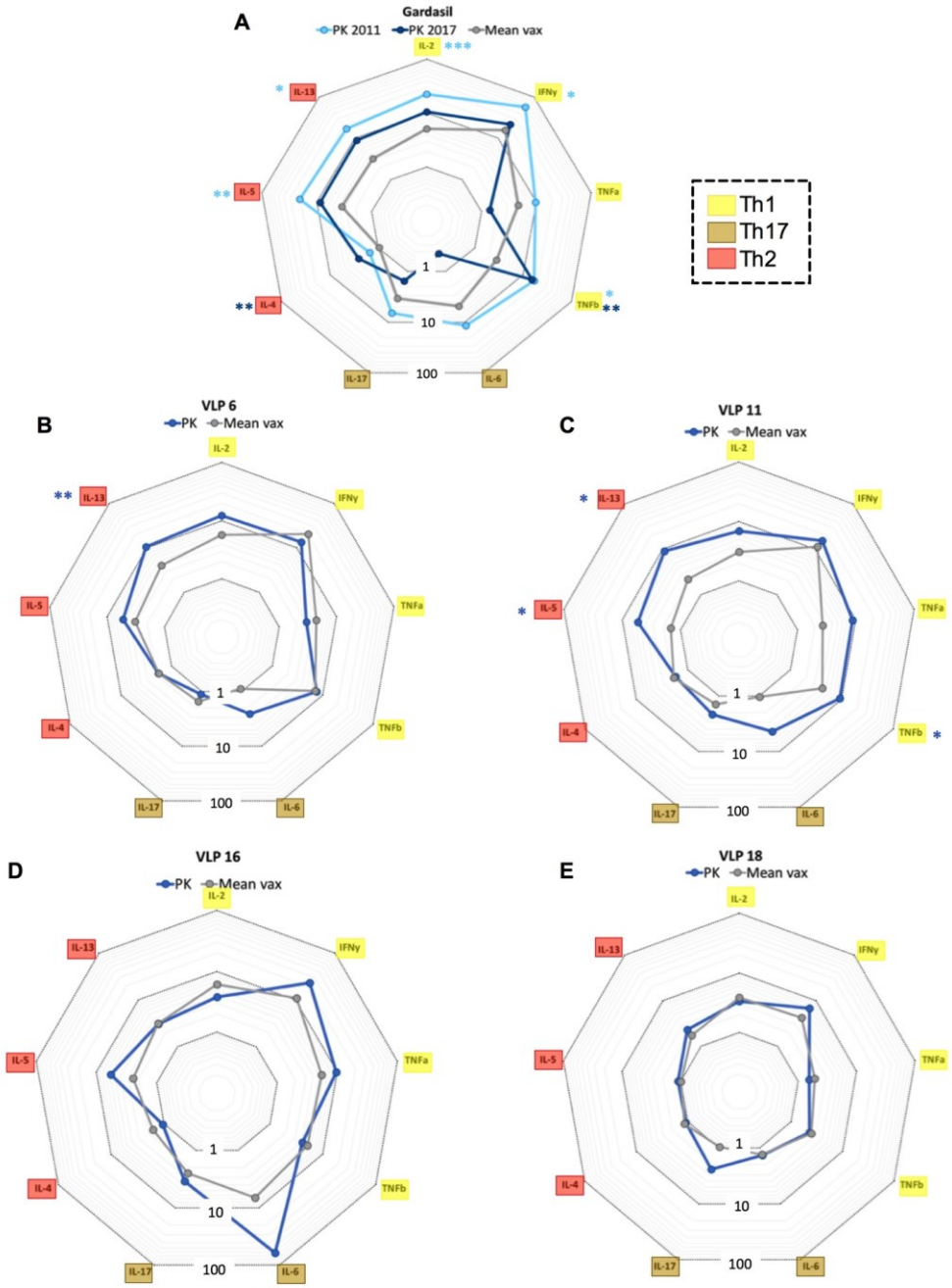



**Figure 2: PK Over-produces Th1 and Th2 cytokines in response to Gardasil vaccine antigens.** PBMC were stimulated for 72 hours with **(A)** Gardasil, **(B)** VLP 6, **(C)** VLP 11, **(D)** VLP 16 and **(E)** VLP 18. Concentrations of 9 cytokines were measured using a multi-plex cytokine ELISA. Data shown is an average of two independent experiments with 2-9 vaccinated controls. For each cytokine and each subject, a ratio between stimulated and unstimulated samples was calculated and is shown on log-scale. Significance: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ and **** = $p < 0.0001$.

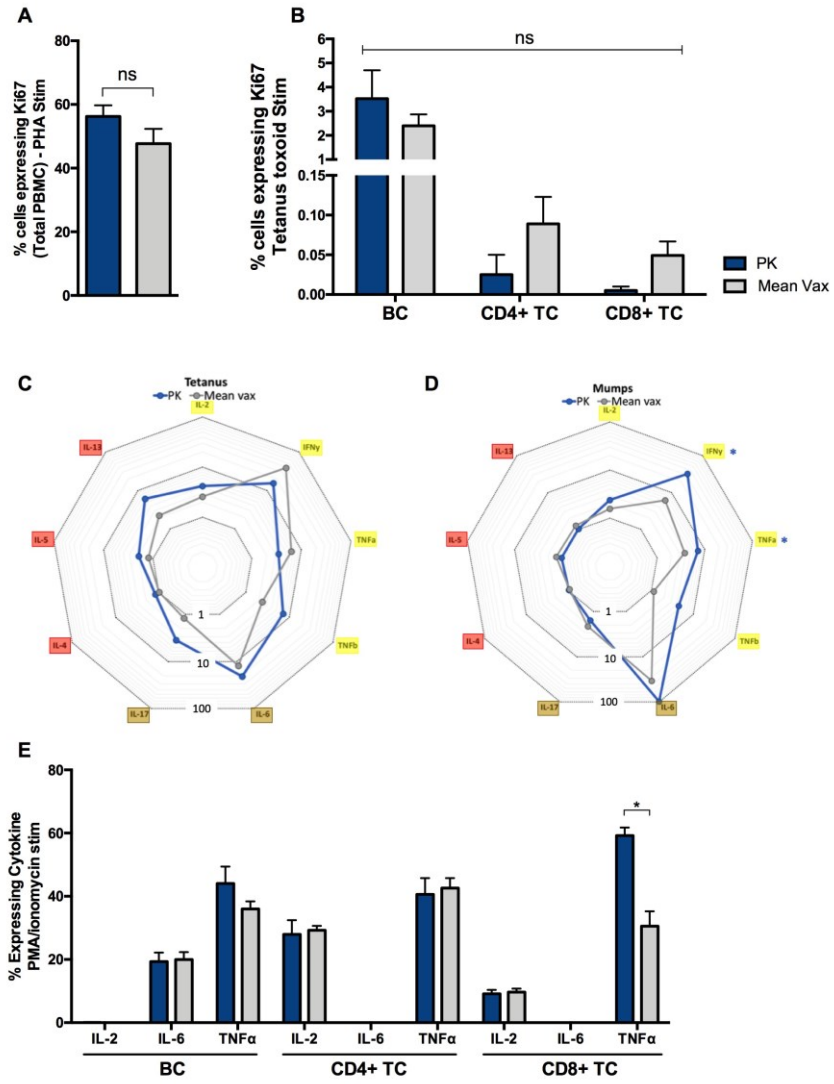



**Figure 3: PK has moderate pro-inflammatory responses to non-HPV antigens.** (**A**) Frequency of total PBMC expressing Ki67 after 72-hour stimulation with PHA. (**B)** Frequency of B cells, $CD4^+$ and $CD8^+$ T cells expressing Ki67 after 72-hour stimulation with tetanus toxin. For **A** and **B,** values are background subtracted, results for PK are from 2-3 independent experiments, and mean vax represents 8 control subjects. **(C-D)** Same as Figure 2, but samples were stimulated with tetanus toxin and mumps respectively. **(E)** Frequency of B cells, $CD4^+$ and $CD8^+$ T cells expressing either IL-2, Il-6 or TNFα after 18-hour stimulation with PMA/ionomycin, as measured by intracellular flow cytometry. Values are background subtracted with unstimulated samples, results for PK are from 2 independent experiments, and mean vax represents 8 control subjects. Significance: * = $p < 0.05$; ** = $p < 0.01$; *** = $p<0.001$ and **** = $p < 0.0001$.

