

The immunodominance of antigenic site Sb on the H1 influenza virus hemagglutinin increases with high immunoglobulin titers of the cohorts and with young age, but not sex

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ABSTRACT

The head domain of the hemagglutinin of influenza viruses plays a dominant role in the antibody response due to the presence of immunodominant antigenic sites that are the main targets of host neutralizing antibodies. For the H1 hemagglutinin, five major antigenic sites defined as Sa, Sb, Ca1, Ca2, and Cb have been described. Although previous studies have focused on defining the hierarchy of the antigenic sites of the hemagglutinin in different human cohorts, it is still unclear if the immunodominance profile of the antigenic sites might change with the antibody levels of individuals or if other demographic factors (such as exposure history, sex, or age) could also influence the importance of the antigenic sites. The major antigenic sites of influenza viruses hemagglutinins are responsible for eliciting most of the hemagglutination inhibition antibodies in the host. To determine the antibody prevalence towards each major antigenic site, we evaluated the hemagglutination inhibition against a panel of mutant H1 viruses, each one lacking one of the “classic” antigenic sites. Our results showed that the individuals from the Stop Flu NYU cohort had an immunodominant response towards the sites Sb and Ca2 of H1 hemagglutinin. A simple logistic regression analysis of the immunodominance profiles and the hemagglutination inhibition titers displayed by each donor revealed that individuals with high hemagglutination inhibition titers against the wild-type influenza virus exhibited higher probabilities of displaying an immunodominance profile dominated by Sb, followed by Ca2 (Sb > Ca2 profile), while individuals with low hemagglutination inhibition titers presented a higher chance of displaying an immunodominance profile in which Sb and Ca2 presented the same level of immunodominance (Sb = Ca2 profile). Finally, while age exhibited an influence on the immunodominance of the antigenic sites, biological sex was not related to displaying a specific immunodominance profile.

Abbreviations: CDC, Centers for Disease Control and Prevention; HA, Hemagglutinin; NA, Neuraminidase; Sa, Specific site a; Sb, Specific site b; Ca1, Constant site a1; Ca2, Constant site a2; Cb, Constant site b; RBS, Receptor binding site; HI, Hemagglutination Inhibition; NYU, New York University; RDE, Receptor destroying enzyme; ELISA, Enzyme linked immunosorbent assay; PBS, Phosphate buffered saline; PBST, Phosphate buffered saline containing 0.1% (vol/vol) Tween-20; OPD, o-phenylenediamine dihydrochloride; ANOVA, Analysis of variance; Wt, Wild-type; ROC, receiver operating characteristic curve; CI, Confidence interval; CIVIC, Collaborative Influenza Vaccine Innovation Centers; CEIRR, Center for Excellence on Influenza Research and Response; NIH, National Institutes of Health.

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1. Introduction

Influenza viruses are the etiologic agents of seasonal influenza, a serious respiratory infection widely distributed around the world [1,2]. It has been estimated that around a billion people become sick with influenza every year, with 3 to 5 million severe cases and 290 000 to 650 000 deaths annually [3]. Although influenza vaccination is the primary intervention to prevent influenza virus infections and their complications, this strategy is not completely effective. According to the Centers for Disease Control and Prevention (CDC) in the United States, from 2004 to 2023, the effectiveness of influenza vaccines ranged from 10 % to 60 %, depending on the season [4]. To overcome this drastic variation in vaccine effectiveness, a better understanding of the immunogenicity of influenza virus vaccines is essential to develop new and better vaccines strategies. The main antigens that compose the viral lipid envelope of influenza viruses are the hemagglutinin (HA) and the neuraminidase (NA) glycoproteins [1]. Although natural infections with influenza virus have been shown to elicit protective antibodies against the HA and NA, during vaccination, the HA protein is the main target that is recognized by the immune system [5,6]. The HA protein mediates entry into the host cells by enabling viral attachment to sialic acid residues on the surface of the cells [1]. Structurally, this protein consists of a globular head domain and a conserved stalk (Fig. 1) [1]. Between these two domains, the globular head plays a dominant role in the antibody response because it contains immunodominant antigenic sites that are the main targets of host neutralizing antibodies [7,8]. Based on the antigenicity of the HA protein, influenza A viruses can be classified into 18 hemagglutinin subtypes divided in two phylogenetic groups: group (1) consists of H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18; while group 2 contains H3, H4, H7, H10, H14, and H15 [1]. More recently, a potential candidate for a new subtype, nominally H19, was discovered in a sequence analysis of cloacal samples from wild birds in Kazakhstan [9]. The genomic evidence showed that the new potential H19 subtype exhibited a 30 % genetic distance, based on the HA coding

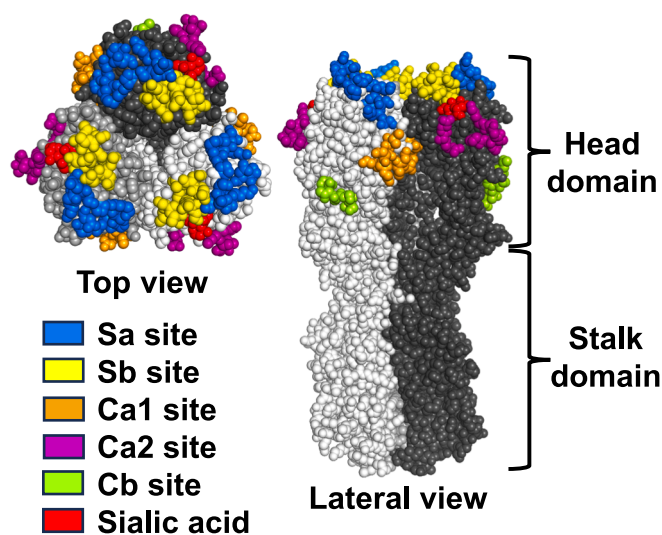


Fig. 1. Spatial distribution of classical head domain antigenic sites from the 2009 pandemic-like H1 HA protein. Top view and lateral view of the HA trimer crystal structure from the A/California/04/2009 (H1N1) virus (PDB:3UBE) [24]. Three monomers are shown in different colors: monomer one in white, monomer two in light gray, and monomer three in dark gray. Classic antigenic sites are highlighted in different colors as follows: Sa in blue, Sb in yellow, Ca1 in light brown, Ca2 in magenta, and Cb in green. Modeling was done with PyMOL (The PyMOL Molecular Graphics System, Version 2.5.1, Schrodinger, LLC). To observe the antigenic sites surrounding the RBS of HA protein, a sialic acid molecule (shown in red) is depicted in the RBS of each monomer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

