1 **Influenza virus infection history drives and shapes antibody responses to influenza vaccination**
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42 ## Abstract

43 The controversial hypothesis that recalled immunological memory limits responses to variant virus strains
44 has been revived by reports linking poor vaccine effectiveness against influenza A(H3N2) viruses with prior
45 vaccination. The impact of memory induced by prior infection is rarely considered and is difficult to ascertain
46 because infections are often subclinical. This study investigated influenza vaccination among adults from the
47 Ha Nam Cohort (Viet Nam), who were purposefully selected to include 72 with and 28 without documented
48 influenza A(H3N2) during the preceding 9-years (ANZCTR 12621000110886). The primary outcome was the
49 effect of influenza A(H3N2) infection on hemagglutination inhibiting antibody responses induced by locally
50 available influenza vaccine administered in Nov-2016. Baseline and post-vaccination sera were titrated
51 against 40 A(H3N2) strains spanning 1968-2018. At each time-point (baseline, d14, d280) geometric mean
52 antibody titres across 2008-2018 strains were higher among participants with recent infection: 34 (29-40),
53 187 (154-227), 86 (72-103) than among participants without recent infection: 19 (17-22), 91 (64-130), 38
54 (30-49). On d14 and d280, mean titre-rises across 2014-2018 strains were 6.1-fold (5.0-7.4) and 2.6-fold (2.2-
55 3.1) for participants with recent infection versus 4.8-fold (3.5-6.7) and 1.9-fold (1.5-2.3) for those without.
56 1/72 vaccinees with recent infection versus 4/28 without developed illness due to A(H3N2) infection in the
57 season after vaccination ($p = 0.021$). The range of A(H3N2) viruses recognized by vaccine-induced antibodies
58 was associated with the prior infection strain. These results suggest that immunological memory from prior
59 infection drives and shapes antibody responses to inactivated influenza vaccine and underpins the capacity
60 for vaccine to induce sufficient antibody for protection.
61

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69 RNA viruses undergo relatively rapid mutation, which can critically impact vaccination strategies[1]. Influenza
70 viruses are particularly prone to substitutions within the major surface protein, hemagglutinin (HA), as a
71 consequence of viral RNA replication without proofreading[2], and selection of human antibody escape
72 mutants. This process, termed antigenic drift, facilitates recurrent influenza infection throughout life. In turn,
73 prevention by vaccination requires repeated administration of vaccine containing regularly updated virus
74 strains. Vaccine effectiveness (VE) has been found to be poor against A(H3N2) viruses from 2010 onward,
75 when VE estimation by subtype became more widely implemented[3]. This could, in part, be due to greater
76 mismatch between vaccine and circulating strains. A(H3N2) viruses have undergone greater antigenic
77 evolution compared to A(H1N1) and B influenza viruses[4], and more often acquire substitutions within
78 antigenic sites when propagated in eggs to produce vaccine[4,5]. It is further speculated that vaccine
79 immunogenicity and effectiveness may be limited by recall of immunological memory against past strains, a
80 hypothesis that was first proposed in the 1960's and termed original antigenic sin[6]. Interest in this
81 phenomenon has been revived by a series of recent reports that antibody responses[7], and VE against
82 A(H3N2) viruses[8-11] are attenuated among people who received vaccine in prior year(s). A meta-analysis
83 indicates that while repeat vaccination effects are more pronounced for A(H3N2) than for other subtypes,
84 there is substantial heterogeneity in effects[12].

85 The cellular and molecular mechanisms underlying the variable effects of prior vaccination and pre-existing
86 immunity remain largely undefined. The antigenic distance hypothesis postulates that when successive
87 vaccine strains are antigenically similar, existing antibodies or memory B cells attenuate vaccine
88 immunogenicity by masking or clearing vaccine antigen, resulting in attenuated VE if the vaccine and
89 epidemic strains differ, but not if they are also similar[13]. Alternately, it is hypothesized that memory B cells
90 induced by prior vaccination dominate and focus responses on epitopes that are conserved between prior
91 and prevailing vaccine strains, compromising responses against epitopes that have changed[14]. This could
92 enhance antibody responses and VE, if epidemic strains retain those conserved epitopes, but could reduce
93 VE if these epitopes have changed[14]. The epitopes recognized by influenza virus neutralizing antibodies are
94 largely located on the globular head of HA, surrounding the receptor binding site[15]. Up to 131 amino acid

95 positions in the head of HA of A(H3N2) viruses have been associated with antigenic variation and assigned to
96 one of five antigenic sites, designated A to E[16,17]. Antigenic sites A and B are immunodominant[16], and single
97 amino acid substitutions in these sites can result in escape from vaccine-induced immunity, particularly if
98 glycosylation sites are introduced[4,18].

