The impact of imprinting and repeated influenza vaccination on adaptive immunity, transcriptomics, and metabolomics.

Protocol Number: FLU2

Sponsored by:

Emory Institutional Funds

Principal Investigator:

Nadine Rouphael, MD

Draft or Version Number:

3.0

13 SEP 2019

NCT Number:

NCT03686514

Statement of Compliance

The trial will be carried out in accordance with Good Clinical Practices (GCP) as required by the following:

- United States Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR Part 46; 21 CFR Part 50, 21 CFR Part 54, 21 CFR Part 56, and 21 CFR Part 312);
- International Conference on Harmonization (ICH) E6; 62 Federal Register 25691 (1997);

Compliance with these standards provides public assurance that the rights, safety and wellbeing of study subjects are protected, consistent with the principles that have their origin in the Declaration of Helsinki.

All key personnel (all individuals responsible for the design and conduct of this trial) have completed Human Subjects Protection Training.

SIGNATURE PAGE

The signature below constitutes the approval of this protocol and the attachments, and provides the necessary assurances that this trial will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements and applicable US federal regulations and ICH guidelines.

Signed: _____ Date: _____

Nadine Rouphael, MD

Protocol Summary

Title:	The impact of imprinting and repeated influenza vaccination on adaptive immunity, transcriptomics, and metabolomics.
Population:	60 participants, age greater than 40 years of age, who are in good health and meet the eligibility criteria.
Clinical Site:	The Hope Clinic of the Emory Vaccine Center
Study Duration:	Approximately 3 years
Subject Duration:	Approximately 6 months
Description of Agent:	One dose delivered intramuscularly of the FDA-approved seasonal influenza vaccine

Objectives:

Primary:

- To characterize the magnitudes and specificities of the hemagglutination inhibition (HAI) antibody response to each influenza A subtype or influenza B lineage included in the seasonal quadrivalent influenza vaccine in adults who had “imprinted” against either influenza A/H3N2 or influenza A/H1N1 and who have been either repeatedly vaccinated or unvaccinated

Secondary:

- To characterize the magnitudes and specificities of the neutralizing antibody (NAb) responses to each influenza A subtype or influenza B lineage included in the seasonal quadrivalent influenza vaccine in adults who had “imprinted” against either influenza A/H3N2 or influenza A/H1N1 and who have been either repeatedly vaccinated or unvaccinated
- To characterize the duration of the immune responses to each subtype of the seasonal quadrivalent influenza vaccination in adults who have “imprinted” against H3N2 or H1N1 and who have been either repeatedly vaccinated or unvaccinated
- To evaluate adaptive cellular immune responses and cytokines before and following vaccination

Exploratory:

- To evaluate systems biology measures (transcriptional and metabolic changes) after seasonal influenza vaccination in subjects from two distinct birth cohorts who have been either repeated vaccinated or unvaccinated
- To evaluate ELISA binding antibodies to the highly conserved HA stalk, NA, and M2 ectodomain regions of the influenza virus
- To evaluate the influences on HAI antibody titers of age, gender, ethnicity, vaccination history, and/or birth cohort

- To evaluate the temporal dynamics of the humoral immune response to influenza vaccination in oral fluid compared to serum.

Endpoints:

Primary:

- The proportion of subjects achieving seroprotection (titer of ≥ 40) or seroconversion (four-fold rise in HAI post-influenza vaccination compared to pre-vaccination, or achieving a titer of >40 if the pre-vaccination titer was <10), against each strain contained in the seasonal quadrivalent influenza vaccine, as measured by HAI antibody response approximately 28 days after vaccination

- Geometric Mean Titers (GMTs) of serum HAI and neutralizing antibodies against each strain of the seasonal quadrivalent influenza vaccine, approximately 28 days after vaccination (Study Day 29)

Secondary:

- The proportion of subjects achieving seroprotection (titer of ≥ 40) or seroconversion (four-fold rise in HAI post-influenza vaccination compared to pre-vaccination, or achieving a titer of >40 if the pre-vaccination titer was <10), against each strain contained in the seasonal quadrivalent influenza vaccine, as measured by NAb responses approximately 28 days after vaccination

- Geometric Mean Titers (GMT) of serum NAb against each strain of the seasonal quadrivalent influenza vaccine, approximately 28 days after vaccination

- For HAI and NAb responses, the proportions of subjects achieving seroprotection (titer of ≥ 40) and seroconversion (four-fold rise in HAI post-influenza vaccination compared to pre-vaccination) against each strain of the seasonal quadrivalent influenza vaccine approximately 180 days after vaccination (Study Day 181)

- Geometric Mean Titers (GMTs) of serum HAI and neutralizing antibodies against each strain of the seasonal quadrivalent influenza vaccine approximately 180 days after vaccination (Study Day 181)

- Level of circulating follicular helper T cells (cTFH) at days 1, 8, and 15

- Level of plasmablasts (Ag-specific) by ELISpot at days 1, 8, and 15

- Level of CD4+ T-cells at days 1, 8, 15 and 29

- Level of cytokine and chemokine levels (including IL-2, IFN-γ, IL-21, CD40L/154, IL10) at days 1, 3, 8 and 15