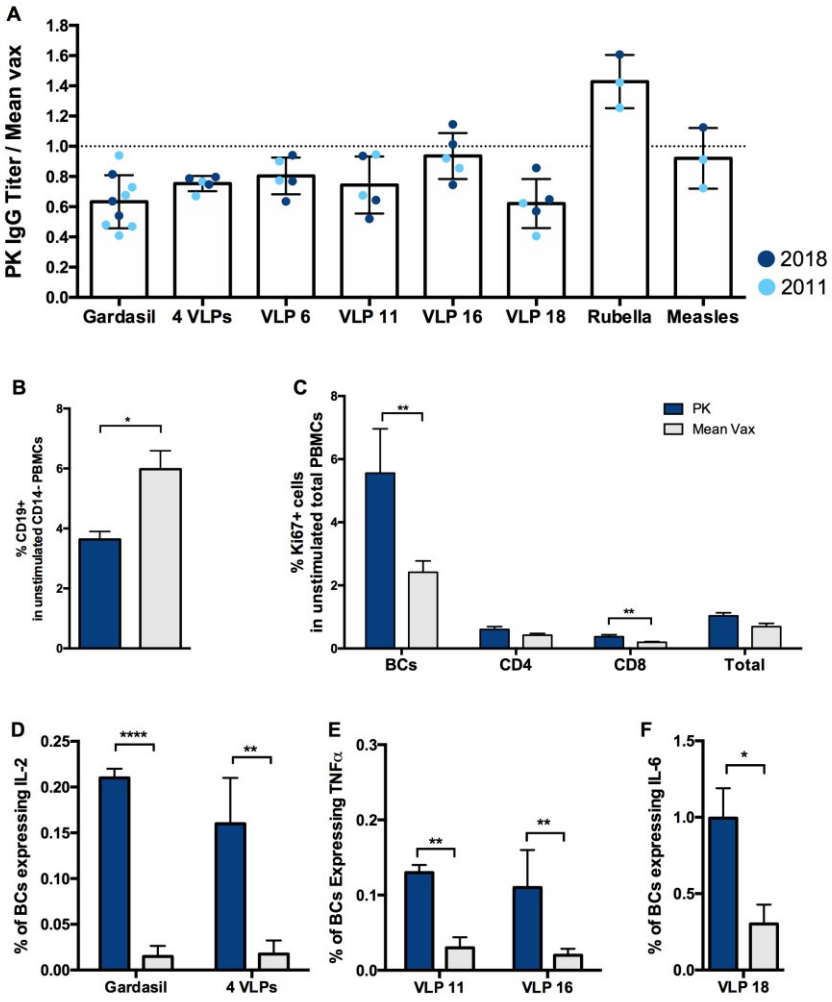



**Figure 4: PK has a weak HPV antibody response despite B cell activation. (A)** Antigen-specific IgG titers in serum were measured with an ELISA. Data is shown as a ratio of PK divided by the mean of vaccinated controls (n = 6) from 3-8 independent experiments. PK replicates include antibody titers in serum collected in 2011 and 2018. **(B)** Frequency of B cells among live, single $CD14^-$ PBMC. Frequencies were measured by flow cytometry. Value for PK is the mean of 5 independent experiments and mean vax represents 8 subjects. **(C)** Frequency of $Ki67^+$ cells among PBMC that were left unstimulated for 72 hours before extracellular and nuclear staining for flow cytometry. **(D-F)** Frequency of $CD19^+$ B cells expressing IL-2 **(D)**, TNFα **(E)** or IL-6 **(F),** as measured with intracellular flow cytometry staining after an 18-hour stimulation Gardasil or HPV VLPs. All data are background subtracted with unstimulated samples. Values for PK are a mean of 2 independent experiments, and mean vax is the average of 8 healthy vaccinated controls. Significance: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ and **** = $p < 0.0001$.

## 4.10 Supplemental Tables

| Gardasil | | Human Protein Match | |
|---|---|---|---|
| **VLP** | **Peptide Sequence** | **Name** | **Gene Ontology - Biological Process** |
| 11 | SVSKSATY | Neuronal acetylcholine receptor subunit alpha-4 | Neuromuscular synaptic transmission |
| 16 | NTNFKEYLRHGEE | High affinity nerve growth factor receptor | Axon guidance<br>Axonogenesis involved in innervation<br>Response to axon injury |
| 16 | LCLIGCKPPIG | Dihydroxyacetone phosphate acyltransferase | Myelin assembly<br>Nervous system development |

**Supplemental Table 1: VLP Peptides with Sequence Homology to Peripheral Nervous System Proteins**

| Immunoglobulin | Concentration* | Reference Values | Remarks |
|---|---|---|---|
| IgG | 9.42 | | |
| IgG1 | 6.990 | 1.510 - 7.920 | |
| IgG2 | 1.610 | 0.260 - 1.360 | Elevated |
| IgG3 | 0.181 | 0.093 - 0.920 | |
| IgG4 | 0.317 | 0.004 - 0.464 | |
| IgA | 1.2 | | |
| IgM | 0.53 | | |
| IgE | 37.9 | | |

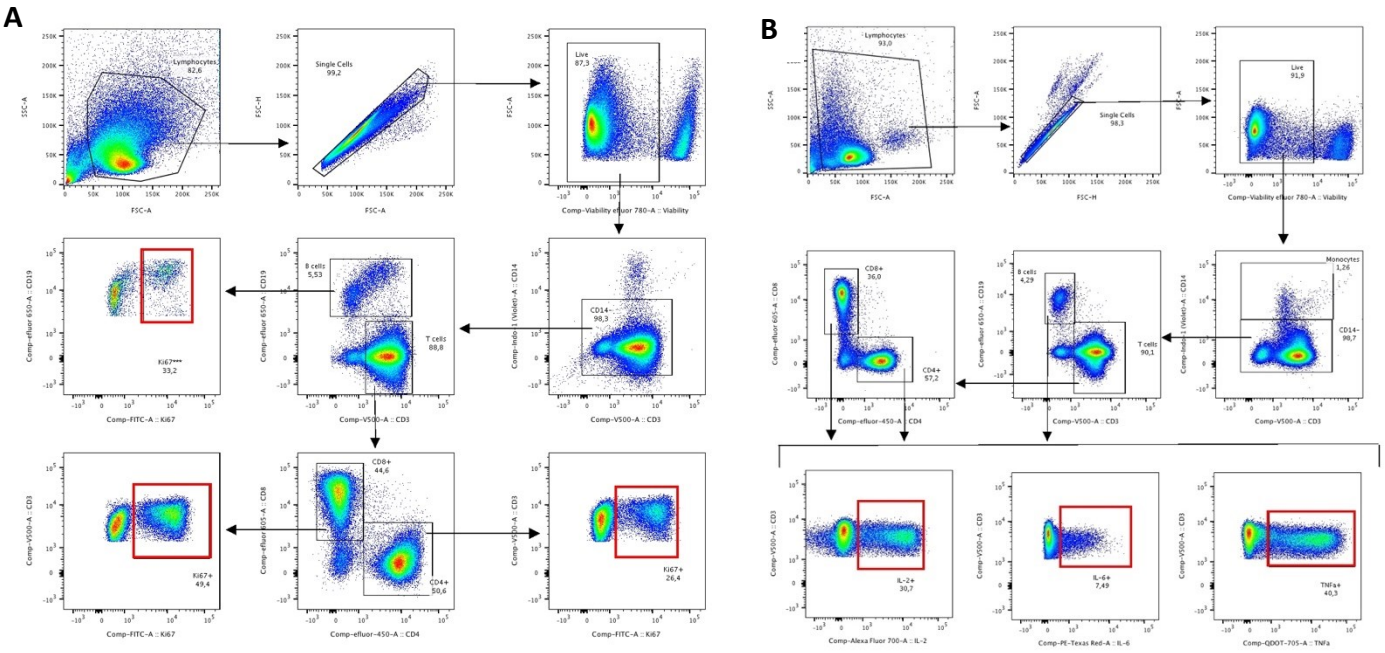
**Supplemental Table 2: Concentrations of Immunoglobulin Subtypes in PK's Serum**