99 Few studies consider how prior influenza infections affect the immunogenicity and protection afforded by
100 influenza vaccines. Understanding infection history is contingent on detecting asymptomatic/subclinical
101 infection, which may account for up to three-quarters of influenza virus infections[19,20]. To this end, we
102 investigated vaccine immunogenicity among participants of a cohort in northern Viet Nam (Ha Nam
103 Cohort), who were influenza vaccine naïve, and who had been monitored for clinical or sub-clinical
104 influenza infection for 9-years.

105 Results

106 Study design

107 The primary study objective was to determine the effect of recent influenza A(H3N2) infection on vaccine-
108 induced antibody responses against A(H3N2) viruses. Therefore, adult participants of the Ha Nam Cohort
109 were purposefully selected based on influenza infection history during the preceding 9-years of Cohort
110 participation (ANZCTR 12621000110886) (Fig. 1a, Supplementary Fig. 1). The Ha Nam Cohort commenced in
111 December 2007 when 945 members of 270 households were enrolled to participate in monitoring for
112 influenza infection via active ILI surveillance, RT PCR on swabs, and serology on blood samples collected
113 annually or biannually to detect seroconversion. The vaccine study commenced Nov-2016. Participants were
114 considered for inclusion if they were aged ≥ 18-years and if they had completed all investigations to detect
115 influenza infections since Dec-2007. We aimed to recruit 50 with, and 50 without, documented A(H3N2)
116 infection, however, only 32/161 eligible participants lacked A(H3N2) infection during the preceding 9-years
117 (Supplementary Fig. 1). We therefore selected 82/129 participants with prior A(H3N2) infection, based on
118 proximity of age to the 32 participants without prior infection and sex (Supplementary Fig. 1, Supplementary
119 Table 1). 100/114 selected participants consented, including 28/32 without prior A(H3N2) infection and

120 72/82 with prior A(H3N2) infection. Age and sex distributions were similar between participants with and
121 without prior A(H3N2) infection (Fig. 1a), and 40/72 (55%) versus 14/28 (50%) had a prior A(H1N1) virus
122 infection.

123 Participants received licensed, locally available, influenza vaccine (Vaxigrip, Sanofi Pasteur), containing
124 inactivated, egg-grown A/Hong Kong/4801/2014 (H3N2), A/California/7/2009 (H1N1)pdm09, and
125 B/Brisbane/60/2008 viruses. Blood samples were collected pre- and post-vaccination (d4, d7, d14, d21,
126 d280), and post-infection, if detected in the subsequent season (Fig. 1a). Sera were tested by
127 Hemagglutination Inhibition (HI) assay to determine antibody titres against 40 A(H3N2) viruses that
128 circulated between 1968, when A(H3N2) viruses emerged in humans, and 2018, 4 years after the vaccine
129 strain (Fig 1b, Supplementary Table 2). The primary outcome was fold-rise in antibody titre, determined
130 from geometric mean ratios (GMRs) on d14 and d280 post-vaccination, comparing vaccinees with and
131 without A(H3N2) infection during the preceding 9-years.

132 Description of prior A(H3N2) infections and circulating strains

133 Prior A(H3N2) virus infection was detected as ILI, confirmed by RT PCR, for 16/72 participants, and as
134 seroconversion without ILI for 56/72. 51/72 had one recent prior A(H3N2) infection, 18/72 had two, and
135 3/72 had three prior infections (Supplementary Table 3). The year that participants were last infected with
136 an A(H3N2) virus ranged from 2008 to 2015. A(H3N2) viruses circulating during these years belonged to a
137 range of genetic clades, and were distinct from the vaccine strain - A/Hong Kong/4801/2014 (HK14e), which
138 belonged to clade-3c2a (Fig. 1b, Supplementary Table 3). Twenty-six antigenic site positions differed
139 between at least one prior strain and HK14e (Fig. 1c, d). Viruses circulating in 2014 (HN14/Sw13-like, clade-
140 3c3a) differed substantially from HK14e in sites A and B where 7 amino acids were substituted, 6 to amino
141 acids that had different properties or that affected a glycosylation site. Viruses circulating in 2012 (HN12-
142 like, clade-3c1) and earlier differed more from HK14e in site C where 5 positions were substituted to an
143 amino acid with different properties or affecting a glycosylation site (Fig. 1d).