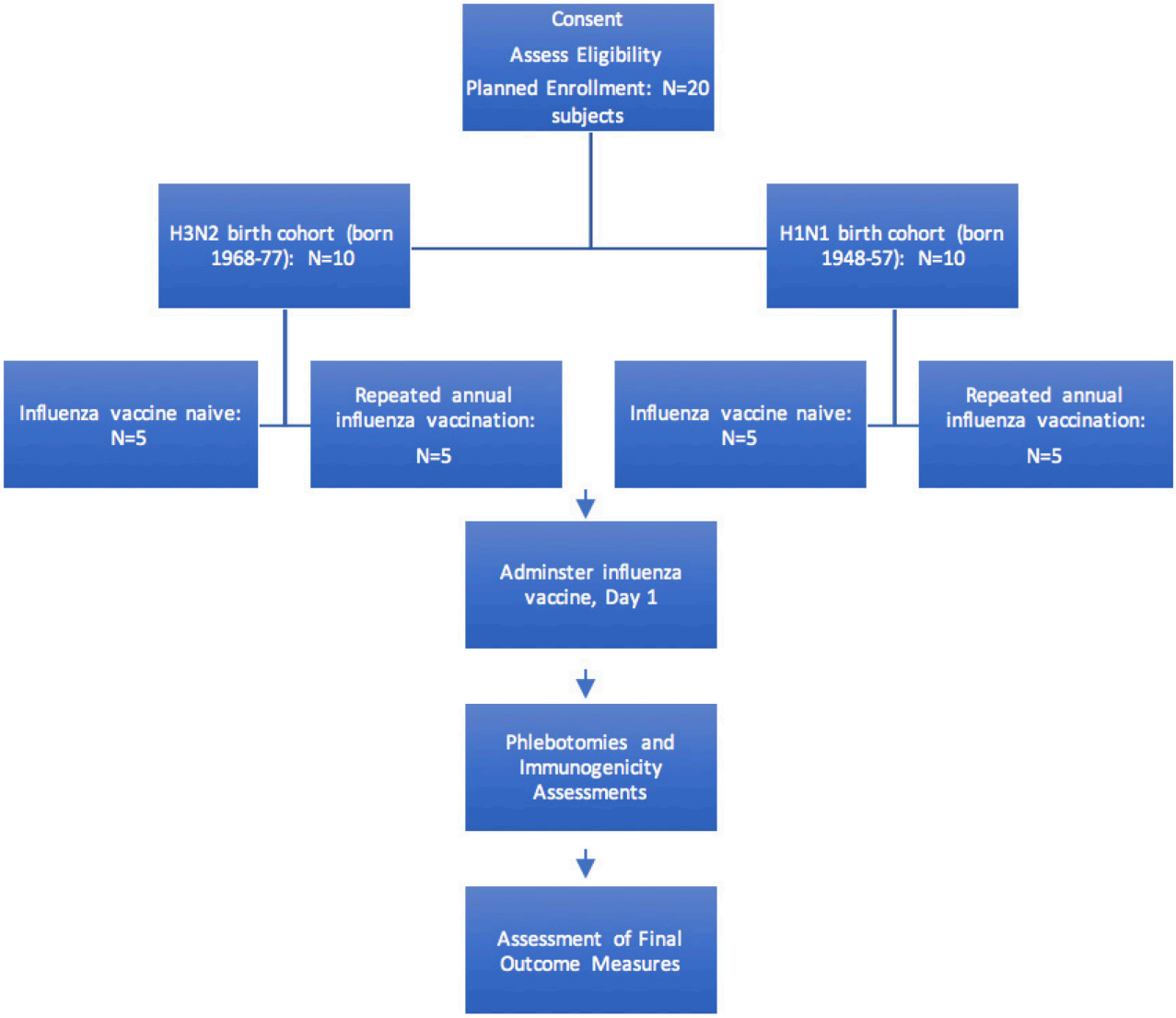
Exploratory:

- Differentially expressed genes (DEG) as measured by transcriptomics comparing baseline to Study Days 3 and 8

- Differentially abundant metabolites (DAM) comparing baseline to Study Days 3 and 8
- Levels of ELISA binding antibodies to the highly conserved HA stalk, NA, and M2 ectodomain, before and approximately 28 days after vaccination
- Multivariate analyses of HAI and neutralizing antibody titers with regards to age, gender, ethnicity, vaccination history, and birth cohort
- Levels of IgA and IgG binding to a panel of influenza antigens and HAI titers to each component of the quadrivalent influenza vaccine in the crevicular fluid vs serum at days 1, 8, 15, 29, and 180

Figure 1. Schematic of Study Design

Per each influenza season:


Consent
Assess Eligibility
Planned Enrollment: N=20 subjects
H3N2 birth cohort (born 1968-77): N=10
H1N1 birth cohort (born 1948-57): N=10
Influenza vaccine naive: N=5
Repeated annual influenza vaccination: N=5
Influenza vaccine naive: N=5
Repeated annual influenza vaccination: N=5
Adminster influenza vaccine, Day 1
Phlebotomies and Immunogenicity Assessments
Assessment of Final Outcome Measures

1 KEY ROLES

Principal Investigators: Nadine Rouphael, MD

Co-investigator: Amy Sherman, MD

Institution:

The Hope Clinic of the Emory Vaccine Center

2 BACKGROUND INFORMATION AND SCIENTIFIC RATIONALE

2.1 Background Information

Seasonal influenza outbreaks continue to cause substantial disease burden, with an estimated 3-5 million cases of severe illness, and 250,000 to 500,000 deaths worldwide each year.[i] In the United States, the CDC reports that influenza has resulted in 9.2-35.6 million illnesses with 12,000-56,000 deaths annually since 2010.[ii] There is an urgent need to better understand the immunologic responses to current licensed vaccines in order to develop a more effective vaccine that does not rely on annual updates, provides broad protection, and is durable; i.e., a universal influenza vaccine.

In 1960, Francis described the "doctrine of original antigenic sin."[iii] He observed that the antibodies produced by a child to the first influenza A sub-type infection continued to dominate throughout his or her life, and governed the immune response to all subsequent influenza exposures thereafter. Gostic et al. provided evidence that individuals indeed have a lifelong "imprint" from his or her first influenza A virus (IAV) exposure, and thus have a reduced risk of suffering from severe disease due to infection with a novel strain of IAV within the same phylogenetic group (HA group 1 contains subtypes H1, H2, and avian H5, while HA group 2 contains seasonal H3 and avian H7). Amino acid homology for the conserved HA stem region is significantly higher within, as opposed to between groups (see Figure 1)[iv].