*All concentrations in g/L, except IgE in IU/mL

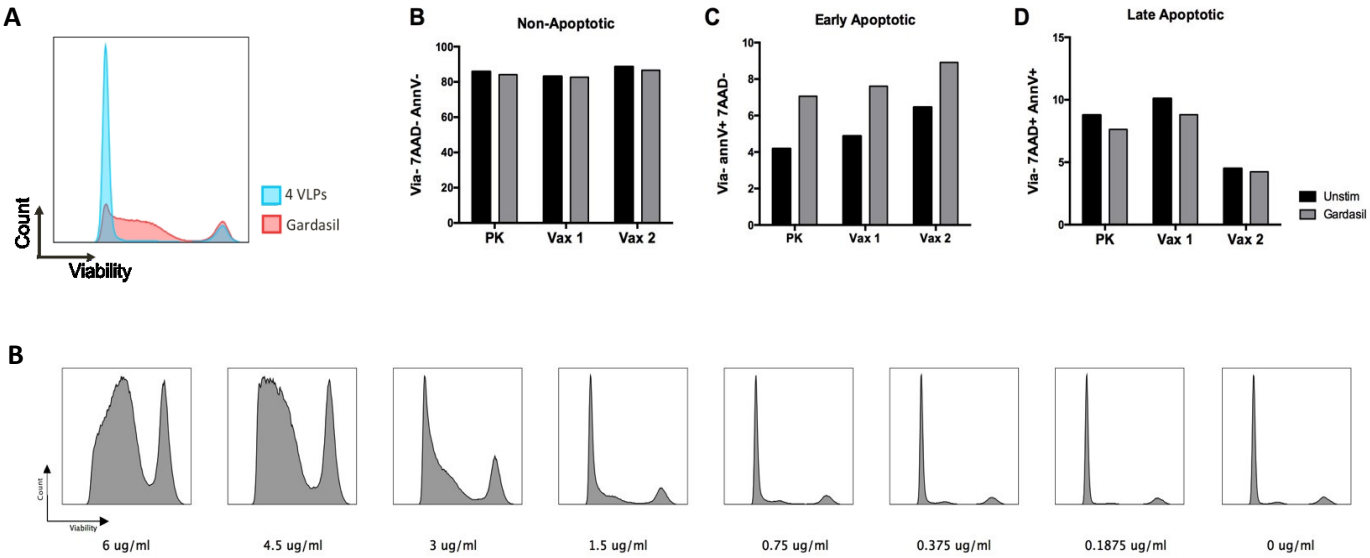
| Class | Allele | Autoimmune Associations |
|---|---|---|
| A | 26:01 | Behcets disease[72] |
| B | 55:01 | Psoriasis[73] |
| C | 17:01:01:02 | **Rare**[51-53] |
| DQA1 | 01:02 | Systemic lupus erythematosus [74] & chronic fatigue syndrome[75] |
| DQB1 | 05:02 | Myasthenia gravis[76] |
| DRB1 | 07:01 | Autoimmune hepatitis type 2[43], crohn's disease[31,44,45], primary biliary cirrhosis[46], Guillain-Barre syndrome[47], vitiligo[48,49], dermatomyositis[50] |
| | 16:01 | Acute rheumatic fever post-infection[77] |

**Supplemental Table 3: PK's HLA Alleles of Interest**

## 4.11 Supplemental Figures




**Supplemental Figure 1: Flow Cytometry Gating Strategy.** Terminal gates are depicted in red. Debris and doublets were removed by selecting single cells from total events plot of forward scatter versus side scatter**.** Absence of viability dye was used to identify live cells. $CD14^+$ cells were identified as monocytes. $CD14^-$ cells were separated into T and B cell populations using CD3 and CD19 expression respectively. T cells were separated into $CD4^+$ and $CD8^+$ sub-populations. **(A)** The Ki67 positive populations were identified in each cell population, using a PHA-stimulated positive control and fluorescence-minus-one (FMO) controls to set gates. **(B)** IL-$2^+$, IL-$6^+$ and TNF$\alpha^+$ populations were identified in each cell population, using PMA/ionomycin-stimulated samples as a positive control and FMOs as negative controls.




**Supplemental Figure 2: Gardasil has a negative effect on PBMC cell viability.**

**(A)** PBMC viability curve 72 hours after stimulation with either Gardasil (containing AAHS) or the 4 VLPs. **(B)** Effect of decreasing concentrations of Gardasil on PBMC viability. Concentrations refer to amount of total HPV antigen used. **(C-D)** Annexin V and 7AAD staining were used to determine the apoptotic state of PBMC that fell within the 'live' peak of viability curves. Bar graphs show frequencies of non-apoptotic ($7AAD^{-}AnnV^{-}$) **(C)**, early-apoptotic ($7AAD^{-}AnnV^{+}$) **(D)** and late-apoptotic ($7AAD^{+}AnnV^{+}$) **(D)** PBMC for PK and 2 vaccinated controls.

# CHAPTER V

## General Discussion & Conclusions

## 5.1 Summary of Main Findings

The work described in this thesis highlights four key stages of the vaccine development process. In chapter II, we examined aspects of the humoral and cellular immune responses elicited by both oral and intra-muscular formulations of a plant-derived candidate virus-like particle (VLP) vaccine for norovirus. Analyzing the capacity of lymphocytes to proliferate, produce cytokines and secrete antibodies allowed us to better characterize the response induced by different vaccine routes, and contributed to deciding which formulations will move onto clinical development. In chapter III, we demonstrated the non-inferiority of the MedJet jet injector in delivering seasonal influenza vaccine to healthy volunteers. In addition to documenting the capacity of the MedJet to elicit HAI titers that were equivalent to a traditional needle-and-syringe, we also showed equivalence of the cellular responses induced (functionality, poly-functionality and distribution of memory subsets), suggesting that this device can be considered for mass vaccination campaigns. In chapter IV, we performed a detailed immunologic investigation of a case of bilateral phrenic nerve paralysis that occurred in close temporal association with Gardasil immunization. Our findings suggest that the vaccine may have acted as an environmental trigger of autoimmunity in a genetically pre-disposed individual and highlight the importance of assessing causality in rare AEFI. In appendix I, the optimization, validation and implementation of a low-cost, serum-sparing enzyme linked immunoassay to measure population immunity to rubella are detailed. Through this work, we found socio-demographic inequalities in immunity to rubella among pregnant Canadian women and suggested that post-partum booster programs are successful in mitigating some of these inequalities. Together, this work demonstrates the values both of developing new vaccines and of evaluating existing ones.