region nucleotide sequence, to the H9 subtype of group (1) [9]. Specifically, for H1, five main antigenic sites have been identified in the head domain. These are: the specific site a (Sa) (Fig. 1, blue), the specific site b (Sb) (Fig. 1, yellow), the constant site a1 (Ca1) (Fig. 1, light brown), the constant site a2 (Ca2) (Fig. 1, magenta), and the constant site b (Cb) (Fig. 1, green) [7,8]. According to the spatial distribution of these antigenic sites in the structure of the HA protein, Sa and Sb are located on the top of the HA protein, while Ca1, Ca2, and Cb are proximate to the stalk domain (Fig. 1). The receptor binding site (RBS), which is marked by a sialic acid on Fig. 1 (red spheres), is located between Sb, Ca2, and Sa. Since the hemagglutination inhibition (HI) titer is an established correlate of protection against influenza virus infections [10], two previous studies employed an HI assay to evaluate the hierarchy of immunodominance for the antigenic sites of the 2009 pandemic-like H1N1 influenza virus strain, A/Michigan/45/2015 [11,12]. In the first paper, using antisera of different animals, Liu *et al.* [11] showed that the hierarchy of the antigenic sites varies depending on the species evaluated. While HI active antibodies in mice were targeted to sites Sb and Ca2, antibodies of ferrets were directed to site Sa, and antibodies of guinea pigs did not show any preference to a specific antigenic site. Finally, the HI active antibodies of adult humans exhibited a hierarchy dominated by Sa and Sb sites [11]. In the second study, Sánchez-de Prada *et al.* [12] reported that the hierarchy of the antigenic sites in humans was dominated by Sb, followed by Ca2 [12]. The reason for these contrasting results [11,12] might be related to the different size and characteristics of the human cohorts analyzed in both studies. Additionally, it is well known that the immunogenicity of the influenza vaccines can be affected by the characteristics of the vaccine as well as by host factors such as age, biological sex, health conditions, and the immune history of the individual [13]. However, it is unclear how these different features could also be impacting the observed antibody response towards specific antigenic sites in the HA protein. Finally, whether the protection from influenza virus infections could correlate with displaying a specific immunodominance profile of the antigenic sites also remains unknown.

This study evaluates the antibody response of human serum samples obtained from the Stop Flu NYU cohort towards the antigenic sites of the H1 HA of the A/Michigan/45/2015 strain and analyzes the correlation between displaying a specific immunodominance profile and the HI titers of the individuals. We also explore if demographic factors, such as age or sex, could influence the immunodominance of the antigenic sites exhibited by each person.

2. Methods

2.1. Human cohorts

A total of 39 different human serum samples were obtained from the Stop Flu NYU cohort (clinical study identifier: S21-01215). Fourteen samples were obtained during the 2021–2022 season, while 25 samples were obtained during the 2022–2023 season. The NYU Vaccine Center received informed consent from participants prior to their inclusion in the study. Participants donated blood before vaccination with the seasonal influenza vaccine. Serum samples were stored at -80°C until use. Prior to the HI assay, samples were treated with *Vibrio cholerae* receptor-destroying enzyme (RDE) (Denka Seiken, Chuo-ku, Tokyo, Japan) as described previously [14]. RDE treatment resulted in human serum samples with an initial 10-fold dilution.

2.2. Panel of recombinant H1 viruses

The methods and description of the generation of mutant viruses (H1- Δ Sa, H1- Δ Sb, H1- Δ Ca1, H1- Δ Ca2, and H1- Δ Cb) have been previously published [11]. The mutant viruses were constructed by substitution of the classic antigenic sites (Sa, Sb, Ca1, Ca2, and Cb) of the H1 A/Michigan/45/2015 strain with the corresponding heterologous

sequences of H5 (A/Vietnam/1203/2004) or H13 (A/black-headed gull/Sweden/1/1999) (Sup. Fig. S1). Each mutant virus contained five or more amino acid substitutions within one antigenic site, while the other four sites were not altered. Viruses were grown in 10-day-old embryonated chicken eggs for 48 h at 37 °C, then hemagglutination assays were performed to confirm the growth of each virus before freezing at – 80 °C. Before the experiments, the sequence of the HA segments from each virus was confirmed by Sanger sequencing.

2.3. HI assay

Chicken red blood cells (LAMPIRE Biological Laboratories, Pipersville, PA, USA) were washed in phosphate-buffered saline (PBS) and resuspended at a concentration of 0.5 % red blood cells. 2-fold serial dilutions of the RDE-treated human samples were done in 25 µl across a 96-well V-bottom plate. Allantoic fluid containing wild-type or mutant H1 viruses was diluted to eight HA-units and then incubated in equal volumes with antisera (25 µl each) for 30 min at 25 °C. Fifty microliters of chicken red blood cells (0.5 % in PBS) were then added. Finally, the plates were incubated for 30 min at 4 °C and the HI titers were visually determined.

As described previously [11], based on the HI titers for each mutant, we calculated the HI immunodominance index following equation (1). For this equation, a value of 5 was assigned to samples with no HI activity [11].

$$HI \text{ immunodominance index} = \frac{HI \text{ titer of wild type virus}}{HI \text{ titer of mutant virus}} \quad (1)$$

2.4. Enzyme linked immunosorbent assay (ELISA)

The recombinant HA protein of the H1 A/Michigan/45/2015 strain used for ELISA was produced as described previously [15]. Immulon 4 HBX 96-well microtiter plates (VWR International, Radnor, PA, USA) were coated with 50 µL/well of the recombinant HA proteins at 2 µg/mL in 1x coating buffer (SeraCare Life Sciences, Milford, MA, USA). The plates were incubated overnight at 4 °C. On the day of the experiment, all plates were washed three times with 220 µL of PBS containing 0.1 % (vol/vol) Tween-20 (PBST). Afterwards, 220 µL of blocking solution (3 % goat serum, 0.5 % non-fat dried milk powder, and 96.5 % PBST) were added to each well and incubated for 1 h at room temperature. After discarding the blocking solution, individual serum samples were serially diluted 3-fold in the blocking solution, followed by a 2-hour incubation at room temperature. Then, ELISA plates were washed three times with PBST and 50 µL of anti-mouse IgG-horseradish peroxidase-conjugated antibody (Cytiva, Marlborough, MA, USA) was added at a dilution of 1:3000 in the blocking solution. After 1 h of incubation at room temperature, plates were washed 3 times with PBST and developed using SigmaFast o-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, Saint Louis, MO, USA) for 10 min. Reactions were stopped by adding 50 µL of hydrochloric acid (3 M). The absorbance of the plates at 492 nm was measured on a FilterMax F3 multi-mode microplate reader (Molecular Devices, San Jose, CA, USA). For each ELISA plate, the average plus 3 standard deviations of absorbance values of blank wells was used as a cutoff to determine endpoint titers using the GraphPad Prism program version 10.0.2 for Mac OS X, GraphPad Software (Boston, MA, USA, <https://www.graphpad.com>).

2.5. Statistics

The simple logistic regression and the Fisher's exact test were performed with the GraphPad Prism program version 10.0.2 for Mac OS X, GraphPad Software (Boston, MA, USA, <https://www.graphpad.com>). Statistical significance between groups was determined by either a Mann-Whitney-Wilcoxon test or a Kruskal-Wallis one-way analysis of variance (ANOVA); a p-value ≤ 0.05 was considered significant.