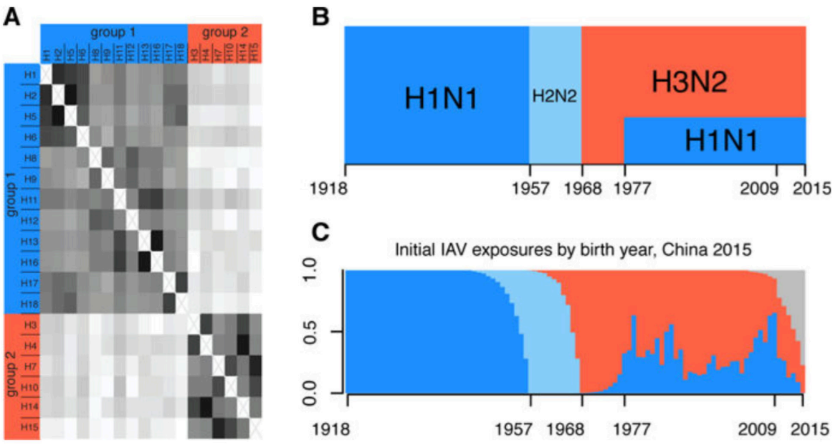


Fig. 1.
HA groups and reconstruction of 20th century HA imprinting. (**A**) HA groups 1 and 2, and pairwise amino acid similarities in the HA stem region. Darker colored cells indicate higher similarity (see Fig. S1). Each within-group subtype pair is more similar (83.2%–97.8%) than any between-group pair (75.9%–81.7%). (**B**) History of seasonal IAV circulation, and (**C**) estimated fraction of each birth cohort in China with initial exposure to each subtype. Estimated patterns in other countries (not shown) are identical up to 1977, and very similar thereafter. Pandemic years are marked on the horizontal axis. Blue represents group 1 HA viruses, red represents group 2, and grey represents naïve children who have not yet experienced an IAV infection.

Figure 1: Gostic et. al

Fonville et al. also demonstrated imprinting in a large Vietnamese cohort of unvaccinated individuals, and showed that the highest serologic titers were for influenza viruses that had circulated when the individuals were around six years of age (likely the time frame of their first infection).[v] Thus, birth-year cohorts can be defined based on an individual's initial childhood exposure to H1N1, H3N2, or another IAV subtype. Other studies have also alluded to the significance of imprinting in terms of patterns seen during influenza outbreaks. Gagnon et al. reported that individuals born during the H2N2 pandemic had higher mortality in 2009 and 2013-2014 to pandemic H1N1.[vi] A recent retrospective study by Flannery et al. examined data from the Flu VE Network study, and demonstrated that patients' initial infections with specific A(H1N1) virus clades did influence vaccine efficacy after exposure to A(H1N1). Birth cohorts were based upon estimated immunologic priming with A(H1N1) viral clades circulating

from 1918-1957 and from 1977-2015,[vii] similar to Gostic above.

Several reports have described decreased vaccine efficacy for H3N2 in patients who have received repeated seasonal vaccination. Petrie et al. described a cohort from 2014-2015 who had received influenza vaccination in the preceding season and had overall a minimal response to vaccination in terms of efficacy.[viii] Similar findings were described in previous years (2012-2013 and 2013-2014).[ix,x] Our research group recently completed the FLU1 study, which demonstrated a dramatic impact of repeated seasonal influenza vaccination on the post-vaccination T and B cell responses, and a fold-increase in HAI antibodies (reductions in all), in repeated vaccinated persons relative to subjects who had not been repeatedly vaccinated (Figure 2; manuscript in preparation).

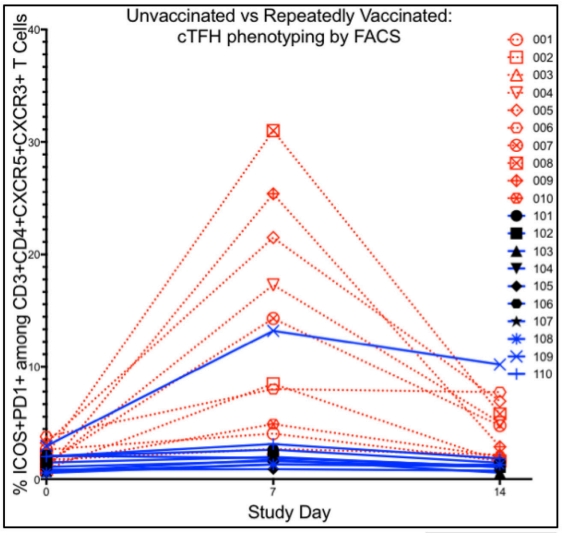


Fig. 2A

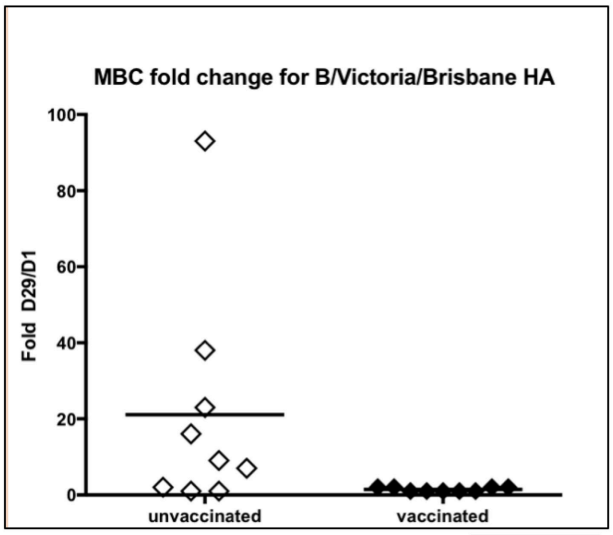


Fig. 2B

Figure 2
A. Blood circulating T follicular helper (cTFH) cell responses in unvaccinated (red) or repeatedly vaccinated (blue) groups. Responses were much lower in the repeatedly vaccinated group. cTFH responses at days 0 (pre-vaccination), 7 and 14 are shown.
B. Memory B cell responses in the repeatedly vaccinated group (solid black diamonds) were much lower than in the unvaccinated group (open diamonds). The fold increase at day 29 after vaccination, relative to day 1 (pre-vaccination), is shown. [Manuscript in preparation]

These findings underscore the importance of several factors that shape host immunity to influenza, and highlight a need for further studies employing newer methods to explore the complicated interplay of pre-existing circulating antibodies, imprinting, vaccination, and responses to novel influenza strains. There is a need for a prospective evaluation to better elucidate the immunological mechanisms involved.