144 ## Effect of recent A(H3N2) virus infection on antibody responses to the vaccine A(H3N2) strain

145 Analysis of the kinetics of antibody production against the HK14e vaccine strain (Fig. 2a) shows that
146 vaccination induced robust antibody production within 7 days. Titres were highest on d14 and remained at
147 least 4-fold higher than pre-vaccination titres on d280 for 54% of participants. Geometric Mean Titres
148 (GMTs) were higher among participants who had recent A(H3N2) virus infection at all time-points. GMRs for
149 participants with and without prior infection were 9.8-fold (7.3-13.1) versus 9.3-fold (5.8-14.8) on d14, and
150 4.0-fold (3.0-5.3) versus 2.5-fold (1.6-4.0) on d280. Proportions seropositive (titre ≥ 40) and seroconverted
151 (titre rise ≥ 4-fold) were also higher among participants with recent infection (Table 1). Titres and titre rises
152 were at least as high for older compared to younger adults, particularly for those with recent prior A(H3N2)
153 infection (Supplementary Fig. 2). Recent A(H3N2) virus infection had little effect on the proportion of
154 participants seropositive against A(H1N1)pdm09 in the vaccine (Table 1). These results indicate that recent
155 A(H3N2) virus infection enhanced the capacity of the vaccine to induce and maintain A(H3N2)-reactive, but
156 not A(H1N1)-reactive, antibodies. Therefore, effects of recent infection were likely to be mediated by
157 subtype-specific memory B cells, rather than by broadly cross-reactive B or T cells.

158 ## Cross-reactivity of vaccine-induced antibodies

159 The strain-coverage of antibodies induced by vaccination was examined, using generalized additive models
160 (GAMs) to estimate titre and titre rise landscapes for viruses arranged by circulation year. Pre-vaccination
161 antibody titres were relatively high against strains encountered early in life, as well as against 1993-2002
162 strains (Fig. 2b, Supplementary Fig. 3). Titre rise from d7 through d280 post-vaccination diminished as virus
163 genetic and temporal distance from HK14e increased and was negligible against the oldest strains (Fig. 2c, d,
164 Supplementary Fig. 4). This back-boosting could reflect low-avidity antibody binding to past strains when
165 antibody concentrations are high since titre rise extended across more strains on d14 after vaccination than
166 on d280 (Fig. 2d). However, back-boosting was largely limited to strains circulating after participant's birth
167 years (Supplementary Fig. 5). This is consistent with previous findings [21], and suggests that back-boosting
168 reflects recall of memory B cells induced by prior infections.

169 ## Effect of recent A(H3N2) infection on antibody responses across A(H3N2) strains

170 Antibody cross-reactivity was compared between participants with and without recent A(H3N2) infection
171 confirmed by seroconversion, RT PCR, or the combination. Pre-vaccination titres differed mainly against
172 strains circulating since 2008, corresponding with participant's infection status since Dec-2007 (Fig. 3a,
173 Supplementary Table 4, 5). Differences increased post-vaccination, most notably against strains circulating
174 after the vaccine strain, and among participants with RT PCR confirmed infection (Fig. 3b,c, Supplementary
175 Table 4). GMTs across 2008-18 strains on d14 and d280 were 187 (154-227) and 86 (72-103) for participants
176 with recent infection versus 91 (64-130) and 38 (30-49) for participants without recent infection. Titre rise
177 was similar against prior strains, but was higher against vaccine and subsequently circulating strains among
178 participants with recent A(H3N2) infection (Fig. 3d, Supplementary Table 6). GMRs averaged against vaccine
179 and subsequently circulating strains differed most on d280 when titres were on average 2.6-fold (2.2-3.1)
180 higher than baseline titres among participants with recent infection, compared to 1.9-fold (1.5-2.3) higher
181 among participants without recent infection (Fig. 3e). This forward boosting was accompanied by higher
182 rates of seroconversion against subsequent A/Kansas/14/2017 (Ka17) and A/Brisbane/60/2018 (Br18) strains
183 (Table 1). Effects of recent infection were observed across all ages (Supplementary Fig. 5), and whether
184 participants had 1 or 2-3 recent infections (Supplementary Fig. 6). However, titres and titre rises tended to
185 increase with proximity of prior infection (Supplementary Fig. 7). Importantly, participants with prior
186 infection had higher GMTs on d280 against viruses from clades that were causing infections in the cohort by
187 that time (Fig. 3b, Supplementary Table 4). We have shown previously that titres of 40 can be associated
188 with substantial protection in this cohort[22]. These results indicate that memory from recent infection
189 enhances the magnitude and breadth of A(H3N2)-reactive antibodies induced by vaccination.