## 5.2 General Discussion

To put the work described above into the larger context of vaccinology in the 21st century, it is useful to consider the 'lifecycle' of a vaccine (Figure 1)[1,2]. Before a vaccination program is implemented, the incidence of the targeted disease is often high, and the public is usually more concerned about the infection itself than they are with potential complications associated with vaccination (ie: adverse events). Most vaccines are introduced into general use having been studied fewer than 100,000 people[3]. As a result, adverse events that are truly associated with vaccination at rates below 1: 20,000-30,000 are unlikely to be detected pre-licensure[4,5]. After licensing however, vaccine coverage typically rises quickly, and the incidence of the targeted disease decreases accordingly. It is only when millions of people are being vaccinated that rare adverse events can be detected with any confidence. These events are often mild and far less severe than the consequences/complications of the disease the vaccine prevents, but their identification and wide (often irresponsible) publication can decrease confidence in vaccination programs. This is particularly true when decreasing prevalence of the disease makes the benefit of vaccination less apparent, leading to low tolerance for risks associated with the vaccine. At some point in the lifecycle of a vaccine, if the risk of a serious adverse event does in fact out-weigh the risk of natural infection, vaccination programs can be discontinued. This occurred when the smallpox vaccine was discontinued in the US and the UK in 1971, eight years before global eradication, when the vaccine was discontinued world-wide[6]. To date, smallpox is the only directly transmitted disease to be eradicated in humans[7], and thus the smallpox vaccine is the only immunization to have completed the entire vaccine lifecycle.

For other vaccines, loss of confidence has resulted in resurgence of the disease. For example, the whole cell pertussis vaccine was introduced in Japan in 1957 and led to a substantial drop in the incidence of pertussis[8]. In the 1970s, concern about neurological AEFI led to a loss of trust in the vaccine, and the government eliminated the program[8]. Vaccine coverage then decreased from 80% in 1974 to 10% in 1976, leading to a dramatic increase in the number of cases and 41 deaths attributed to pertussis in 1979[8]. In 1981, the whole cell pertussis vaccine was replaced by an acellular vaccine, leading once again, to a drastic decrease in incidence[8]. Although safer, acellular pertussis vaccines have since been shown to confer a shorter duration of immunity than the previous version of the vaccine, and pertussis outbreaks continue to occur in vaccinated populations[2,9,10]. In the case of pertussis, the vaccine community has not yet found a good

balance between efficacy and safety and new vaccines and/or vaccination strategies (ie: maternal immunization) are urgently needed.

In the original – and highly optimistic - conception of the 'vaccine lifecycle', it was hypothesized that the reappearance of a disease in a population that had stopped vaccinating would highlight the importance of vaccination, causing an increase in uptake, a decrease in disease incidence and ultimately elimination[1,2]. However, recent outbreaks of measles[11,12] and mumps[13], coupled with ever-growing anti-vaccine sentiments[14] have challenged this perspective. Furthermore, despite the tremendous progress that has been made, infections continue to be the leading cause of death in children and adolescents globally[15]. Attempts to develop vaccines for emerging infectious diseases with epidemic potential (Ebola, Zika, MERS)[16], and the on-going failure to develop effective vaccines against tuberculosis, HIV and malaria[17] have also challenged the vaccine lifecycle as traditionally conceived. These challenges suggest that the field of vaccinology needs to adapt to a new paradigm. There are three fundamental problems that must be solved.

First, new vaccines are needed to control major public health targets (tuberculosis, HIV and malaria), neglected diseases and hyper-variable pathogens[18]. Effective vaccines will ultimately be developed based on new technologies that move beyond the empirical 'isolate-inactivate-inject' approach[18]. Promising strategies include glycoconjugate vaccines, nucleic acid vector delivery systems and protein subunit vaccines[19]. These efforts will also likely rely on faster development platforms, novel adjuvants and bio-markers of vaccine efficacy (ie: correlates or surrogates of protection) to facilitate regulatory approval[18,20]. In addition to scientific innovation, we need to advocate for funding to support vaccine development for both 'hot-topic' pathogens like HIV and Ebola but also relatively-neglected pathogens like Lyme diseases and West Nile Virus[21]. Increased capacity for development of vaccines to protect against infectious diseases with pandemic potential must also be generated. This is complicated by constantly-changing epidemiology during pandemics, ethical concerns and regulatory procedures that have typically been established for 'peacetime' situations[16,20].

Second, we need to improve access to existing vaccines in low- and middle-income countries (LMIC). A major obstacle to achieving this goal is the lack of logistic support and infrastructure necessary to maintain cold chains[22]. In addition to improving cold-chain capacity, the development of temperature-stable vaccines would circumvent this logistical issue, permitting

increased access[23]. The cost of vaccines is another major barrier. GAVI, the global vaccine alliance, has had a major impact on alleviating the financial burden of national vaccination programs. Created in 2000, Gavi is a public-private partnership that provides funding for new and underused pediatric vaccines in over 75 LMIC[24-26]. Recently, Gavi has facilitated the implementation of vaccines for *Haemophilus influenza* b, rotavirus and invasive pneumococcus[27]. It is estimated that use of Gavi-supported vaccines will avert 23.3 million deaths from 2011-2020[28]. Continued support from Gavi, and programs like it, will be crucial as more life-saving vaccines are developed.

Third, we must improve and maintain confidence in vaccines and vaccination programs. The ultimate goal in this respect is for the public to both accept vaccines and view them as necessary for their well-being[18]. To achieve this, we must apply the scientific method to developing, evaluating and implementing effective vaccine education campaigns[29]. We therefore must accept that parental and personal vaccine decisions are rarely made based exclusively on the rational analysis of facts[30] and work within this framework. Multi-disciplinary approaches that move beyond the proximate determinants of vaccine uptake have been suggested as a way forward[31]. While anti-vaccine sentiments are not a new phenomenon[32], it is imperative that the vaccine community adopt new approaches to demonstrating the safety of vaccines and communicating these findings.

## 5.3 Concluding Remarks

Estimating the full public health value of vaccines is a daunting task. Evidence from the past century suggests that vaccines have saved more lives than any other medical intervention[7], and that the indirect socio-economic effects on individuals, populations and health systems are enormous[27]. Yet, challenges remain. Hopefully the work detailed in this thesis highlights the need for, and contributes to, the continued evaluation of vaccines and immunization infrastructure.