2.2 Rationale

Study Question:

The immune response to the influenza vaccine is affected by many parameters, including prior imprinting to a specific influenza strain based on birth cohort, as well as prior influenza vaccination. The FDA-approved, quadrivalent seasonal influenza vaccine that will be administered contains four distinct strains, two IAVs and two influenza B viruses (IBVs). The approved seasonal influenza vaccine will be given for each season of influenza: 2018-2019, 2019-2020, and 2020-2021.

We propose that the A(H1N1) birth cohort (individuals born between 1948-1957) will produce more robust HAI antibody and cellular responses to the H1N1 component of the vaccine as compared to the other three vaccine components, while the H3N2 birth cohort (individuals born between 1968-1977) will produce more robust HAI antibody and cellular responses to the H3N2 component of the vaccine.

This study is not powered to test a formal null hypothesis. Rather, it is a hypothesis-generating investigation that will hopefully lead to larger trials based on the findings. The study will be conducted over the course of three years to increase the total sample population size and to validate our findings over different influenza seasons.

The goal of this study, and the subsequent studies it will lead to, is to understand the impact on the human immune system's response to the quadrivalent vaccine in individuals who have "imprinted" on specific influenza strains. We will also consider the effects of repeated prior annual influenza vaccination on the immune system. Collection of crevicular fluid, which may contain high concentrations of pathogen-specific IgA and IgG, will also be performed to investigate the relative dynamics between serum and saliva influenza-specific humoral immune responses.

2.3 Potential Risks and Benefits

The study utilizes FDA-approved influenza vaccines. See package insert for full risks and benefits of the vaccine.

2.3.1 Potential Risks

The potential harms to participants are those associated with intramuscular injection of the FDA-approved influenza vaccine, possible reactions to the vaccine, having blood drawn, and breach of confidentiality.

The potential harms of receiving the influenza vaccine include, but are not limited to: pain and redness at the injection site, muscle aches, fatigue, and headache. These risks are taken directly from the vaccine package insert from the FDA-approved influenza vaccine, with serious adverse reactions including anaphylaxis and Guillain-Barre Syndrome. For further details, see the attached package insert. The risks of these potential harms are considered to be outweighed by the potential benefits gained by vaccination, as well as the value of the new information yielded from the blood samples collected following vaccination.

Blood sample collection and intramuscular injection involve transient discomfort and may cause fainting, the risk of which is mitigated by having the subject lie down prior to these procedures if needed, and remain seated in the clinic for 20 minutes after vaccination. The blood draw site may bruise, and this can be alleviated by holding pressure to this site following the blood draw. The sites of blood draw and intramuscular injection are potential sites of infection, but this risk is made very unlikely by the use of sterile technique. Saliva collection is low risk but has the potential to cause minimal gum bleeding; a sponge connected to a plastic stick will be used to collect saliva from the area where the gums meet the teeth (crevicular fluid).

2.3.2 Known Potential Benefits

Participants will receive the FDA-approved influenza vaccine recommended by the United States Advisory Committee on Immunization Practices (ACIP) for anyone 6 months and older, known to help protect against infection by influenza viruses. Participants will also receive financial reimbursement to account for time, travel, missed work, and inconvenience.

3 OBJECTIVES

3.1 Study Objectives

Primary:

- To characterize the magnitudes and specificities of the hemagglutination inhibition (HAI) antibody response to each influenza A subtype or influenza B lineage included in the seasonal quadrivalent influenza vaccine in adults who had “imprinted” against either influenza A/H3N2 or influenza A/H1N1 and who have been either repeatedly vaccinated or unvaccinated

Secondary:

- To characterize the magnitudes and specificities of the neutralizing antibody (NAb) responses to each influenza A subtype or influenza B lineage included in the seasonal quadrivalent influenza vaccine in adults who had “imprinted” against either influenza A/H3N2 or influenza A/H1N1 and who have been either repeatedly vaccinated or unvaccinated
- To characterize the duration of the immune responses to each subtype of the seasonal quadrivalent influenza vaccination in adults who have “imprinted” against H3N2 or H1N1 and who have been either repeatedly vaccinated or unvaccinated.
- To evaluate adaptive cellular immune responses and cytokines before and following vaccination

Exploratory:

- To evaluate systems biology measures (transcriptional and metabolic changes) after seasonal influenza vaccination in subjects from two distinct birth cohorts who have been either repeated vaccinated or unvaccinated
- To evaluate ELISA binding antibodies to the highly conserved HA stalk, NA, and M2 ectodomain regions of the influenza virus
- To evaluate the influences on HAI antibody titers of age, gender, ethnicity, vaccination history, and/or birth cohort
- To evaluate the temporal dynamics of the humoral immune response to influenza vaccination in oral fluid compared to serum.

3.2 Study Outcome Measures

Primary:

- The proportion of subjects achieving seroprotection (titer of ≥ 40) or seroconversion (four-fold rise in HAI post-influenza vaccination compared to pre-vaccination, or achieving a titer of >40 if the pre-vaccination titer was <10), against each strain contained in the seasonal quadrivalent influenza vaccine, as measured by HAI antibody response approximately 28 days after vaccination

- Geometric Mean Titers (GMTs) of serum HAI and neutralizing antibodies against each strain of the seasonal quadrivalent influenza vaccine, approximately 28 days after vaccination (Study Day 29)

Secondary:

- The proportion of subjects achieving seroprotection (titer of ≥ 40) or seroconversion (four-fold rise in HAI post-influenza vaccination compared to pre-vaccination, or achieving a titer of >40 if the pre-vaccination titer was <10), against each strain contained in the seasonal quadrivalent influenza vaccine, as measured by NAb responses approximately 28 days after vaccination

- Geometric Mean Titers (GMT) of serum NAb against each strain of the seasonal quadrivalent influenza vaccine, approximately 28 days after vaccination

- For HAI and NAb responses, the proportions of subjects achieving seroprotection (titer of ≥ 40) and seroconversion (four-fold rise in HAI post-influenza vaccination compared to pre-vaccination) against each strain of the seasonal quadrivalent influenza vaccine approximately 180 days after vaccination (Study Day 181)