190 ## Effect of the strain causing prior infection on the cross-reactivity of vaccine induced
191 ## antibodies

192 As described above, HN14-like (clade-3c3a) viruses that were detected in Cohort from 2013 to 2015 were
193 notably distinct within antigenic sites from viruses detected earlier (Fig. 1d), and may therefore have

194 induced memory B cells that cross-react with distinct epitopes of the HK14e vaccine strain. To investigate
195 this, participants with prior HN14-like virus infection were compared to those infected with HN09-like
196 viruses only, HN12-like viruses only, or with HN08-like then HN12-like viruses (Supplementary Table 7).
197 Antibody landscapes, modelled against a 2-dimensional map of virus antigenic distances, differed between
198 participants infected with HN14-like viruses versus earlier viruses (Fig 4). Most notably, d14 post-vaccination
199 landscapes were relatively skewed towards HN14 and other clade-3c3a viruses among the group with prior
200 HN14-like virus infection (Fig. 4b). Similar trends were observed for landscapes on d280 post-vaccination
201 (Supplementary Fig. 8), and for titre rise landscapes (Supplementary Fig. 9). These results suggest that
202 memory recall may drive antibody production towards epitopes that are shared between the vaccine strain
203 and prior infecting strains.

204 HN14-like viruses differed mainly from HK14e within antigenic site B (Fig. 1d). To investigate whether this
205 affects antibody production against site B of the HK14e vaccine, sera were titrated against reverse
206 engineered viruses bearing wild-type HK14e HA or HA containing a substitution in site B (Fig. 4c). Y159S was
207 chosen because substitutions at this position are known to have large antigenic effects[18] and because Sw13e
208 has an S at position 159 and is antigenically distinct from HK14e (Fig. 1d and Fig. 4a). Characterization using
209 ferret antisera indicated that the Y159S virus was antigenically distinct from HK14e, as well as from Sw13e
210 (clade-3c3a) (Supplementary Table 8). Antibody titres against wild-type versus Y159S virus were compared
211 using microneutralization (MN) and HI assays, which were strongly correlated (Fig. 4 d, e). MN titres were
212 higher against wild-type compared to Y159S virus regardless of the prior infecting strain (Fig. 4f, h), which
213 could in part be because the infectious dose of virus in the assay was marginally higher for the Y159S virus
214 (Supplementary Fig. 10). Nevertheless, differences between wild-type and Y159S virus titres were greater
215 among participants with prior HN09 and/or HN12-like virus infection than among those with prior HN14-like
216 virus infection (Fig. 4f-i, Supplementary Fig. 11). HI titres at baseline were on average 1.6-fold higher against
217 Y159S virus among participants with prior HN09/HN12-like virus infection (Fig. 4g). This ratio increased post-
218 vaccination, to around 3-fold on d14 (Fig. 4i), and 2.6-fold on d280 (Supplementary Fig. 12), indicating that
219 vaccination induced antibodies against site B of HK14e among participants with prior HN09 and/or HN12

220 infection. In contrast, post-vaccination HI titres were equivalent against wild-type and Y159S virus among
221 participants with prior HN14-like virus infection (Fig 4h, i), indicating that less of the antibody induced was
222 directed against immuno-dominant site B. Therefore, antibodies may have been induced against sub-
223 dominant sites, such as site C. In turn, antibodies targeting sub-dominant may be expected to have broader
224 cross-reactivity since these sites are better conserved across past and future strains (Supplementary Table
225 9).