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## 5.5 Figures

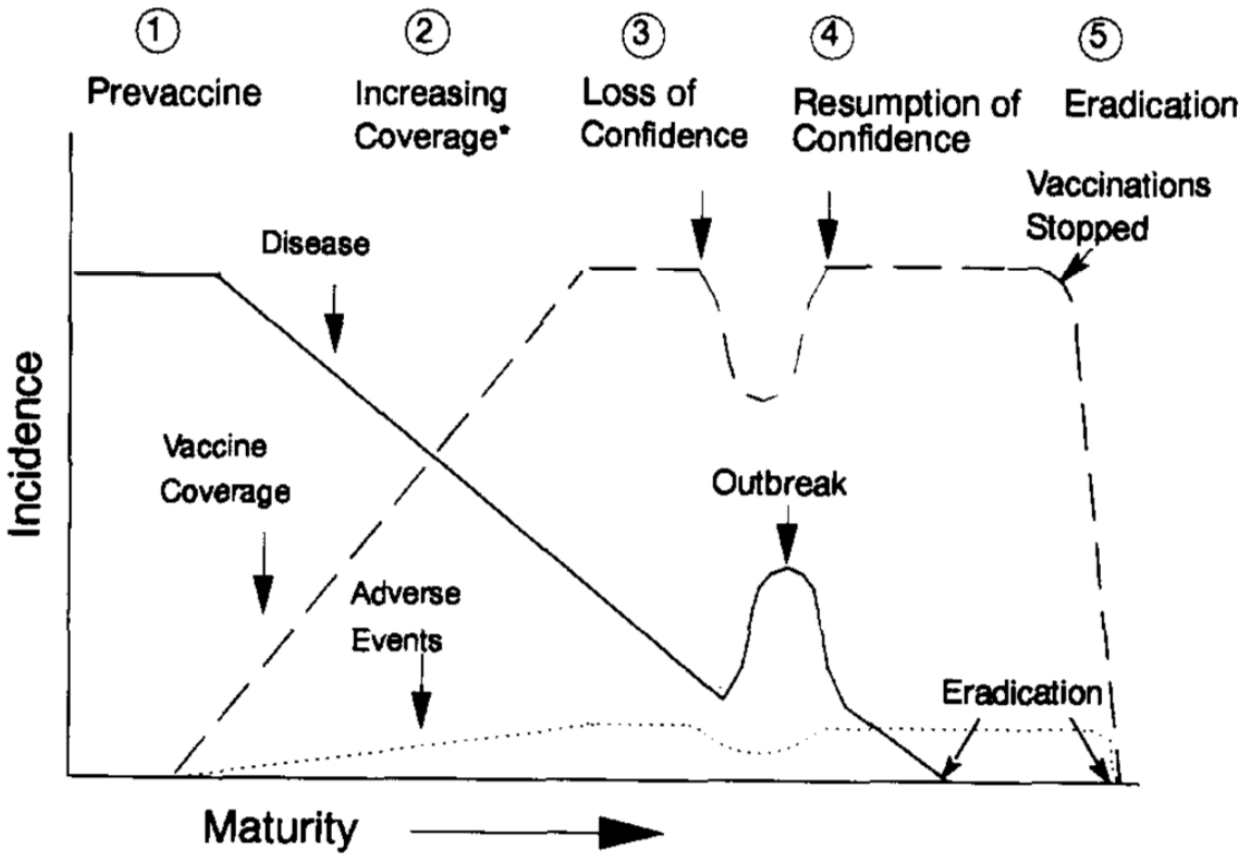


**Figure 1: Graphical representation of the lifecycle of a vaccine.** Potential stages during the evolution of a vaccine program, graphed as a function of the incidence of diseases, vaccine coverage and adverse events over time.

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## APPENDIX I

# Seroprevalence of rubella antibodies and determinants of susceptibility to rubella in a cohort of pregnant women in Canada, 2008-2011

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## AP.1 Preface

Once a vaccine has been shown to be efficacious and safe, it is licensed by national regulatory agencies. However, the evaluation process must continue even after licensure. These evaluations allow manufacturers and public health bodies to understand how the vaccine is working in a real-world context (ie: effectiveness), assess the duration of vaccine-induced immunity and identify social and geographical heterogeneity in vaccine uptake. As long as a reasonable serologic correlate of immunity is known, seroepidemiology, the measurement of antigen-specific antibodies in serum collected from a cross-sectional cohort, allows us to measure population immunity to vaccine-preventable diseases. These studies are particularly important in the era of vaccine-hesitancy and in populations with substantial immigration from countries with poor vaccine coverage. Serosurveys allow public health agencies to improve health by creating targeted supplemental vaccination campaigns to under-immunized populations. In the following manuscript, the development and implementation of a practical assay to measure population immunity to rubella is described.

## AP.2 Abstract

Long term control of rubella and congenital rubella syndrome (CRS) relies on high population-level immunity against rubella, particularly among women of childbearing age. In Canada, all pregnant women should be screened so that susceptible new mothers can be offered vaccination for rubella before discharge. This study was undertaken to estimate rubella susceptibility in a cohort of pregnant women in Canada and to identify associated socio-economic and demographic factors. Biobanked plasma samples were obtained from the Maternal-Infant Research on Environmental Chemicals (MIREC) study, in which pregnant women were recruited between 2008 and 2011. Socio-demographic characteristics and obstetric histories were collected. Second trimester plasma samples (n=1,752) were tested for rubella-specific IgG using an in-house enzyme-linked immunosorbent assay. The percentage of women with IgG titers <5 IU/mL, 5-10 IU/mL, and ≥10 IU/mL were 2.3%, 10.1%, and 87.6%, respectively. Rates of seronegativity, defined <5 IU/mL, were 3.1% in women who had no previous live birth and 1.6% in women who had given birth previously. Among the latter group, seronegativity was higher in women with high school education or less (adjusted OR (aOR) 5.93, 95% CI 2.08-16.96) or with a college or trade school diploma (aOR 3.82, 95% CI 1.45-10.12), compared to university graduates, and those born outside Canada (aOR 2.60, 95% CI 1.07-6.31). In conclusion, a large majority of pregnant women were found to be immune to rubella. Further research is needed to understand inequalities in vaccine uptake or access, and more effort is needed to promote catch-up measles-mumps-rubella vaccination among socioeconomically disadvantaged and immigrant women of childbearing age.

## AP.3 Introduction

Rubella, one of the classic childhood exanthems, is caused by rubella virus, a positive-sense, single-stranded RNA virus of the *Togaviridae* family [1-3]. In children, the disease is characterized by a self-limiting rash and fever. Up to 50% of infections are subclinical[4]. Complications such as meningoencephalitis, thrombocytopenia and post-infectious encephalomyelitis occur but are very rare[1]. In adults and particularly in post-pubertal women, rubella infection is an important cause of arthralgia/arthritis[3]. The most severe complications of rubella in adult women occur during pregnancy when infection can lead to miscarriage, stillbirth,

or congenital rubella syndrome (CRS), a constellation of congenital anomalies including microphthalmia and other eye defects, sensorineural deafness, heart defects, and brain damage such as microcephaly[1]. The rate of vertical transmission and CRS is highest when maternal infection occurs in the first 10 weeks of pregnancy and decreases afterwards[2].

In Canada, routine vaccination with rubella-containing vaccine has been publicly-funded in most provinces since the 1970s, and by 1983 a combined measles-mumps-rubella vaccine (MMR) was incorporated into all provincial and territorial routine vaccination programs. As a result, the average annual incidence of rubella fell from 18.9 cases per 100,000 in 1979-1983 to 5.0 cases per 100,000 in 1984-1997[5]. In parallel, the rate of CRS fell from 3.0 cases per 100,000 live births in 1979-1983 to 0.8 cases per 100,000 live births in 1984-1997[5]. After the introduction of a second dose of MMR vaccine into all provincial and territorial vaccination programs between 1996 and 1997, the average annual rubella and CRS incidence rates decreased further to 0.1 cases per 100,000 and 0.2 cases per 100,000 live births in 1998-2008, respectively[5]. Canada achieved elimination of both rubella and CRS by 2005, with an annual average of only 4.3 rubella cases reported per year between 2006 and 2011 and no reported cases of CRS due to exposure in Canada since 2000[5,6].

The long-term control of rubella and CRS relies on maintaining high coverage with a rubella-containing vaccine. Seroconversion after a single dose of live attenuated rubella vaccines, including measles, mumps and rubella (MMR) combination vaccines, have consistently exceeded 95%[7] and vaccine-induced anti-rubella titres can be remarkably robust[8]. Nonetheless, antibody titres tend to fall after vaccination[9] and at least some of those who have been previously vaccinated mount primary responses upon revaccination[10]. Although global inclusion of a rubella-containing vaccine in routine childhood vaccination programs has increased steadily in the last two decades, vaccination programs in one third of the world's low- and middle-income countries did not include a rubella-containing vaccine in 2009[11]. Of all World Health Organization (WHO) Regions, only the Americas have interrupted the endemic transmission of rubella so far[1]. Finally, widely publicized and fraudulent claims linking MMR vaccination to autism[12] may have negatively affected vaccine uptake, though their actual impact remains difficult to measure. All of these factors highlight the need to maintain high vaccination coverage.