- Geometric Mean Titers (GMTs) of serum HAI and neutralizing antibodies against each strain of the seasonal quadrivalent influenza vaccine approximately 180 days after vaccination (Study Day 181)

- Level of circulating follicular helper T cells (cTFH) at days 1, 8, and 15

- Level of plasmablasts (Ag-specific) by ELISpot at days 1, 8, and 15

- Level of CD4+ T-cells at days 1, 8, 15 and 29

- Level of cytokine and chemokine levels (including IL-2, IFN-γ, IL-21, CD40L/154, IL10) at days 1, 3, 8 and 15

Exploratory:

- Differentially expressed genes (DEG) as measured by transcriptomics comparing baseline to Study Days 3 and 8

- Differentially abundant metabolites (DAM) comparing baseline to Study Days 3 and 8

- Levels of ELISA binding antibodies to the highly conserved HA stalk, NA, and M2 ectodomain, before and approximately 28 days after vaccination

- Multivariate analyses of HAI and neutralizing antibody titers with regards to age, gender, ethnicity, vaccination history, and birth cohort

- Levels of IgA and IgG binding to a panel of influenza antigens and HAI titers to each component of the quadrivalent influenza vaccine in the crevicular fluid vs serum at days 1, 8, 15, 29, and 180

4 STUDY DESIGN

This study will be a prospective pilot study conducted over the course of three years (with three specific influenza seasons studied). For each year (2018-2019, 2019-2020, and 2020-2021), two cohorts of 10 participants each, who are in good health and meet all eligibility criteria, will be recruited. The H3N2 cohort (N=30 total, 10 per year) will consist of participants born between 1968-1977, and the H1N1 cohort (N=30 total, 10 per year) will consist of participants born between 1948-1957. The H3N2 cohort and the H1N1 cohort will consist of participants who have received the influenza vaccine <2 of the past 5 years, and also participants who have received the influenza vaccine >3 of the past 5 years. The participants in the two cohorts will be similar with regard to prior seasonal vaccination history. Enrollments will be stratified based on vaccination history to ensure balance between the two birth cohorts.

Each participant will make a total of six visits to the Hope Clinic. Day 1 will include the informed consent process, and screening to ensure the subject meets all inclusion criteria and meets no exclusion criteria. For the consenting and eligible subject, the visit will also include pre-vaccination phlebotomy for baseline immunogenicity laboratory assays, which will include assays for: HAI Ab and NAb, plasmablasts, memory B cells, cT_{FH} cells, T cell responses, cytokine/chemokine levels (i.e., IL-2, IFN-γ, IL-21, CD40L/154, IL10), and systems biology analysis. Baseline oral fluid (approximately 1-2cc) will also be collected for evaluation of levels of IgA and IgG (See Appendix B)[xi]. After phlebotomy and saliva collection, the participants will receive the FDA-approved seasonal influenza vaccine. Subsequent study visits on Days 3, 8, 15, 29, and 180 will include collection of blood and saliva for immunogenicity assays. Immune response assay results will be charted for each participant and summarized for each study arm. Statistical tests for differences in responses between study arms will be performed.

5 STUDY POPULATION

5.1 Selection of the Study Population

The target sample size will be 60 participants total, 20 participants per year. Up to 100 participants will potentially be screened for study enrollment over the course of the three years. Males and females who were born between 1968-1977 (H3N2 birth cohort) and 1948-1957 (H1N1 birth cohort) will be recruited. Prior vaccination history will be obtained by vaccination records, if available, and by participant report. The participants in the two cohorts will be similar with regard to prior seasonal vaccination history, and enrollments will be stratified based on vaccination history to ensure balance between the two birth cohorts. Participants will be screened for eligibility according to the inclusion/exclusion criteria. Informed consent will be obtained for study participation.

5.2 Inclusion/Exclusion Criteria

Subjects eligible to participate shall meet all of the following **inclusion criteria:**

1. Capable of informed consent and provision of written informed consent before any study procedures.
2. Capable of attending all study visits according to the study schedule.
3. Males or females born between 1968-1977 or 1948-1957.
4. Are in good health, as determined by medical history and targeted physical exam related to this history.
5. Oral temperature is less than 38°C.
6. Resting pulse rate is between 50 and 100 beats per minute.
7. Female subjects of childbearing age must have a negative urine pregnancy test within 24 hours before study vaccination.

8. Have either received the influenza vaccine at least 3 of the past 5 years or received the influenza vaccine 2 or less of the past 5 years.

    a. For the 2018-2019 influenza season: received the influenza vaccine at least 3 of the 5 past years between September 2013-June 2018 or have received the influenza vaccine in 2 or less of the past 5 years
    b. For the 2019-2020 influenza season: received the influenza vaccine at least 3 of the 5 past years between September 2014-June 2019 or have received the influenza vaccine in 2 or less of the past 5 years
    c. For the 2020-2021 influenza season: received the influenza vaccine at least 3 of the 5 past years between September 2015-June 2020 or have received the influenza vaccine in 2 or less of the past 5 years

Subjects eligible to participate shall not meet any of the following **exclusion criteria**:

1. Have an acute illness within 72 hours before vaccination.

2. Have any condition that, in the opinion of the principal investigator, would place the subject at an unacceptable risk of harm or confound the interpretation of the study results.

3. Have any acute or chronic medical condition that, in the opinion of the principal investigator, would make vaccination unsafe or interfere with the evaluation of immune response to study vaccination.

4. Have a suppressed immune system as a result of illness, immunosuppressive medication, chemotherapy, or radiation therapy within 3 years prior to study vaccination.

5. Have known HIV, hepatitis B, or hepatitis C infection.

6. Have a known history of autoimmune disease.

7. Have taken oral or parenteral corticosteroids of any dose within 30 days before study vaccination.

8. Have taken high-dose inhaled corticosteroids within 30 days before study vaccination.

9. Have received, or plan to receive, any licensed live vaccine within 30 days, or any licensed inactivated vaccine within 14 days, prior to, or after, study vaccination.

10. Have planned receipt of any unlicensed or investigational medications, biologics, or vaccines for the duration of subject study participation.