3. Results

3.1. Human cohort description

Thirty-nine different serum samples were obtained from human donors before seasonal vaccination (pre-vaccination sera). Fourteen serum samples were obtained before the 2021–2022 season, while 25 serum samples were obtained before the 2022–2023 season (Sup. Table S1). These individuals are a small group of patients who are part of the Stop Flu NYU cohort study (clinical study identifier: S21-01215), which is a longitudinal study focused on the analysis of the human antibody response to influenza viruses by collecting blood from human donors for up to seven years [16]. The average age of the 39 participants in this cohort was 43.7 years, with a standard deviation (SD) of 16.9 years (Table 1). In terms of sex, the cohort was composed of 20 female donors (51 %) and 19 male donors (49 %) (Table 1 and Sup. Table S1). Although it was not possible to know the previous influenza virus infection exposure of the participants, 26 participants (67 %) reported that they had received the influenza vaccine at least 5 years in a row (Repeated vaccination), while 13 (33 %) of them had received the vaccine sporadically (Non-repeated vaccination) (Table 1 and Sup. Table S1).

3.2. Analysis of the immunodominance profile of pre-vaccination sera towards H1 hemagglutinin

To evaluate the immunodominance profile of the people in this cohort, we followed the same strategy described by Liu *et al.* [11] and Sánchez-de Prada *et al.* [12]. This strategy was based on an HI assay using a panel of mutant H1 viruses expressing the HA protein of the A/Michigan/45/2015 strain [11,12]. For each mutant virus, one antigenic site was replaced with a heterologous antigenic site from either H5 or H13 HAs (Sup. Fig. S1). To evaluate the importance of each antigenic site, we compared the serum HI titers to the wild-type (Wt) H1 virus that has all antigenic sites (H1-Wt) versus the HI titers to the mutant viruses that lack one site. In this analysis, a significant reduction in HI titers against a specific mutant virus compared to the H1-Wt would indicate a loss of activity and therefore the presence of antibodies targeting that antigenic site. The HI titers against the mutants lacking the sites Sb (H1-ΔSb) or Ca2 (H1-ΔCa2) were significantly lower than the H1-Wt (Fig. 2a and Sup. Fig. S2), suggesting that Sb and Ca2 were the most immunodominant antigenic sites. On the other hand, the HI titers against the H1-Wt virus were similar to those against mutants lacking the sites Sa (H1-ΔSa), Ca1 (H1-ΔCa1), and Cb (H1-ΔCb), indicating that these three sites did not show immunodominance (Fig. 2a and Sup. Fig. S2). To rule out the possible contribution of anti-H5 antibodies to the HI activity, we also used a chimeric virus (cH5/1 HA and N1 NA virus) in which the complete head domain of the A/Michigan/45/2015 strain was replaced by the head domain of H5, to which humans are typically naïve. None of the serum samples from the cohort showed HI activity against the chimeric cH5/1 virus (Fig. 2a), suggesting that antibodies against the head domain of the A/Michigan/45/2015 strain did not cross-react with the H5 head domain. Finally, to analyze the influence of non-classical HA-head epitopes on the overall HI activity, we also used a mosaic virus in

Table 1
Summary of the demographics of human donors.

N	39
Age (mean ± SD)	43.7 ± 16.9
Female (n, %)	20, 51 %
Male (n, %)	19, 49 %
Repeated vaccination (n, %)	26, 67 %
Non-repeated vaccination (n, %)	13, 33 %

Percentages were calculated using the total N of the cohort. SD: Standard deviation

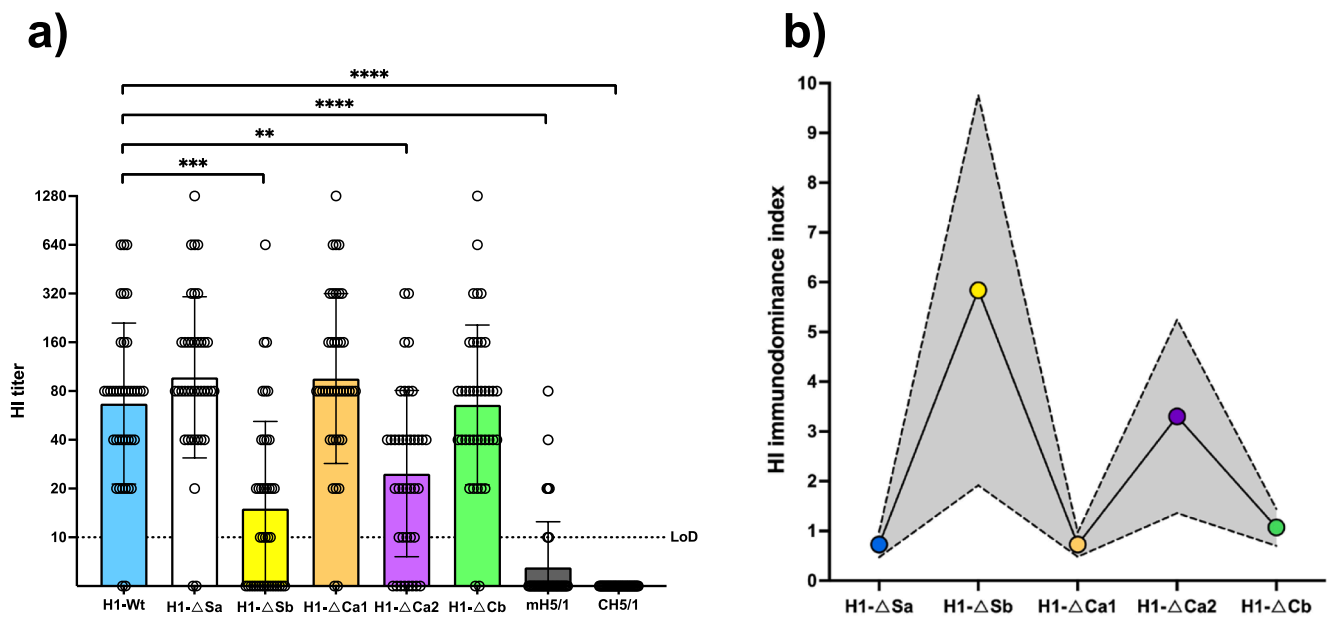


Fig. 2. HI titers and immunodominance profile of donors in the Stop Flu NYU cohort. a) HI titers of serum samples from 39 adult donors were quantified against a panel of H1 viruses. Geometric mean titers and geometric SD are shown for each virus. Circles represent the averaged HI titers of a single donor. Experiments were performed in technical duplicates. The statistical significance between the different viruses was determined using a Kruskal-Wallis one-way ANOVA. Asterisks indicate the significance level, with ** = p-value \leq 0.01, *** = p-value \leq 0.001, and **** = p-value \leq 0.0001. LoD: Limit of detection. b) HI immunodominance index for the H1 mutants: H1-ΔSa, H1-ΔSb, H1-ΔCa1, H1-ΔCa2, and H1-ΔCb. The HI immunodominance index was calculated for individual donors using the HI titers from a in equation (1) (Section 2.3 of Methods). The solid line represents averaged HI dominance indices, and shading shows SD.

which all antigenic sites were replaced with the sequences of H5 (mH5/1 HA and N1 NA virus), we discovered that the HI activity against this virus was mostly eliminated (Fig. 2a), indicating that the immunodominant sites were successfully removed in this virus.