As CRS is a severe consequence of rubella infection during pregnancy, rubella immunity in post-pubertal women is of particular interest. The Society of Obstetricians and Gynaecologists of Canada (SOGC) recommends that i) every opportunity be taken to assess rubella immunity in women of childbearing age (e.g., pre-conception consultation); ii) all pregnant women be screened to determine their rubella serostatus; and iii) susceptible women be immunized either pre-conception or post-partum before hospital discharge[13]. While rubella seroprevalence studies have previously been conducted in Canada, they focussed on specific provinces and lacked detailed information on risk factors for rubella susceptibility. Moreover, although screening studies on pregnant women conducted in Ontario[14] and Alberta[15] identified those tested more than once for rubella IgG during the study period, neither could distinguish women who had at least one previous live birth from those who had not. Knowing the seroprevalence of rubella antibodies among women who have had a previous live birth is of particular importance, as it provides a proxy for compliance with recommendations to screen pregnant women and to immunize at-risk mothers post-partum.

This study was undertaken to i) determine the seroprevalence of rubella IgG antibodies in a cohort of pregnant women in Canada (overall, for those who had no previous live birth, and for those who had at least one); and ii) to identify the socio-economic and demographic factors associated with higher susceptibility to rubella infection.

## AP.4 Methods

### AP.4.1 The MIREC study

The Maternal-Infant Research on Environmental Chemicals (MIREC) study was undertaken to examine potential adverse health effects of prenatal exposure to specific environmental chemicals on pregnancy and infant health. The study participants were pregnant women recruited during their first trimester between 2008 and 2011 in ten Canadian cities within six provinces (British Columbia, Alberta, Manitoba, Ontario, Québec, and Nova Scotia)[16]. Enrolment occurred between the 6$^{th}$ and 13$^{th}$ week of pregnancy, at which time participants completed a questionnaire documenting their socio-demographic characteristics and obstetrical history. Maternal blood samples were collected in each trimester and at delivery. Plasma from the second trimester were used in this study because of their availability in the biobank. Samples were centrifuged within two hours of collection, aliquotted, and stored at -20°c until tested.

### AP.4.2 Laboratory Methods

Plasma samples were tested for rubella-specific IgG using an in-house enzyme-linked immunosorbent assay (IH-EIA) based upon a highly purified GMP-quality rubella virus lysate antigen (Rubella K2S: Microbix, Mississauga, ON). Briefly, 96-well microtiter round-bottom plates (Greiner bio-one, Monroe, NC) were coated overnight with 50 µL of rubella virus antigen at a concentration of 0.25 µg/well in a carbonate buffer (pH 9.6) at 4°C. After washing three times in PBS-T (phosphate-buffered saline [pH 7.4] containing 0.05% [vol/vol] Tween 20), 300 µL of blocking buffer (ELISA Blocker Blocking Buffer – Thermofisher Scientific, Ottawa, ON) was added per well, and the reaction mixture was incubated for two hours at room temperature to block nonspecific binding. Plates were washed three times with PBS-T, and then 10 µL of control or sample was diluted in 240 µL of blocking buffer, added to each well and incubated at 37°C for one hour. After washing three times with PBS-T, 100 µL of mouse anti-human IgG conjugated to horseradish peroxidase (Fisher Scientific, Ottawa, ON) diluted 1:50,000 in blocking buffer was added to each well, and the reaction mixture was incubated for one hour at 37°C. After washing four times, 100 µL of substrate, 3,3',5,5'-tetramethylbenzadine was added to each well, and the reaction mixture was incubated in the dark at room temperature for 20 minutes. The reaction was stopped with the addition of 50 µL of 5% sulfuric acid. The optical density of each control and sample was read at 450 nm.

Each microtiter plate contained a 7-point standard curve constructed using duplicate, serial 2-fold dilutions of the WHO RUBI-1-94 starting at a concentration of 40 IU/mL (range 40 – 0.625 IU/ml). An internal anti-rubella virus IgG quality control sample diluted with negative human serum to 20 IU/mL (based on the Architect assay: Abbott Diagnostics, Abbott Park, IL) was tested at least once in each plate.

In preliminary work to optimize the IH-EIA, variance between duplicate wells was less than 15% and inter-assay variance was 23%. The IH-EIA was validated essentially as described by Dimech et al.[17] against a commercial EIA (Architect: Abbott Diagnostics) using a panel of human sera and the WHO international anti-rubella immunoglobulin standard (RUBI-1-94). The Architect assay is a micro-particle chemiluminescent enzyme immune-assay that is FDA-approved for clinical diagnostic use. A total of 126 samples, including 33 with IgG titers <10 IU/mL, were analyzed in both assays and the ability of IH-EIA to detect sero-negative samples was compared to the commercial platform. Overall, the positive and negative percent agreements

between the two assays were 86% and 92% respectively, and the negative and positive predictive values were 82% and 94% respectively.

### AP.4.3 Data Analysis

Data were analyzed using SAS Enterprise Guide 5.1. Participant year of birth was categorized as follows: those born after the initiation of MMR vaccination at 12 months of age in Canada (i.e. from 1983 onwards), those born between 1978 and 1982, those born between 1974 and 1977, and those born between 1960 and 1973.

The distribution of anti-rubella IgG titers was assessed as one of three categories: rubella susceptible/seronegative (<5 IU/mL), indeterminate susceptibility (5 to <10 IU/mL), and rubella immune/seropositive (≥ 10 IU/mL). Geometric means of rubella IgG titers with their 95% confidence intervals were calculated by birth year categories, and compared using generalized linear models (GLM). This analysis was repeated after excluding women who had had a previous live birth (to prevent effect modification by post-partum vaccination) and women born outside Canada (to examine the effect of MMR introduction).

For the analysis of factors associated with rubella susceptibility, the threshold of <5 IU/ml suggested by Lai et al.[15] was used to identify those who were definitively seronegative. In contrast, the use of a higher threshold (i.e. <10 IU/mL) to identify all those who *may not* be immune is clinically appropriate[18] as they could benefit from vaccination.

Associations between sociodemographic factors and rubella seronegativity were determined by simple and multiple logistic regressions. Factors with *p* values below 0.10 in simple regressions were included in multiple regression models and retained in models as long as their *p* values remained below 0.10. The standard errors of parameter estimates in the multiple regression models were compared to those in the simple regression models to find co-linearity, but none were identified. Unadjusted odds ratios (OR) and adjusted odds ratios (aOR) and their respective 95% confidence intervals were calculated. This analysis was carried out for the entire study population, and then separately for women who had no previous live births and those who had at least one live birth, to account for effect modification by post-partum vaccination.

### AP.4.4 Ethics

This study was reviewed and approved by Health Canada and the Public Health Agency of Canada's Research Ethics Board (REB). The MIREC study had previously been reviewed and approved by the REBs of the Centre Hospitalier Universitaire Sainte-Justine, all recruitment sites and Health Canada and Public Health Agency of Canada. The consent provided by participants allowed for the use of anonymized data and bio-banked biological samples for further research.

## AP.5 Results

Of the 1,928 participants enrolled in the MIREC study, 1,752 had a second-trimester plasma sample available for testing. Their age at enrollment ranged between 18 and 48 years, 64% of them were university graduates, and 55% had had at least one previous live birth (Table 1).

Anti-rubella IgG antibody titers in the second trimester of pregnancy ranged from 0.9 to 897 IU/mL. The percentage of women with IgG titers < 5 IU/mL, between 5 and 10 IU/mL, and ≥10 IU/mL were 2.3%, 10.1%, and 87.6%, respectively (Table 2).