11. Have received immunoglobulin or other blood products, with the exception of Rho D immunoglobulin, within 90 days prior to study vaccination.

12. Have donated blood or blood products within 30 days before study vaccination, or within 60 days after study vaccination, or plan to donate blood within 30 days of the last blood draw.

13. Have known hypersensitivity or allergy to eggs, egg protein, chicken protein, or other compounds of the study vaccine.

14. Have a history of severe reactions following vaccination with influenza virus vaccines.

6 STUDY PROCEDURES/EVALUATIONS

6.1 Study Procedures

Complete medical history will be obtained by interview of study subjects on Day 1 (Visit 1) prior to the study vaccination. Subjects will be asked about a known history of significant medical disorders, cancer, immunodeficiency, allergies, psychiatric illness, substance use, and autoimmune diseases.

Medication history will include a review of all current medications and any medications taken in the last 30 days before study vaccination. Medications included in this history will include prescription medication, over-the-counter medication, vitamins, supplements, and prohibited treatments listed in the above Inclusion/Exclusion Criteria section.

On Day 1 (Visit 1) and before the study vaccination, a targeted physical examination, if indicated by the patient's medical history, may be performed by the principal investigator or a study investigator who is licensed to make medical diagnoses. At Visits 2-6, a targeted physical examination may be performed, if indicated.

Vital signs (oral temperature, pulse rate, and blood pressure) will be measured on Day 1 (Visit 1) before study vaccination. Subjects must neither smoke nor eat or drink anything hot or cold 10 minutes prior to measuring oral temperature.

Height and weight will be measured on Day 1 (Visit 1) before study vaccination for the calculation of body mass index.

Subjects will be observed in the clinic for at least 20 minutes after study vaccination on Day 1 (Visit 1).

6.1.1 Laboratory Evaluations/Assays

Urine pregnancy tests will be performed by the site laboratory on the same day as and prior to study vaccination (Day 1). Results must be negative and known prior to study vaccination.

6.1.2 Special Assays or Procedures

The immunologic assays will be performed according to the existing standard operating procedures in place at the Hope Clinic laboratory and at Emory Core Laboratories.

6.1.3 Specimen Collection, Preparation, Handling and Analyses

Blood and saliva will be collected at each of the six study visits as per the Study Schedule in Appendix A.

The phlebotomy tubes and saliva collection tubes will be transported immediately from the research clinic to the research laboratory for sample processing (both are located within the Hope Clinic building) or analysis. The processed specimens will be cryopreserved per the laboratory standard operating procedures.

7 STUDY SCHEDULE

7.1 Screening/Enrollment/Baseline, Visit 01, Day 01

Study participants will be given a description of the study and have an opportunity to have their questions and concerns addressed by the study principal investigator or designee.

Subjects will be given the informed consent form to read and discuss with study staff, and if they wish to enroll they will sign the document before any study vaccination, procedures, or lab draws are performed.

A medical history and targeted physical exam, if necessary, as outlined in the Study Procedures section will be conducted in order to assess for compliance with study inclusion and exclusion criteria.

A list of any medications taken by participants in the past 30 days and those to be taken during any time throughout the study duration will be documented on the appropriate study form.

A urine pregnancy test will be conducted for female participants, and a negative result will be required and be documented on the appropriate study form prior to any study interventions.

Vital signs, including pulse rate, blood pressure, oral temperature, and height and weight will be measured and documented on the appropriate study form.

Approximately 90 mL of blood will be collected for baseline study labs, and two tubes of saliva will be obtained as outlined in the Study Procedures section.

A single intramuscular injection of the FDA-approved seasonal influenza vaccine will be administered in the deltoid muscle of the preferred arm, and subjects will be asked to

remain at the clinic for 20 minutes to monitor for any reactions or adverse events. The injection site location and any reactions or adverse events will be documented on the appropriate study form.

Subjects will be informed to contact the study clinic should they experience any severe reactions to the vaccine, and they will be asked to visit the clinic for evaluation should the principal investigator deem the reaction in need of evaluation.

7.2 Follow-up and Final Visits

7.2.1 Visit 02 Day 03, Visit 03 Day 08, Visit 04 Day 15, Visit 05 Day 29, Visit 06 Day 180

Interim medical history and targeted physical exam and vital signs (including pulse rate, blood pressure, and oral temperature), if necessary, will be conducted.

Medications taken during the interim from the previous visit will be documented on the appropriate study form.

On visits 02, 03, 04, 05 and 06 approximately 32 mL, 32 mL, 48 mL, 90 mL and 90 mL of blood will be collected, respectively. Two tubes of saliva will also be obtained on visits 03, 04, 05 and 06.

7.3 Early Termination Visit (if needed)

The following will be performed at an early termination visit, if necessary, for subjects who withdraw or who are withdrawn from the study:

Interim medical history and targeted physical exam, if necessary, will be conducted.

Medications taken during the interim from the previous visit will be documented on the appropriate study form.

Vital signs, including pulse rate, blood pressure, and oral temperature will be measured and documented on the appropriate study form.

Approximately 90 mL of blood will be collected for immune response study assays as outlined in the Study Procedures section.

7.4 Visit Windows

Visit 01 (Day 01) and Visit 02 (Day 03) will not be allowed a window.

Visit 03 (Day 08) will be allowed a window to include Day 07, Day 08 and Day 09.

Visit 04 (Day 15) will be allowed a window to include Day 14, Day 15, and Day 16.

Visit 05 (Day 29) will be allowed a window to include Day 28, Day 29, Day 30 and Day 31.

Visit 06 (Day 181) will be allowed a window to include Days 166-180, and Days 181-196.

8 STATISTICAL CONSIDERATIONS

8.1 Introduction

The goal of this study is to assess the difference in immune response to the seasonal influenza vaccine between two distinct birth cohort populations, one of which has imprinted on H3N2 and the other on H1N1. This is a pilot study designed to occur over the course of three years, and demonstrate the potential utility of a subsequent larger scale study.