226 Influenza virus infection during the post-vaccination season was associated with prior
227 infection status and post-vaccination antibody titre

228 Five ILI cases with RT PCR confirmed A(H3N2) virus infection were detected 275-340 days after vaccination,
229 coinciding with case detection in the Ha Nam Cohort as a whole. Infecting strains belonged to clades 3c2a2
230 and 3c2a1b (Fig. 1c). Four cases were from the group that lacked recent A(H3N2) infection (4/28, 14%),
231 whereas only one was from the group with recent infection (1/72, 1.4%, odds ratio 0.084, 95% CI 0.009 -
232 0.793, $p = 0.021$). In contrast, A(H3N2) ILI cases accounted for similar proportions of vaccinees with recent
233 A(H1N1) virus infection (3/54, 5.6%) and without recent A(H1N1) virus infection (2/37, 5.4%). Post-
234 vaccination antibody titres were relatively low among vaccinees who developed A(H3N2) ILI (Fig. 5a versus
235 b), and titre rises were transient, and did not increase from day 7 to day 21 (Fig. 5c versus d, Supplementary
236 Fig. 13). Titre rise was greater after infection than after vaccination in these participants, and increased
237 further from day 7 to day 21 (Fig. 5b, d), indicating that infection was more immunogenic than vaccination.
238 One participant was infected with an A/Switzerland/8060/2017 (Sw17)-like (clade-3c2a2) virus despite prior
239 RT PCR-confirmed infection, and relatively high post-vaccination titres against the vaccine strain (Fig. 5e).
240 However, titres against Sw17 were low and were not boosted by vaccination. Eleven vaccinees lived in a
241 household with a participant who developed A(H3N2) ILI in the season after vaccination. Notably, four of six
242 who were not infected had recent prior infection (Supplementary Table 10). Taken together, these results
243 suggest that adults who were infected with an A(H3N2) virus up to 9-years prior to vaccination were better

244 protected against antigenically drifted A(H3N2) viruses, and that protection was mediated by subtype-
245 specific rather than cross-reactive immune responses.

246 Discussion

247 In the current study adults who had undergone active investigation to detect influenza virus infections since
248 Dec-2007 received inactivated influenza vaccine in Nov-2016, and antibody titres were assessed against
249 A(H3N2) viruses spanning 1968-2018. Antibody titres against older strains were associated with year of birth
250 whereas titres against post-2007 strains were associated with recent A(H3N2) infection status. However,
251 titres were also relatively high against 1993-2002 strains. This deviates from antigenic sin and seniority
252 hypotheses, which suggest that strains encountered earlier in life are higher in the antibody hierarchy
253 because later infections back-boost antibodies against earlier strains and/or because immune responses to
254 earlier strains mitigate responses to later strains[6,23]. Antibody titres against the vaccine A(H3N2) strain, as
255 well as recent past strains, rose substantially within 7 days of vaccination, indicating that memory B cells
256 were recalled. In contrast, young children produce negligible antibody within 7 days of their earliest
257 influenza infections [24], and the antibody induced mostly targets the HA of the strain that caused infection [25].
258 Participants with an A(H3N2) virus infection during 9-years prior to vaccination had higher antibody titres,
259 and better-maintained titre rise against the vaccine virus and future circulating viruses. Similarly, A(H3N2) ILI
260 was predominantly detected among vaccinees who lacked prior A(H3N2) virus infection indicating that both
261 vaccine immunogenicity and effectiveness are enhanced by immunological memory associated with prior
262 infection.

263 The boosting effect of prior infection, observed here, contrast with reports of negative effects of prior or
264 repeated vaccination[7-11], suggesting that the type of prior exposure is highly relevant. It was also notable
265 that vaccine responses were at least as good among older compared to younger adults, contrasting with
266 studies in more highly vaccinated populations[26,27]. The recommended annual interval between influenza
267 vaccinations is typically shorter than between influenza infections. However, titres and titre-rises following
268 vaccination tended to increase with proximity of prior infection indicating that timing does not directly