3.3. The immunodominance profiles of donors in the cohort can be grouped into three types

To better represent the importance of the antigenic sites for each donor, we calculated the HI immunodominance index of the antigenic sites, as described previously [11]. The HI immunodominance index is a parameter that represents the reduction of HI titers for each mutant with respect to the Wt virus. For each donor, we divided the HI titer to the H1-Wt by the HI titer to the respective mutant (Equation (1), Section 2.3 of Methods). Hence, higher indices indicate a higher immunodominance of the mutated site. When we averaged the HI immunodominance index of the 39 donors of the cohort for every antigenic site, we found the Sb site exhibited the highest HI immunodominance index, followed by Ca2, and then the remainder of the antigenic sites (Sa, Ca1, and Cb) (Fig. 2b). A closer look at the different HI immunodominance profiles displayed by each donor in the cohort (Sup. Fig. S3) showed that according to the HI immunodominance indices of Sb and Ca2, it was possible to classify the different donors according to three main profiles. Twenty-two donors (Donors: 3, 4, 5, 7, 9, 11, 12, 18, 19, 20, 21, 22, 24, 27, 28, 31, 32, 33, 34, 35, 36, and 39) displayed an immunodominance profile dominated by Sb, followed by Ca2 (Sb > Ca2 profile), and then the rest of the antigenic sites (Sup. Fig. S3). Thirteen donors (Donors: 1, 2, 6, 8, 13, 14, 15, 23, 25, 29, 30, 37, and 38) presented an immunodominance profile in which Sb and Ca2 showed the same HI immunodominance index (Sb = Ca2 profile) (Sup. Fig. S3). Two donors (Donors: 10 and 17) exhibited an immunodominance profile dominated by the Ca2 site (Sb < Ca2 profile) (Sup. Fig. S3). Because there were only two donors displaying an Sb < Ca2 profile, they were not included in the analyses described in the next sections. In addition, since donors 16 and 26 did not show HI activity against any of the virus tested (Sup. Fig. S2), these two individuals were also not included for the next analyses.

3.4. Individuals with high HI titers against the H1-Wt virus show a higher probability of displaying an Sb > Ca2 profile

To examine the influence of antibody titer level on displaying either an Sb > Ca2 profile or an Sb = Ca2 profile, we performed a simple logistic regression using the log of the HI titers against the H1-Wt virus to measure the probability of displaying an Sb > Ca2 profile or an Sb = Ca2 profile. The model obtained from this analysis exhibited a p-value of 0.019 for a likelihood-ratio test, and a receiver operating characteristic curve (ROC) of 0.7308. Based on this model, it was discovered that donors exhibited greater probabilities of displaying an Sb > Ca2 profile as the HI titers against the H1-Wt increased (blue line in Fig. 3a). While low titers were associated with a higher probability of exhibiting the Sb = Ca2 profile (red line in Fig. 3a). On the other hand, the odds ratio calculated by the logistic regression model showed that for every log of the HI titer against the H1-Wt virus increased, the donors were \approx 10 times more likely to display an Sb > Ca2 profile (Fig. 3b). In line with this finding, the odds of displaying an Sb = Ca2 profile declined \approx 10 times for every log of the HI titer against the H1-Wt increased (Fig. 3b).

3.5. Donors displaying an Sb > Ca2 profile exhibit higher total IgG titers than donors with an Sb = Ca2 profile

When we compared the total serum IgG titers against the recombinant HA protein of the H1-Wt virus for the donors displaying either an Sb > Ca2 or an Sb = Ca2 profile, we discovered that, similar to the results with the HI titers, donors displaying an Sb > Ca2 profile exhibited significantly higher total IgG titers against the HA protein of the H1-Wt virus than the donors displaying an Sb = Ca2 profile (Fig. 4a). Moreover, we found that 77 % of the donors displaying an Sb > Ca2 profile had a repeated vaccination history, while 23 % had a non-repeated vaccination history (Fig. 4b). Similar to this distribution, 69 % of donors with an Sb = Ca2 profile had a repeated vaccination history, while 31 % exhibited a non-repeated vaccination history (Fig. 4b).

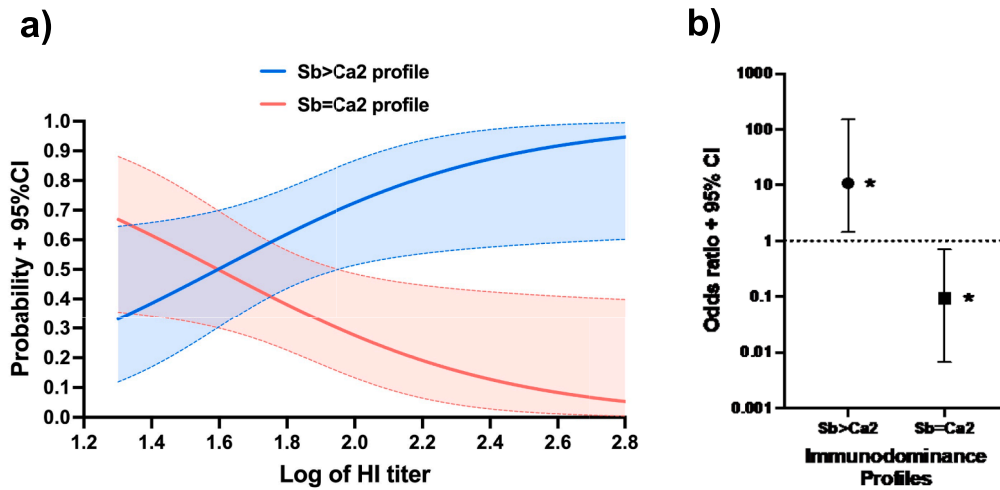


Fig. 3. Probability distribution and odds of displaying an Sb > Ca2 or Sb = Ca2 profile based on the log of HI titers against the H1-Wt virus. a) Probability distribution of displaying an Sb > Ca2 profile (blue) or an Sb = Ca2 profile (red) based on the log of the HI titers against the H1-Wt. The probability was calculated through a simple logistic regression analysis using the log of HI titers from individuals displaying either an Sb > Ca2 or an Sb = Ca2 profile (total n = 35). Donors with no HI activity or displaying an Sb < Ca2 profile were not considered for this analysis. Solid lines represent the mean probability, and shading shows the 95 % confidence interval (CI). b) Odds of displaying an Sb > Ca2 profile or an Sb = Ca2 profile calculated from the simple logistic regression shown in a). The odds ratio and the 95 % CI are depicted. Asterisks indicate the significance level, with * = p-value ≤ 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