Anti-rubella IgG antibody titers were lower in younger participants (Table 3), with the greatest gap between those born in 1974-1977 compared to those born in 1978-1982. Similar trends were observed after excluding women who had had at least one live birth (i.e., potentially vaccinated post-partum) and those born outside Canada (i.e., possibly vaccinated according to a different schedule or not vaccinated at all). There was no significant difference between those born on or after 1983 and those born immediately before that milestone (Table 3).

Adjusting for year of birth, education, and history of previous live birth, the odds of rubella seronegativity were significantly (i) lower in women born in 1978-1982 compared to those born in 1960-1973 (aOR 0.35, 95% CI 0.14-0.90), (ii) higher in women with a trade school or college diploma compared to university graduates (aOR 2.15, 95% CI 1.03-4.51), and (iii) higher in women with one or more live births compared with those with none (aOR 0.47, 95% CI 0.24-0.92) (Table 4). Among those who had had no previous live births (and therefore no opportunity for postpartum vaccination), the odds of rubella susceptibility were even greater for those with i) a high school education or less [5.93 (2.08-16.96)] or ii) a college or trade school diploma [3.82 (1.45-10.12)] compared to university graduates. Similarly, the association between being born outside Canada and the risk of rubella seronegativity was statistically significant in women with no previous live births, whereas it was non-significant for the entire population (Table 5). In

women who had had a previous live birth, none of the demographic factors analyzed were associated with rubella seronegativity (Table 6).

## AP.6 Discussion

Plasma concentrations of anti-rubella IgG in pregnant women were clearly lower in those born after the introduction of monovalent (early 1970s) and subsequently combined rubella-containing vaccines (i.e. MMR, 1983). This trend is consistent with decreased circulation of wild-type rubella virus in Canada following vaccine introduction[5]. A similar pattern has been observed in countries as diverse as Spain[19] and Peru[20]. As higher vaccination rates are achieved and fewer exposures to wild-type virus occur, populations are increasingly dependent upon vaccine-induced immunity alone. Even though rubella-containing vaccines are highly immunogenic, they generally produce a lower and less durable antibody response than natural infection[21]. To date however, decreasing antibody levels in highly vaccinated populations have not led to major outbreaks of rubella or an increased incidence of CRS[22].

The overall percent of pregnant women immune to rubella in this study (88% with ≥10 IU/mL) was lower than the 90% measured in Ontario pregnant women in 2006-2010[14], but higher than the 84.1% measured in Alberta in 2009-2012[15]. Rubella seropositivity in our study was also slightly lower than the 91.5% measured in the United States in non-pregnant women aged 20-49[23]. The different assays used in these studies may have influenced rubella seroprevalence results, particularly at low antibody titers[17]. Nonetheless, all of these studies send the same message: that some women of child-bearing age may be susceptible to rubella.

Although not truly national in scope, our rubella seroprevalence study is the first to be conducted across multiple Canadian provinces representing 92.5% of the country's total population in 2016[24] and with good geographic coverage.

Unfortunately, the number of foreign-born women in this study (total 327, seronegative 11) was too small to undertake a detailed analysis by country or region of birth as the numbers of women from specific regions would not allow valid inferences. However, because of varying rubella vaccination programs and disease incidence rates, differences between countries or regions of birth can be expected. The SOGC recommends vaccinating all immigrant and refugee women at their first encounter with the Canadian health care system, unless they have documentation of effective vaccination or natural immunity[13]. A chart review of 1,987 Canadian-

born and 3,796 foreign-born pregnant women found that, among the latter, those born in the Middle East or in North Africa were at higher risk of seronegativity than those Canadian-born, while those born in Sub-Saharan Africa were at lower risk. Women from other parts of the world were not statistically different from those Canadian-born[25]. In a study of 1,480 immigrants in Montreal, rubella seronegativity rates (<10 IU/mL) ranged from 5% to 30% depending upon the region of birth, with the lowest rates in Sub-Saharan Africa immigrants and the highest in those born in East Asia-Pacific countries[26]. These observations, together with our current findings, reinforce the message that more effort is needed to deliver catch-up vaccinations in immigrant women.

The association between lower educational attainment and increased rubella susceptibility may suggest socio-economic inequalities in rubella vaccine uptake. This possibility is consistent with inequalities observed in the general uptake of childhood vaccines in Canada[27]. More research is needed to determine the underlying causes of these inequalities, and to measure the relative contributions of vaccine hesitancy and systemic barriers.

Little is currently known regarding either the completeness of pre-natal screening or the uptake of postpartum rubella vaccination in Canada. In the 1990s, a chart review of prenatal rubella screening and its follow-up in 2,551 women who delivered in Québec hospitals found that among the 1.6% initially found to be seronegative, 33.5% were definitely vaccinated post-partum, 29.5% were definitely not vaccinated, and vaccination was not required for various reasons for 6%. The vaccination status of the remaining 31% could not be ascertained from the charts[28]. In the absence of more recent published data, it is unknown whether compliance with this health intervention changed over time. However, the difference in seronegativity rates in the current study between the women who had had a previous live birth and those who had not (1.6% vs 3.1% respectively) suggest that, while clearly not functioning optimally, the SOGC recommendation to vaccinate post-partum is having at least some impact.

This study has several limitations. MIREC was not designed to study vaccination or vaccine-preventable diseases so the vaccination history of participants was not recorded. Moreover, the study sample is not fully representative of the Canadian population as it is primarily a convenience sample, and was restricted to six out of ten provinces with no representation from the three territories. Further, the proportion of university graduates in this study, 64%, was much higher than the 35% measured in new mothers in a population-based survey conducted in 2006-

2007[29]. Therefore, the rates derived from our data may not be generalizable to all pregnant women in Canada.

## AP.7 Conclusion

Despite a general decrease over time in anti-rubella IgG titers after the introduction of rubella-containing vaccines, a large majority of pregnant women in the cohort were found to be immune to rubella. Among those who had at least one previous live birth, very few were susceptible to infection suggesting that post-partum vaccination recommendations, while not fully complied with, are having a positive impact. Lower educational attainment (possibly an indicator of low socio-economic status) and birth outside Canada were risk factors for rubella susceptibility. Further research is warranted to understand the socioeconomic inequalities in vaccine uptake or access, and more effort is needed to promote catch-up measles-mumps-rubella vaccination among socioeconomically disadvantaged and immigrant women of childbearing age.

## AP.8 Acknowledgements

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## AP.10 Tables

**Table 1**
Socio-demographic characteristics and birth history of MIREC study participants with a second-trimester plasma sample available for testing (n = 1,752).

| Characteristic | n | % |
|---|---|---|
| *Province of residence* | | |
| Nova Scotia | 263 | 15.0 |
| Quebec | 334 | 19.1 |
| Ontario | 922 | 52.6 |
| Manitoba | 77 | 4.4 |
| Alberta | 17 | 1.0 |
| British Columbia | 139 | 7.9 |
| *Year of birth* | | |
| 1960–1973 | 470 | 26.8 |
| 1974–1977 | 478 | 27.3 |
| 1978–1982 | 581 | 33.2 |
| 1983–1992 | 223 | 12.7 |
| *Age at enrollment* | | |
| 18–29 | 521 | 29.7 |
| 30–34 | 640 | 36.5 |
| 35–48 | 591 | 33.7 |
| *Education* | | |
| High school or less | 232 | 13.2 |
| College or trade school diploma | 403 | 23.0 |
| University graduate | 1,115 | 63.6 |
| Not stated | 2 | 0.1 |
| *Household Income* | | |
| $1 - $60,000 | 371 | 22.1 |
| $60,001 - $100,000 | 620 | 36.9 |
| $100,001 or more | 689 | 41.0 |
| Not stated | 72 | 4.3 |
| *Born outside Canada* | | |
| No | 1425 | 81.3 |
| Yes | 327 | 18.7 |
| *Number of previous live births* | | |
| 0 | 781 | 44.6 |
| 1 | 704 | 40.2 |
| ≥2 | 267 | 15.2 |