269 account for the different effect of prior infection. Several groups have demonstrated that neutralizing
270 antibodies can become focused on limited virus epitopes that have remained conserved across successively
271 encountered strains[28,29]. It is hypothesized that recalled memory B cells dominate and focus responses on
272 epitopes that are well conserved in successively encountered strains, which could either enhance or
273 compromise protection depending upon whether these targeted epitopes undergo mutation in subsequent
274 strains[13,14]. In the current study, the strain-coverage of antibodies, and capacity to generate antibodies
275 against a prominent site B epitope were shaped by the prior infecting strain, consistent with memory B cell
276 dominance. These findings present a paradox whereby memory B cell recall is pivotal for inactivated egg-
277 based influenza vaccine to elicit sufficient antibody for protection, but may also be problematic in terms of
278 the capacity for vaccination to update immunity by generating memory B cells and antibodies against
279 epitopes that have mutated in a new vaccine strain. To generate antibodies and memory B cells against
280 variant epitopes, influenza vaccines must either induce memory B cells to undergo further affinity
281 maturation[30] or induce naïve B cell differentiation. Memory B cells may have a competitive advantage
282 because they have undergone affinity maturation, so may compete more successfully for antigen in order to
283 engage T cell help for further differentiation, and are additionally less reliant than naïve B cells on T cell help
284 for activation[31,32]. Inactivated influenza vaccines deliver antigen transiently, and induce minimal innate co-
285 stimulation, hence may have little capacity to activate naïve B cells and generate new B cell clones and
286 antibodies in the presence of vaccine-reactive memory B cells.

287 Infection induced higher antibody titres against a broader antigenic range of A(H3N2) viruses than
288 vaccination among individuals who developed A(H3N2) ILI in the season after vaccination. This indicates that
289 infection may have greater potential to expand the antibody repertoire than vaccination. In turn, as the
290 epitope range of the memory B cell pool increases, the potential to recognize epitopes in a new vaccine
291 strain will also increase, providing a mechanism for the differential effects of prior infection and vaccination.
292 Similarly, in ferrets and mice, priming with inactivated influenza vaccine induces little to no antibody, and no
293 protection against variant virus strains, whereas priming by infection induces more antibody and substantial
294 protection against variant strains[33,34]. These differences in antibody responses may reflect a greater capacity

295 for influenza virus infection, as opposed to vaccination, to activate both the innate and adaptive immune
296 systems[35], and in turn activate naïve B cells. Additionally, antigen may be retained for longer periods after
297 infection than vaccination, and may be available to engage naïve B cells after the memory B cell response
298 starts to contract[36].

299 The study has several limitations. The objective, to investigate the effect of prior A(H3N2) infection on
300 vaccine immunogenicity against A(H3N2) viruses, required an observational design, and sample size was
301 constrained by the rarity of people who lacked A(H3N2) infection over 9 years. Therefore, inferences are
302 suggestive rather than conclusive. It would be possible to perform larger studies if looking at effects of
303 infection in the prior season only, however the results presented here indicate that it is important to
304 consider infections over a number of years. We used seroconversion, in addition to ILI surveillance to
305 determine participant's prior infection status. While this should limit misclassification, it was also clear that
306 titres and titre rises were higher among participants with infection confirmed by RT PCR than by
307 seroconversion. This could reflect the potential for serologically confirmed infections to be false positive or
308 negative [37]. However, recent studies indicate that antibody titres can increase with severity of influenza
309 infection [38].

310 Taken together, the results of this study indicate that prior A(H3N2) virus infection may increase the titre
311 and breadth of antibody responses induced by a new A(H3N2) vaccine strain, and thereby enhance
312 protection despite antigenic drift. However, the range of strains against which antibodies are induced may
313 be dictated by the prior infecting strain, consistent with a memory-dominated response. Such memory
314 dominance may need to be overcome in future vaccine strategies to increase protection against drifted
315 A(H3N2) viruses.

316 # Online Methods

317 ## Study Design and Participants

318 Participants were purposefully selected from a household-based influenza cohort established in Thanh Ha
319 Commune, Thanh Liem District, Ha Nam Province, northern Viet Nam (Supplementary. Fig 1). In brief, the Ha
320 Nam Cohort enrolled 270 households in Dec-2007. Households members were asked to participate in active
321 ILI surveillance, to provide swabs if they developed ILI, and to provide blood samples annually or biannually,
322 at times spanning influenza transmission peaks[19]. Swabs were assessed by RT PCR to detect influenza virus
323 RNA, and sera were assessed to determine hemagglutination inhibition (HI) antibody titres against
324 circulating strains. Infection was defined as detection of RT PCR-confirmed ILI or a four-fold or greater rise in
325 antibody titre (seroconversion).