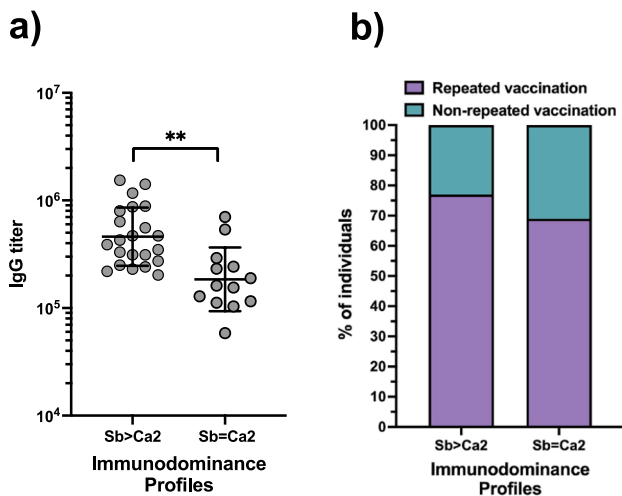


Fig. 4. IgG titers and vaccination history of donors displaying either an Sb > Ca2 or Sb = Ca2 profile. a) Total IgG titers against the HA protein of the H1-Wt virus from donors displaying an Sb > Ca2 profile or an Sb = Ca2 profile. Endpoint geometric mean titers and geometric SD are shown for each profile. Dots represent the averaged endpoint IgG titers of a single donor. The experiment was performed in technical duplicates. The statistical significance between the different profiles was determined by a Mann-Whitney-Wilcoxon test. Asterisks indicate the significance level, with ** = p-value ≤ 0.01. b) Vaccination history of donors displaying an Sb > Ca2 profile or an Sb = Ca2 profile. Repeated vaccination was defined as donors who received the influenza vaccine for at least 5 years in a row. Non-repeated vaccination was defined as donors who received the vaccine sporadically.

3.6. The age on the odds of displaying an Sb > Ca2 profile

We first compared the age of the individuals in the cohort with the specific HI titers against the H1-Wt virus. For this analysis, we grouped the donors in the cohort according to three age categories: young adults (20–39 years old), middle-aged adults (40–59 years old), and old adults (≥60 years old). The average age of the groups was: 28.9 years ± 4.9 for young adults, 49.1 years ± 6 for middle-aged adults, and 68.4 years ±

5.3 for old adults (Fig. 5a). The difference in average age among the different age groups was demonstrated to be statistically significant by a Kruskal-Wallis one-way ANOVA. When we compared the HI titers against the H1-Wt virus for the different groups, we found that although there is a downward trend in the HI titers as the age of the group increases (Fig. 5b), this decline in the HI titers was not statistically significant. Given that the HI titers seemed to be independent of the age of individuals in the cohort, we compared the ages of donors displaying an Sb > Ca2 versus an Sb = Ca2 profile. The mean age of the donors with an Sb > Ca2 profile (38.9 years ± 15.6) was significantly lower than that of the donors displaying an Sb = Ca2 profile (51.7 years ± 16.7) (Fig. 5c). Next, we performed a simple logistic regression to assess the precise influence of age on displaying an Sb > Ca2 profile or an Sb = Ca2 profile. This analysis produced a model with a p-value of 0.0267 for a likelihood ratio test, and an ROC of 0.7028. According to this model, as age increased, the probability of displaying an Sb > Ca2 profile declined (blue line in Fig. 6a), while the probability of exhibiting an Sb = Ca2 profile increased (red line in Fig. 6a). The odds ratios calculated from this model indicated that for every year that age increased, there was a slight but significant increase in the odds of displaying an Sb = Ca2 profile, while at the same time there was a slight but significant decrease in the odds of displaying an Sb > Ca2 profile for every year that age increased (Fig. 6b).

3.7. Displaying a specific immunodominance profile (either an Sb > Ca2 profile or an Sb = Ca2 profile) is not related to sex

Next, we compared the age, vaccination history, and HI titers against the H1-Wt virus of females and males in the cohort. Females exhibited an average age of 43 years ± 18.7, while males had an average age of 44.5 years ± 15.5 (Fig. 7a). In terms of the vaccination history, we found that in both females and males, a large part of the donors (65 % of females and 68.5 % of males) had a repeated vaccination history, while only a small part (35 % of females and 31.5 % of males) exhibited a non-repeated vaccination history (Fig. 7b). In addition, the comparison of HI titers against the H1-Wt virus revealed that females and males presented similar HI titers (Fig. 7c). Given that age, vaccination history, and HI titers were similar between females and males, we were able to analyze the direct effect of sex on displaying an immunodominance profile without any bias caused by age, vaccination history, or HI titers.

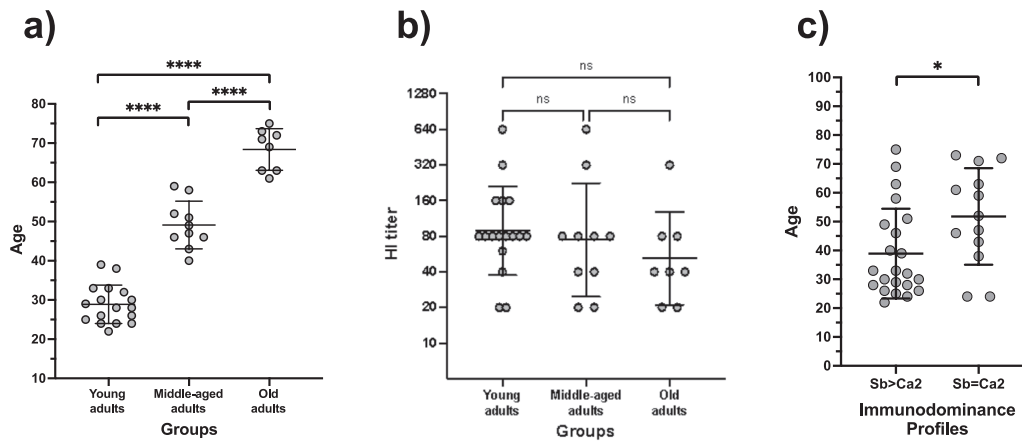


Fig. 5. Comparison of age with the HI titers of donors in the Stop Flu NYU cohort and the age distribution of donors displaying either an Sb > Ca2 or Sb = Ca2 profile. a) Age of young adults, middle-aged adults, and old adults in the Stop Flu NYU cohort. Young adults were defined as donors from 20 to 39 years old, middle-aged adults were defined as donors from 40 to 59 years old, and old adults were defined as donors with ≥ 60 years old. The mean and the SD of age are depicted for each group. Dots represent individual donors. b) HI titers of serum samples from the different age groups shown in a. HI titers were quantified against the H1-Wt virus. Geometric mean titers and geometric SD are shown for each group. Dots represent the averaged HI titers of a single donor. Experiments were performed in technical duplicates. For a and b, the statistical significance between the different groups was determined using a Kruskal-Wallis one-way ANOVA. Asterisks indicate the significance level, with **** = p-value ≤ 0.0001. ns: non-significant. c) Age of donors displaying an Sb > Ca2 profile or an Sb = Ca2 profile. The mean and the SD of age are depicted for each profile. Dots represent individual donors. The statistical significance between the different profiles was determined by a Mann-Whitney-Wilcoxon test. Asterisks indicate the significance level, with * = p-value ≤ 0.05.