8.2 Study Question

This study is not powered to test a formal null hypothesis. Rather, it is a hypothesis generating investigation that will hopefully lead to larger trials based on the findings. It is proposed that the H1N1 birth cohort (individuals born between 1948-1957) will produce a more robust H1N1 HAI antibody response to the H1N1 component of the seasonal vaccine, while the H3N2 birth cohort (individuals born between 1968-1977) will produce a more robust H3N2 HAI antibody response to the H3N2 component of the vaccine. The quadrivalent seasonal influenza vaccine that will be administered will contain four distinct strains.

8.3 Study Outcome Measures

Primary:

- The proportion of subjects achieving seroprotection (titer of ≥ 40) or seroconversion (four-fold rise in HAI post-influenza vaccination compared to pre-vaccination, or achieving a titer of >40 if the pre-vaccination titer was <10), against each strain contained in the seasonal quadrivalent influenza vaccine, as measured by HAI antibody response approximately 28 days after vaccination

- Geometric Mean Titers (GMTs) of serum HAI and neutralizing antibodies against each strain of the seasonal quadrivalent influenza vaccine, approximately 28 days after vaccination (Study Day 29)

Secondary:

- The proportion of subjects achieving seroprotection (titer of ≥ 40) or seroconversion (four-fold rise in HAI post-influenza vaccination compared to pre-vaccination, or achieving a titer of >40 if the pre-vaccination titer was <10), against each strain contained in the seasonal quadrivalent influenza vaccine, as measured by NAb responses approximately 28 days after vaccination

- Geometric Mean Titers (GMT) of serum NAb against each strain of the seasonal quadrivalent influenza vaccine, approximately 28 days after vaccination

- For HAI and NAb responses, the proportions of subjects achieving seroprotection (titer of ≥ 40) and seroconversion (four-fold rise in HAI post-influenza vaccination compared to pre-vaccination) against each strain of the seasonal quadrivalent influenza vaccine approximately 180 days after vaccination (Study Day 181)

- Geometric Mean Titers (GMTs) of serum HAI and neutralizing antibodies against each strain of the seasonal quadrivalent influenza vaccine approximately 180 days after vaccination (Study Day 181)

- Level of circulating follicular helper T cells (cTFH) at days 1, 8, and 15

- Level of plasmablasts (Ag-specific) by ELISpot at days 1, 8, and 15

- Level of CD4+ T-cells at days 1, 8, 15 and 29

- Level of cytokine and chemokine levels (including IL-2, IFN-γ, IL-21, CD40L/154, IL10) at days 1, 3, 8 and 15

Exploratory:

- Differentially expressed genes (DEG) as measured by transcriptomics comparing baseline to Study Days 3 and 8

- Differentially abundant metabolites (DAM) comparing baseline to Study Days 3 and 8

- Levels of ELISA binding antibodies to the highly conserved HA stalk, NA, and M2 ectodomain, before and approximately 28 days after vaccination

- Multivariate analyses of HAI and neutralizing antibody titers with regards to age, gender, ethnicity, vaccination history, and birth cohort

- Levels of IgA and IgG binding to a panel of influenza antigens and HAI titers to each component of the quadrivalent influenza vaccine in the crevicular fluid vs serum at days 1, 8, 15, 29, and 180

8.4 Sample Size Considerations

The study is a hypothesis-generating study with a convenient sample size.

8.5 Participant Enrollment and Follow-Up

Subjects will be enrolled and followed for 180 days, with six clinic visits.

8.6 Analysis Plan

The study investigators will perform descriptive statistics to describe the participants enrolled in each group, including demographic parameters and influenza vaccination history.

Immune responses assessed in serum samples obtained immediately prior to vaccination (Day 1) and after vaccination will be summarized in terms of HAI or neutralizing antibody titers, and other humoral and cellular assay results. Individual antibody results are reported as a titer with values of $10*2^k$, where k=0, 1, 2, etc. The lower limit of detection of both assays is 1:10; values below the limit of detection are reported as '<10', and for analysis are imputed as one-half the limit of detection (10/2 = 5).

Seroconversion is defined as either a pre-vaccination HAI titer <1:10 and a post-vaccination HAI titer ≥1:40 or a pre-vaccination HAI titer ≥1:10 and a minimum four-fold rise in post-vaccination HAI titer. HAI summaries include tabular and graphical displays of GMT, percentage of subjects with seroconversion, and percentage of subjects with titers ≥1:40. Summaries of antibody data will include tabular and graphical displays of GMT, and percentage of subjects with titers ≥1:40 for all visits as well as percentage of subjects with a four-fold increase (to titer ≥1:40) for post-vaccination visits. Seroprotection is defined as achieving a day 29 HAI titer of ≥40.

Group-specific or pair-wise comparisons will be considered for the percentage of subjects achieving seroconversion or seroprotection at 28 days after the study vaccination as well as the magnitude of HAI titer at the same time point.

For each comparison of interest, a Chi-square test will be used to compare the percentage of subjects with seroconversion, while a non-parametric test (Kruskal-Wallis or Wilcoxon Mann-Whitney) will be used for comparisons of magnitude of HAI titer, other adaptive immune response measures, and the individual 'omics (systems biology) data. Statistical significance will be considered at a level of p= 0.05, without adjustment for multiple comparisons.

Systems biology outcomes will be assessed and analyzed using standard bioinformatics software packages to compare Days 1 (baseline) with Days 03 and 08 for each individual subject, and will be summarized for the subjects within each study arm.

9 QUALITY CONTROL AND QUALITY ASSURANCE

The study will undergo internal quality control and quality assurance per the Hope Clinic standard operating procedures.

10 ETHICS/PROTECTION OF HUMAN SUBJECTS

10.1 Ethical Standard

The site principal investigator will ensure that this trial is conducted in full conformity with principles of the Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research of the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research (April 18, 1979) and codified in 45 CFR 46, 21 CFR 50 and 56, and ICH E6; 62 Federal Regulations 25691 (1997), if applicable.

10.2 Institutional Review Board

Prior to enrollment of subjects into this trial, the protocol and informed consent form will be reviewed and approved by the Emory IRB. The responsible official for the IRB will sign the IRB letter of approval of the protocol prior to the start of this study.