326 The primary objective was to determine whether vaccine induced antibody titre rises against A(H3N2)
327 viruses differ between participants with and without documented prior A(H3N2) infection. Sample size was
328 based on the assumption that geometric mean ratios (GMRs) of post- to pre-vaccination antibody titres
329 would differ by 0.7 with standard deviation of 1.0 giving an effect size of 0.7. It was estimated that 33 per
330 group would provide 80% power to detect this effect with 95% confidence. This was inflated to 50 per group
331 to account for loss to follow-up, and to facilitate comparison of people infected with A(H3N2) in different
332 years since 2007.

333 Inclusion criteria were age ≥ 18 years, and continued participation in cohort investigations in order to
334 ascertain prior infection status. Participants with a history of allergic reactions were excluded. 371 of 556
335 adults registered interest in participating in a vaccine study at the time of re-consent for the Ha Nam Cohort
336 in July 2016 (Supplementary Fig. 1). 161 had participated continually in ILI surveillance and in all blood
337 sample collections, including 32 with and 129 without A(H3N2) infection since Dec-2007 (Supplementary Fig.
338 1). For each of the 32 participants without recent A(H3N2) infection, two to three participants with prior
339 infection were selected based on proximity of their ages and sex to obtain a similar ratio of males to females
340 (Supplementary Table 1). 100/114 selected participants consented to the vaccine study between October 1

341 and November 6, 2016. This included 28/32 without recent infection and 72/82 with recent A(H3N2)
342 infection. Ages and proportions female were similar among non-selected, selected and consenting
343 participants (Supplementary Fig. 1). Selected participants were from 79 of 210 household remaining in the
344 Cohort, with three households contributing three participants each, and 16 households contributing two
345 participants each.

346 Participants received licensed, locally available Trivalent Inactivated influenza Vaccine (TIV; Vaxigrip, Sanofi
347 Pasteur) in November 2016. Blood samples were collected before and 4, 7, 14, 21, and 280 days after
348 vaccination. Blood samples were also collected 7 and 21 days after confirmed influenza illness occurring in
349 the season after vaccination.

350 Study protocols were approved by ethics committees of the University of Melbourne (1646470), the
351 National Institute of Hygiene and Epidemiology in Viet Nam (IRB-VN01057 – 08/2016), and the Oxford
352 Tropical Medicine Research Unit (30-16). All participants provided informed consent, conducted in
353 Vietnamese. The study was not prospectively registered as a clinical trial because participants were not
354 assigned to intervention versus control groups. However, study protocols were retrospectively included on
355 the Australian New Zealand Clinical Trials Registry (ACTRN #12621000110886).

356 Virus propagation and characterization

357 Viruses were propagated in mammalian cell lines and/or in 10 to 12 day old embryonated chicken eggs
358 (Supplementary Table 2). Madin Darby Canine Kidney (MDCK) cells and MDCK cells transfected with 2,6-
359 sialtransferase (SIAT) were grown DMEM (Gibco) containing penicillin/streptomycin and 10% fetal bovine
360 serum (Bovagen). A number of viruses acquired neuraminidase (NA) substitutions, which that have been
361 associated with erythrocyte agglutination via NA [39], when propagated in MDCK-cells (Supplementary Table
362 2). HA titres of most of these viruses decreased when Oseltamivir was added to inhibit NA, but HI titres did
363 not uniformly increase in the presence of Oseltamivir. Therefore, viruses were plaque-selected on SIAT cells
364 to produce stocks that lacked NA T148X or D151X substitutions, and that were more sensitive for detecting
365 HI antibodies (Supplementary Table 11, Supplementary Fig. 14).

366 Reverse genetics (RG) viruses were produced using the eight plasmid system based on A/Puerto Rico/8/1934
367 (PR8) [40]. The Y159S substitution was introduced into the HA of HK14e using primers: forward-
368 CTTAAACAGCAAATACCCAGCATTGAACGTGACT and reverse-TATTTGCTGTTTAAGTGGGTCAACCAATTT. Wild-
369 type and Y159S HA were cloned into the vector PHW2000 [40]. 7:1 reassortant viruses were generated using
370 plasmids encoding PR8 internal and NA genes, and HA of HK14e or HK14e-Y159S. Plasmids were transfected
371 into co-cultured 293T/SIAT cells, then recovered viruses were propagated in eggs. RG viruses were assessed
372 by HI assay using antisera raised against HK14e and Sw13e, and a human mAb (Q129C) that recognizes site B
373 of A/Victoria/361/2011 (generously provided by Alain Townsend, MRC Weatherall Institute of Molecular
374 Medicine, UK).