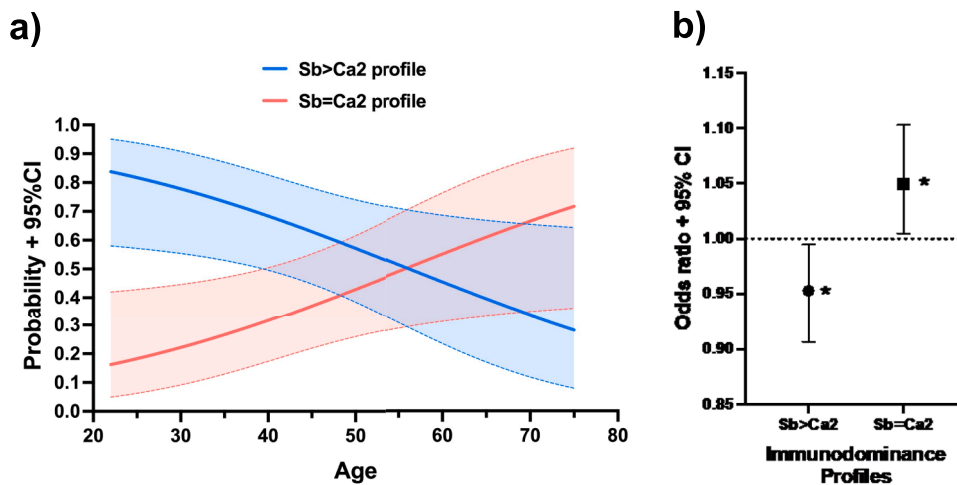


Fig. 6. Probability distribution and odds of displaying an Sb > Ca2 or Sb = Ca2 profile based on the age of the donors from the Stop Flu NYU cohort. a) Probability distribution of displaying an Sb > Ca2 profile (blue) or an Sb = Ca2 profile (red) based on the age of the donors. The probability was calculated through a simple logistic regression analysis using the age of individuals displaying either an Sb > Ca2 or an Sb = Ca2 profile (total n = 35). Donors with no HI activity or displaying an Sb < Ca2 profile were not considered for this analysis. Solid lines represent the mean probability and shading shows the 95 % CI. b) Odds of displaying an Sb > Ca2 profile or an Sb = Ca2 profile calculated from the simple logistic regression shown in a. The odds ratio and the 95 % CI are depicted. Asterisks indicate the significance level, with * = p-value ≤ 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

By looking at the distribution of the Sb > Ca2 profile and the Sb = Ca2 profile in females and males, we found that 50 % of the female donors displayed an Sb > Ca2 profile while 50 % exhibited an Sb = Ca2 profile (Fig. 7d). However, in the case of males, we found that the larger part of male donors displayed an Sb > Ca2 profile (76 %) while only a small part (24 %) of male donors exhibited an Sb = Ca2 profile (Fig. 7d). To validate if the sex of the individuals and their immunodominance profile were related, we performed a Fisher’s exact test. The p-value calculated by this test was 0.1642 (Fig. 7d), indicating that there was no statistically significant association between sex and displaying a specific immunodominance profile.

4. Discussion

The characterization of the immune response to influenza virus has revealed that the HA head domain plays a critical role in the antibody responses elicited by infection and vaccination [5,6]. The importance of the HA head domain is related to the presence of immunodominant antigenic sites located in this domain of the protein [7,8]. Given that these antigenic sites are prone to rapid mutation [17], influenza vaccines need to be redesigned annually. The development of vaccines with increased breadth and duration of protection represents a formidable challenge. A critical aspect of overcoming this problem is to better understand the antibody response to the different antigenic sites of the HA protein.

Here, we studied the hierarchy of importance of the classical

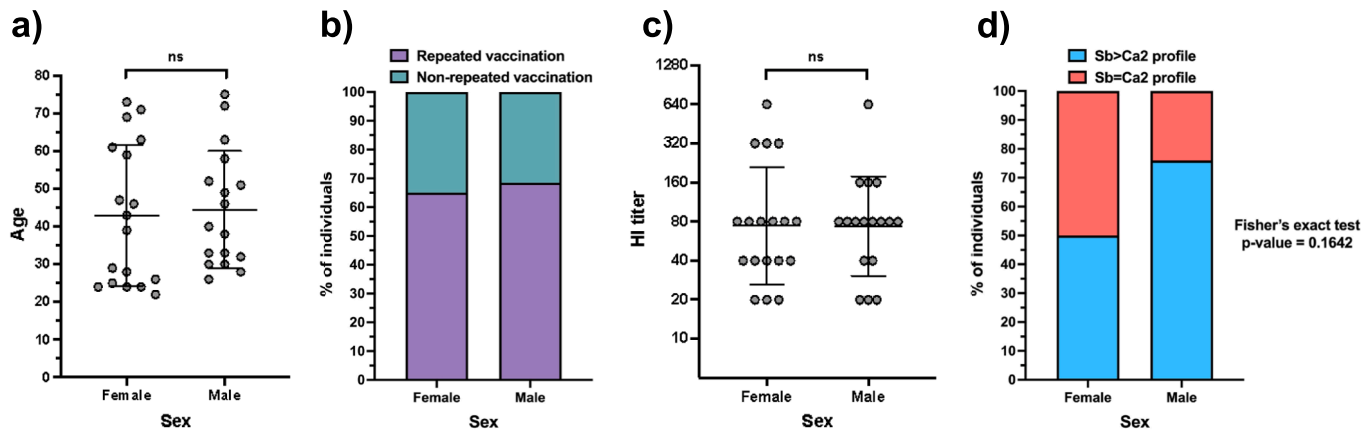


Fig. 7. Age, HI titers, vaccination history, and distribution of Sb > Ca2 and Sb = Ca2 profiles in females and males in the Stop Flu NYU cohort. a) Age of females and males in the Stop Flu NYU cohort. The mean and the SD of age are depicted for each sex. Dots represent individual donors. b) Vaccination history of females and males in the Stop Flu NYU cohort. Repeated vaccination was defined as donors who received the influenza vaccine for at least 5 years in a row. Non-repeated vaccination was defined as donors who received the vaccine sporadically. c) HI titers against the H1-Wt virus determined on serum samples from females and males in the Stop Flu NYU cohort. Geometric mean titers and geometric SD are shown for each sex. Dots represent the averaged HI titers of a single donor. Experiments were performed in technical duplicates. For a and c, the statistical significance between females and males was determined by a Mann-Whitney-Wilcoxon test. ns: non-significant. d) Percentage of females and males in the Stop Flu NYU cohort that displayed either an Sb > Ca2 or an Sb = Ca2 profile. A Fisher's exact test was performed to analyze distribution of Sb > Ca2 and Sb = Ca2 profiles in females and males. The p-value calculated by the Fisher's exact test is depicted on the right.