10.3 Informed Consent Process

The site principal investigator and designees will choose subjects in accordance with the eligibility criteria detailed in Section 5. Before any study procedures are performed, subjects must sign an informed consent form that complies with the requirements of 21 CFR Part 50 and 45 CFR 46 and the local IRB.

Informed consent is a process that is initiated prior to an individual agreeing to participate in a study and continuing throughout the individual's study participation. Before any study procedures are performed, subjects will receive a comprehensive explanation of the proposed study procedures and study interventions/products, including the nature and risks of the trial, alternate therapies, any known adverse effects, and the other elements that are part of obtaining proper informed consent. Subjects will also receive a detailed explanation of the proposed use and disclosure of their protected health information, including specifically their serum samples. Subjects will be allowed sufficient time to consider participation in the study, after having the nature and risks of the trial explained to them, and have the opportunity to discuss the trial with their family, friends or legally authorized representative or think about it prior to agreeing to participate.

Informed consent forms describing in detail the study interventions/products, study procedures, risks and possible benefits are given to subjects. The informed consent form must not include any exculpatory statements. Informed consent forms will be IRB-approved and subjects will be asked to read and review the appropriate document. Upon reviewing the appropriate document, the site principal investigator (or designee) will explain the research study to subjects and answer any questions that may arise. Subjects must sign the informed consent form, and written documentation of the informed consent process is required prior to starting any study procedures/interventions being done specifically for the trial, including administering study product.

Study personnel may employ IRB-approved recruitment efforts prior to obtaining the subjects consent; however, before any study procedures are performed to determine protocol eligibility an informed consent form must be signed. Subjects will be given a copy

of all informed consent forms that they sign.

By signing the informed consent form, subjects agree to complete all evaluations required by the trial, unless the subject withdraws voluntarily, or is withdrawn or terminated from the trial for any reason.

The rights and welfare of subjects will be protected by emphasizing to subjects that the quality of their medical care will not be adversely affected if they decline to participate in or withdraw from this trial.

10.4 Exclusion of Women, Minorities, and Children (Special Populations)

This trial will be inclusive of all adults who meet the Subject Inclusion/Exclusion Criteria, regardless of religion, sex, or ethnic background.

10.5 Subject Confidentiality

Subjects' records and specimens will have code numbers and will not be identified by name. Subject confidentiality is strictly held in trust by the principal investigator and the Hope Clinic personnel directly involved in the study. This confidentiality is extended to cover testing of biological samples, in addition to the clinical information relating to participating subjects. The study protocol, documentation, data, and all other information generated will be held in strict confidence.

10.6 Future Use of Stored Specimens

Subjects participating in this study are agreeing to allow the investigators to keep any remaining samples for possible use in future research studies, such as examining additional immunological assessments or testing for antibodies against other viruses or bacteria. Some samples may be stored at the Hope Clinic. Samples may be shared with other investigators at other institutions. The samples will not be sold or used directly for production of any commercial product. No human genetic tests will be performed on samples. Each sample will be encoded (labeled) only with a barcode and a unique tracking number to protect subjects' confidentiality. There are no benefits to subjects in the collection, storage and subsequent research use of specimens. Reports about future research done with subjects' samples will NOT be kept in their health records.

The subject's agreement to allow future use of their specimens can be changed at any time prior to the end of the study by notifying the study doctors or nurses in writing. However, if the subject originally consents to future use and subsequently changes his/her decision, any data from a previously collected sample may still be used for this research.

11 LITERATURE REFERENCES

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SUPPLEMENTS/APPENDICES

Appendix A.

Study Visit Number	V01	V02	V03	V04	V05	V06
Study Day Relative to Study Vaccination	D1	D3	D8 (+/-1D)	D15 (+/-2D)	D29 (+/-3D)	D181 (+/- 14D)
Obtain Informed Consent#	X*					
Review Eligibility Criteria	X*					
Medical History+	X*	X	X	X	X	X
Concomitant Medications	X*%	X	X	X	X	X
Vital Signs	X*&$	(X)$	(X)$	(X)$	(X)$	(X)$
(Oral Temperature, Pulse Rate, and BP)	X*&$	(X)$	(X)$	(X)$	(X)$	(X)$
Height and Weight	X*					
Targeted Physical Examination	(X)*	(X)	(X)	(X)	(X)	(X)
Urine Pregnancy Test	X*@					
Study Vaccination	X					

20-minute Evaluation Period After Study Vaccination	X					
Saliva Collection for Study Assays (~1-2mL/visit)	X		X	X	X	X
Venous Blood Collection for Study Assays	X	X	X	X	X	X
Serological Assays	10	-	-	-	10	10
Cellular Immunology and 'Omics Assays	64	16	16	32	64	64
Future Research	16	16	16	16	16	16
Per Visit Blood Volume Total (mL)[!]	90	32	32	48	90	90
Cumulative Blood Volumes[!]	90	122	154	202	292	382

[#]Prior to study procedures
*****Prior to study vaccination.
[%]All current medications and medications taken within 30 days prior to day 1 of the study.
[&]Vital signs assessed on Day 1 (Visit 1) before the study vaccination will be considered as baseline.
[$]Subjects must not eat or drink anything hot or cold, or smoke within 10 minutes prior to taking oral temperature.
() Targeted physical examination if indicated based on review of complete or interim medical history.
[@]Must be performed on all female subjects of childbearing potential within 24 hours prior to each study vaccination and results must be negative and known prior to each study vaccination.
[+]Complete medical history by interview of subjects to be obtained on Day 1 (Visit 01) prior to the first study vaccination and interim medical history by interview of subjects to be obtained at follow-up visits after the first study vaccination.
[!]Blood volumes are approximate and measured in milliliters (mL).

Appendix B. Saliva collection

Saliva will be obtained per the Study Schedule using Oracol Plus (Malvern Medical) devices, which are specifically designed to sample crevicular fluid (saliva expressed from the area where the teeth meet the gums) as this is the component of saliva that is particularly rich in IgA and IgG. Sample will be collected by the subject by gentle brushing of the crevicular region (gum line) with an Oracol Plus saliva collection device for 60 sec or until saturation. The sponge will be inserted into a labeled plastic test tube, capped, and stored at -80°C until analysis.