**Table 2**
Distribution of anti-rubella IgG titers in pregnant women by history of live birth.

| Rubella IgG titers (IU/mL) | All participating women (n = 1,752) | | Women with no previous live birth (n = 781) | | Women with ≥1 previous live birth (n = 971) | |
|---|---|---|---|---|---|---|
| | n | % (95% CI) | n | % (95% CI) | n | % (95% CI) |
| 0–4.99 | 40 | 2.3 (1.6–3.1) | 24 | 3.1 (2.0–4.5) | 16 | 1.6 (0.9–2.7) |
| 5.00–9.99 | 177 | 10.1 (8.7–11.6) | 90 | 11.5 (9.4–14.0) | 87 | 9.0 (7.2–10.9) |
| ≥10.00 | 1535 | 87.6 (86.0–89.1) | 667 | 85.4 (82.7–87.8) | 868 | 89.4 (87.3–91.3) |

**Table 3**
Participant anti-rubella IgG titers (IU/mL) by birth year, live birth history, and place of birth.

| Year of birth | All participating women (n = 1,752) | | | Women with no previous live birth (n = 781) | | | Canadian-born women with no previous live birth (n = 640) | | |
|---|---|---|---|---|---|---|---|---|---|
| | N | Geometric mean (95% CI) | *p* | N | Geometric mean (95% CI) | *p* | N | Geometric mean (95% CI) | *p* |
| 1960–1973 | 470 | 30.3 (27.9–33.0) | Reference | 167 | 30.1 (26.2–34.7) | Reference | 121 | 29.7 (25.4–34.8) | Reference |
| 1974–1977 | 478 | 29.0 (26.8–31.2) | 0.4097 | 170 | 26.7 (23.5–30.3) | 0.2052 | 131 | 24.9 (21.7–28.6) | 0.0908 |
| 1978–1982 | 581 | 23.8 (22.3–25.4) | <0.0001 | 308 | 22.9 (20.9–25.2) | 0.0012 | 264 | 21.8 (19.8–24.0) | 0.0006 |
| 1983–1992 | 223 | 22.9 (20.4–25.8) | <0.0001 | 136 | 21.4 (18.2–25.1) | 0.0007 | 124 | 20.4 (17.5–23.7) | 0.0004 |

*p* values are for the comparison with the 1960–1973 birth cohort.

**Table 4**
Determinants of rubella IgG seronegativity[a] in participants (n = 1,752).

| Characteristic | N | n | % | Unadjusted OR (95% CI) | Adjusted[b] OR (95% CI) |
|---|---|---|---|---|---|
| *Year of birth* | | | | | |
| 1960–1973 | 470 | 13 | 2.8 | Reference | Reference |
| 1974–1977 | 478 | 10 | 2.1 | 0.75 (0.33–1.73) | 0.76 (0.33–1.77) |
| 1978–1982 | 581 | 7 | 1.2 | 0.43 (0.17–1.08) | 0.35 (0.14–0.90) |
| 1983–1992 | 223 | 10 | 4.5 | 1.65 (0.71–3.82) | 0.98 (0.39–2.47) |
| *Education* | | | | | |
| High school or less | 232 | 9 | 3.9 | 2.46 (1.09–5.55) | 2.38 (0.97–5.84) |
| College or trade school diploma | 403 | 13 | 3.2 | 2.03 (0.99–4.19) | 2.15 (1.03–4.51) |
| University graduate | 1,115 | 18 | 1.6 | Reference | Reference |
| Not stated | 2 | 0 | 0.0 | | |
| *Household Income* | | | | | |
| $1 - $60,000 | 371 | 12 | 3.2 | 2.06 (0.90–4.72) | |
| $60,001 - $100,000 | 620 | 13 | 2.1 | 1.32 (0.59–2.97) | |
| $100,001 or more | 689 | 11 | 1.6 | | |
| Not stated | 72 | 4 | 5.6 | 3.63 (1.12–11.70) | |
| *Born outside Canada* | | | | | |
| No | 1,425 | 29 | 2.0 | Reference | |
| Yes | 327 | 11 | 3.4 | 1.68 (0.83–3.39) | |
| *Previous live birth* | | | | | |
| No | 781 | 24 | 3.1 | Reference | Reference |
| Yes | 971 | 16 | 1.6 | 0.53 (0.28–1.00) | 0.47 (0.24–0.92) |

[a] Seronegativity defined as IgG titers <5 IU/mL.
[b] Model includes all variables for which values are shown in the column.

**Table 5**
Determinants of rubella IgG seronegativity[a] in participants who had no previous live birth (n = 781).

| Characteristic | N | % | Unadjusted OR (95% CI) | Adjusted[b] OR (95% CI) |
|---|---|---|---|---|
| *Year of birth* | | | | |
| 1960–1973 | 167 | 3.6 | Reference | |
| 1974–1977 | 170 | 2.4 | 0.65 (0.18–2.33) | |
| 1978–1982 | 308 | 1.6 | 0.44 (0.13–1.47) | |
| 1983–1992 | 136 | 6.6 | 1.90 (0.66–5.48) | |
| *Education* | | | | |
| High school or less | 90 | 7.8 | 5.47 (1.93–15.49) | 5.93 (2.08–16.96) |
| College or trade school diploma | 164 | 5.5 | 3.77 (1.43–9.93) | 3.82 (1.45–10.12) |
| University graduate | 527 | 1.5 | Reference | Reference |
| *Household Income* | | | | |
| $1 - $60,000 | 170 | 5.3 | 3.41 (1.12–10.34) | |
| $60,001 - $100,000 | 272 | 2.2 | 1.38 (0.42–4.56) | |
| $100,001 or more | 310 | 1.6 | | |
| Not stated | 29 | 13.8 | 9.76 (2.46–38.66) | |
| *Born outside Canada* | | | | |
| No | 640 | 2.5 | Reference | Reference |
| Yes | 141 | 5.7 | 2.35 (0.98–5.60) | 2.60 (1.07–6.31) |

[a] Seronegativity defined as IgG titers <5 IU/mL.
[b] Model includes all variables for which values are shown in the column.

**Table 6**
Determinants of rubella IgG seronegativity[a] in participants who had at least one previous live birth (n = 971).

| Characteristic | N | n | % | Unadjusted OR (95% CI) |
|---|---|---|---|---|
| *Year of birth* | | | | |
| 1960–1973 | 303 | 7 | 2.3 | Reference |
| 1974–1977 | 308 | 6 | 1.9 | 0.84 (0.28–2.53) |
| 1978–1982 | 273 | 2 | 0.7 | 0.31 (0.06–1.52) |
| 1983–1992 | 87 | 1 | 1.1 | 0.49 (0.06–4.05) |
| *Education* | | | | |
| High school or less | 142 | 2 | 1.4 | 0.83 (0.18–3.81) |
| College or trade school diploma | 239 | 4 | 1.7 | 0.98 (0.31–3.17) |
| University graduate | 588 | 10 | 1.7 | Reference |
| Not stated | 2 | 0 | 0.0 | |
| *Household Income* | | | | |
| $1 - $60,000 | 201 | 3 | 1.5 | 0.94 (0.23–3.81) |
| $60,001 - $100,000 | 348 | 7 | 2.0 | 1.28 (0.43–3.84) |
| $100,001 or more | 379 | 6 | 1.6 | Reference |
| Not stated | 43 | 0 | 0.0 | |
| *Born outside Canada* | | | | |
| No | 785 | 13 | 1.7 | Reference |
| Yes | 186 | 3 | 1.6 | 0.97 (0.28–3.45) |

[a] Seronegativity defined as IgG titers <5 IU/mL.