antigenic sites (Sa, Sb, Ca1, Ca2, and Cb) of the H1 protein by evaluating the HI activity of 39 human serum samples from the Stop Flu NYU cohort (clinical study identifier: S21-01215) against a panel of mutant viruses lacking one of the antigenic sites (H1- Δ Sa, H1- Δ Sb, H1- Δ Ca1, H1- Δ Ca2, and H1- Δ Cb). Our results indicated that for the HI activity of the human serum samples, Sb and Ca2 are the most important antigenic sites, since their deletion induced a significant reduction in HI titers against the Wt influenza virus. To detect subtle shifts in HI dominance, we calculated the HI immunodominance index exhibited by each antigenic sites [11]. Our results showed that on average, the individuals from the Stop Flu NYU cohort study exhibited an immunodominance profile dominated by Sb, followed by Ca2, and then the other sites (Sb > Ca2 > Sa, Ca1, and Cb). These results validate the immunodominance hierarchy reported by Sánchez-de Prada *et al.* [12], and contrast with the results reported by Liu *et al.* [11], who found that their cohorts displayed an immunodominance profile where sites Sa and Sb were the most important. One reason could be that, as compared to the work of Sanchez-de Prada *et al.* [12] and the present work, Liu *et al.* [11] experiments were performed with different cohorts from different seasons. Additionally, Liu *et al.* [11] used a small group of human donors ($n = 18$), making their study more likely to have bigger margins of error that could account for the variation in the hierarchy of the antigenic sites. Another possibility is that, due to the small number, the cohorts evaluated by Liu *et al.* [11] contained an unknown bias related to specific demographic factors or exposure histories of the donors. Of note, among the immunodominance profiles reported in the cohort of Liu *et al.* [11], they also found cohorts displaying an Sb > Ca2 profile.

A detailed analysis of the immunodominance profiles displayed by each donor from the Stop Flu NYU cohort showed that according to the HI immunodominance indices of Sb and Ca2, the immunodominance profiles of the donors could be grouped into three main types: Sb > Ca2 profile, Sb = Ca2 profile, and Sb < Ca2 profile. We discovered that donors with high HI titers against the H1-Wt virus exhibited a higher probability of displaying an Sb > Ca2 profile. Moreover, the odds ratio calculated through the simple logistic regression analysis confirmed that the level of HI titers had a significant impact on displaying a specific immunodominance profile, with the odds of displaying an Sb > Ca2 profile increasing ≈ 10 times by each log that the HI titers increased. Given that donors with an Sb > Ca2 profile also showed higher total IgG titers towards the HA protein, it appears that at low titers, the immune

responses towards the HA protein are able to recognize the antigenic sites Sb and Ca2 to the same extent. However, as the antibody levels towards the influenza virus increase, the immune responses shift to be focused primarily on the Sb site, leaving the Ca2 site in a secondary role. This discovery suggests that the hierarchy of the antigenic sites, rather than being static, is very dynamic and can evolve as the antibody responses increase. On the other hand, since there were no differences in the vaccination histories (repeated vs non-repeated vaccination) of the individuals that displayed either an Sb > Ca2 profile or an Sb = Ca2 profile, it seems that vaccination history is not related to displaying a specific immunodominance profile. When we evaluated the age of the different donors, we found that donors displaying an Sb > Ca2 profile were significantly younger than the donors displaying an Sb = Ca2 profile. In line with this finding, a detailed analysis of the influence of age on displaying a specific profile revealed that younger donors had a higher probability of displaying an Sb > Ca2 profile. Nevertheless, since we found that the HI titers of the individuals were not affected by the age of the donors, we can conclude that the influence of HI titers on displaying a specific immunodominance profile is independent of age.

Although previous studies have reported that immune responses to the influenza vaccine can differ between males and females [18–21], here, we did not find that biological sex alone significantly influence the immunodominance profile of the donors in the cohort based on a Fisher's exact test. Although our analysis shows a statistically significant impact of age (and not sex or vaccination history on the human immunodominance profile) future studies with higher sample numbers will be needed to confirm and better evaluate the impact of different demographic factors on the immune response to the classical antigenic sites. In addition, it remains unknown how the interplay of biological sex with different demographic factors could affect the immune response to the classical antigenic sites. Moreover, our study also did not consider if comorbidities associated with the individuals could impact the immunodominance hierarchy of the antigenic sites of influenza virus HA. Similar to these limitations, it is still unknown if the immunodominance profile could be affected by the phenomenon of the original antigenic sin, which refers to the concept that the first antigenic variant encountered early in life conditions lifelong immunity [22,23].

Furthermore, here we used the A/Michigan/2015 strain of the 6B.1 clade, which is the precursor of the currently circulating pdmH1N1 strains. Nevertheless, in order to precisely characterize antibody

prevalence in more details, it will be necessary to construct the panel of mutant viruses in the HA backbone of more recent pdmH1N1 strains.

Finally, studies on the immunodominance of antigenic sites over several years may identify specific epitopes that induce protective antibody responses, which should be included in the design of more effective vaccines.

5. Conclusion

Altogether, the results from this study demonstrate that in the Stop Flu NYU cohort study, the hierarchy of the immunodominant antigenic sites of H1 A/Michigan/45/2015 strain is dominated by the sites Sb and Ca2. Interestingly, the immunodominance profile displayed by each person (either Sb > Ca2 or Sb = Ca2) is strongly associated with the level of HI titers against the A/Michigan/45/2015 strain. Moreover, although biological sex does not appear to influence the hierarchy of the antigenic sites, age seems to play a role in the probability of displaying a specific immunodominance profile. Longitudinal studies with larger cohorts of human donors would help to better evaluate whether one or a combination of different demographic and immune factors can correlate with displaying a particular immunodominance profile of the H1 HA.

6. Data statement

All data are presented in the manuscript and requests for raw data may be submitted to the corresponding authors.

CRediT authorship contribution statement

Jose L. Martínez: . **Nicholas Lemus:** Investigation, Methodology, Writing – review & editing. **Tsoi Ying Lai:** Investigation, Methodology, Writing – review & editing. **Mitali Mishra:** Investigation, Methodology, Writing – review & editing. **Irene González-Domínguez:** Resources, Writing – review & editing. **Eduard Puente-Massaguer:** Resources, Writing – review & editing. **Madhumathi Loganathan:** Resources, Writing – review & editing. **Benjamin Francis:** Resources, Writing – review & editing. **Marie I. Samanovic:** Resources, Writing – review & editing. **Florian Krammer:** Resources, Writing – review & editing. **Mark J. Mulligan:** Resources, Writing – review & editing. **Viviana Simon:** Resources, Writing – review & editing. **Peter Palese:** Conceptualization, Data curation, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing. **Weina Sun:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [The Icahn School of Medicine at Mount Sinai has filed patent application entitled “Mosaic influenza virus hemagglutinin polypeptides and uses thereof” which list Florian Krammer, Peter Palese, and Weina Sun as co-inventors. Florian Krammer has consulted for Merck, Seqirus, Curevac and Pfizer, and is currently consulting for GSK, Gritstone, 3rd Rock Ventures and Avimex and he is a co-founder and scientific advisory board member of CastleVax. The Krammer laboratory is also collaborating with Pfizer on animal models of SARS-CoV-2 and Dynavax on influenza virus vaccines. Mark J. Mulligan reported laboratory research and clinical trials contracts for vaccines or MAB with Lilly, Pfizer, and Sanofi; personal fees for Scientific Advisory Board service from Merck, Meissa Vaccines, Inc. and Pfizer. All other authors have declared that no conflict of interest exists.].

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vaccine.2024.04.037>.

References

- [1] Krammer F., and Palese P. Orthomyxoviridae: The viruses and their replication. In: Knipe DM, HP, and Whelan S., editor. *Fields Virology Emerging virus*. 7th ed. Philadelphia, PA.: Wolters Kluwer/Lippincott Williams & Wilkins Health; 2020. p. 596–648. DOI: 10.1016/j.coviro.2013.07.007.
- [2] Zanobini P, Bonaccorsi G, Lorini C, Haag M, McGovern I, Paget J, et al. Global patterns of seasonal influenza activity, duration of activity and virus (sub)type circulation from 2010 to 2020. *Influenza Other Respir Viruses* 2022;16:696–706. <https://doi.org/10.1111/irv.12969>.
- [3] Paget J, Spreuwenberg P, Charu V, Taylor RJ, Iuliano AD, Bresee J, et al. Global mortality associated with seasonal influenza epidemics: new burden estimates and predictors from the GLaMOR project. *J Glob Health* 2019;9:020421. <https://doi.org/10.7189/jogh.09.020421>.
- [4] Centers for Disease Control and Prevention. Past Seasons Vaccine Effectiveness Estimates. Accessed October 13th 2023. Retrieved from <https://www.cdc.gov/flu/vaccines-work/past-seasons-estimates.html>.
- [5] Chen Y, Wohlbold T, Nai-Ying Z, Huang M, Huang Y, Neu K, et al. Influenza infection in humans induces broadly cross-reactive and protective neuraminidase-reactive antibodies. *Cell* 2018;173. <https://doi.org/10.1016/j.cell.2018.03.030>. 417–29.e10.
- [6] Krammer F. The human antibody response to influenza a virus infection and vaccination. *Nat Rev Immunol* 2019;19:383–97. <https://doi.org/10.1038/s41577-019-0143-6>.
- [7] Gerhard W, Frankel ME, Webster R. Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies. *Nature* 1981;290:713–7. <https://doi.org/10.1038/290713a0>.
- [8] Caton AJBG, Yewdell JW, Gerhard W. The antigenic structure of the influenza virus A/PR/8/34 haemagglutinin (H1 subtype). *Cell* 1982;31:417–27. [https://doi.org/10.1016/0092-8674\(82\)90135-0](https://doi.org/10.1016/0092-8674(82)90135-0).
- [9] Fereidouni S, Starick E, Karamendin K, Genova CD, Scott SD, Khan Y, et al. Genetic characterization of a new candidate hemagglutinin subtype of influenza a viruses. *Emerg Microbes Infect* 2023;12:2225645. <https://doi.org/10.1080/22221751.2023.2225645>.
- [10] Hobson D, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *J Hyg (Lond)* 1972;70:767–77. <https://doi.org/10.1017/s0022172400022610>.
- [11] Liu STH, Behzadi MA, Sun W, Freyn AW, Liu W-C, Broecker F, et al. Antigenic sites in influenza H1 hemagglutinin display species-specific immunodominance. *J Clin Invest* 2018;128:4992–6. <https://doi.org/10.1172/JCI122895>.
- [12] Sánchez-De Prada L, Sanz-Muñoz I, De Lejarazu RO, Eiros JM, García-Sastre A, Aydllo T. Immunodominance hierarchy after seasonal influenza vaccination. *Emerg Microbes & Infect* 2022;11:2670–9. <https://doi.org/10.1080/22221751.2022.2135460>.
- [13] Wen S, Wu Z, Zhong S, Li M, Shu Y. Factors influencing the immunogenicity of influenza vaccines. *Hum Vaccin Immunother* 2021;17:2706–18. <https://doi.org/10.1080/21645515.2021.1875761>.
- [14] Robinson RQ, and Dowdle WR. Influenza viruses. In: Lennette EH, and Schmidt NJ., editor. *Diagnostic procedures for viral and rickettsial infections*. 4th ed. New York, NY.: American Public Health Association, Inc.; 1969. p. 414–33.
- [15] Krammer F, Magine I, Tan GS, Pica N, Krause JC, Palese P. A Carboxy-terminal Trimerization domain stabilizes conformational epitopes on the stalk domain of soluble recombinant hemagglutinin substrates. *PLoS One* 2012;7:e43603. <https://doi.org/10.1371/journal.pone.0043603>.
- [16] NYU Langone Health. The Stop Flu NYU cohort study. Accessed October 24th 2023. Retrieved from <https://clinicaltrials.med.nyu.edu/clinicaltrial/1725/stop-flu-nyu-cohort/>.

- [17] Kirkpatrick E, Qiu X, Wilson PC, Bahl J, Krammer F. The influenza virus hemagglutinin head evolves faster than the stalk domain. *Sci Rep* 2018;8:10432. <https://doi.org/10.1038/s41598-018-28706-1>.
- [18] Furman D, Hejblum BP, Simon N, Jojic V, Dekker CL, Thiebaut R, et al. Systems analysis of sex differences reveals an immunosuppressive role for testosterone in the response to influenza vaccination. *Proc Natl Acad Sci U S A* 2014;111:869–74. <https://doi.org/10.1073/pnas.1321060111>.
- [19] Potluri T, Fink AL, Sylvia KE, Dhakal S, Vermillion MS, Vom Steeg L, et al. Age-associated changes in the impact of sex steroids on influenza vaccine responses in males and females. *npj Vaccines* 2019;4:29. <https://doi.org/10.1038/s41541-019-0124-6>.
- [20] Sanchez-de PL, Ortiz de Lejarazu-Leonardo R, Castrodeza-Sanz J, Tamayo-Gomez E, Eiros-Bouza JM, Sanz-Munoz I. Do vaccines need a gender perspective? *Influenza Says Yes!* *Front Immunol* 2021;12:715688. <https://doi.org/10.3389/fimmu.2021.715688>.
- [21] Shapiro JR, Li H, Morgan R, Chen Y, Kuo H, Ning X, et al. Sex-specific effects of aging on humoral immune responses to repeated influenza vaccination in older adults. *npj Vaccines* 2021;6:147. <https://doi.org/10.1038/s41541-021-00412-6>.
- [22] Davenport FM, Hennessy AV, Francis T. Epidemiologic and immunologic significance of age distribution of antibody to antigenic variants of influenza virus. *J Exp Med* 1953;98:641–56. <https://doi.org/10.1084/jem.98.6.641>.
- [23] Francis T. On the doctrine of original antigenic sin. *Proc Am Philos Soc* 1960;104:572–8. <http://www.jstor.org/stable/985534>.
- [24] Xu R, McBride R, Nycholat CM, Paulson JC, Wilson IA. Structural characterization of the hemagglutinin receptor specificity from the 2009 H1N1 influenza pandemic. *J Virol* 2012;86:982–90. <https://doi.org/10.1128/JVI.06322-11>.