375 HA and NA genes of viruses used for serology and/or from swabs of Ha Nam Cohort participants (isolates or
376 clinical specimens) were sequenced via Sanger sequencing and aligned using the Multiple Alignment using
377 Fast Fourier Transform (MAFFT) algorithm in MegAlign Pro 13 (DNASTAR Lasergene 13). Phylogenetic trees
378 were edited in FigTree Version 1.4.4 (2006-2018, Andrew Rambaut, Institute of Evolutionary Biology,
379 University of Edinburgh. http://tree.bio.ed.ac.uk/). HA antigenic site positions (Fig. 1c), defined by Lee et al[17],
380 that varied between HK14e and at least one recent prior strain were tabulated to determine whether
381 antigenic variation from HK14e was clustered within particular sites, and if this varied between prior
382 infecting strains (Fig. 1d).

383 Viruses circulating since 2007 were antigenically characterized by HI assay using ferret antisera generated for
384 routine virus characterization by the WHO Collaborating Center for Reference and Research on Influenza,
385 Melbourne (Supplementary Table 12). A two-dimensional map of virus antigenic distances was generated
386 from the matrix of two-way titres of each sera against each virus using antigenic cartography software
387 (Racmacs, https://acorg.github.io/Racmacs/).

388 Serological assays

389 Sera were assessed by HI assay to determine antibody titres against influenza viruses. Assays were
390 performed according to WHO Global Influenza Surveillance Network protocols [41] with the exception that
391 volumes were reduced to 25 µL each of diluted sera, virus and 1% erythrocytes (0.33% final). Guinea Pig

392 erythrocytes were used for titration of antibodies against all A(H3N2) viruses, based on initial comparisons of
393 titres obtained using Guinea Pig versus Turkey erythrocytes (Supplementary Fig. 15). Sera were treated with
394 receptor destroying enzyme (RDE, Denka Sieken), adsorbed with 5% erythrocytes, then tested over two-fold
395 serial dilutions from 1:10 through 1:10240. Each individual's complete set of sera were tested against all
396 viruses using the same batch of erythrocytes. Quality control viruses and sera were run with each new batch
397 of samples/erythrocytes and were accepted if HA and HI titres were within 2-fold of initial values. HI titres
398 were read using an automated reader (CypherOne, InDevR). Instrument settings for plate reading were
399 determined by comparison with manual titre reads (Supplementary Fig. 16), then applied to all plates.
400 Antibody titres against reverse engineered viruses were validated by Microneutralization (MN) assay,
401 conducted according to WHO protocols [41] using SIAT cells, and plasma treated as per the HI assay protocol
402 above.

403 ## Outcomes

404 The primary outcome was vaccine immunogenicity, comparing geometric mean ratios (GMRs) of antibody
405 titres among participants with and without recent A(H3N2) virus infection. Geometric mean titres (GMTs),
406 and proportions seropositive (defined as a titre of 40 or more) or seroconverting (defined as a four-fold or
407 greater titre rise) were also compared. The strain-coverage of antibodies induced by vaccination was further
408 compared by fitting antibody titre landscapes across all A(H3N2) viruses tested[21]. Titres were determined at
409 a range of time points, but comparison focused on day 14 post-vaccination, when titre peaks were detected,
410 and on day 280, when titre decay plateaus [42].
411 Post hoc comparisons of participants who had been infected with viruses from distinct genetic clades, and of
412 participants who did or did not develop A(H3N2) ILI in the season after vaccination were also performed.

413 ## Statistical Analysis

414 GMTs and GMRs were estimated from $\log_2$ HI titres and from differences of $\log_2$ titres at post minus pre-
415 vaccination time-points. GMTs and GMRs were calculated for individual viruses (n=40) and for groups of
416 viruses representing prior exposure and post-vaccination periods, which were averaged for each person. To

estimate the size of the effect of recent infection on GMTs and GMRs, a mixed effects linear regression model was used, which included a random effects term to account for within-person correlations of antibody titres over time, and an interaction term for time of serum collection by recent infection status. Fisher's exact test was used to compare proportions with and without prior infection who seroconverted on day 14; maintained a 4-fold titre rise on day 280; or who became infected post-vaccination.

To construct and compare antibody landscapes, generalized additive models (GAMs) and lowess models were used to fit $\log_2$ titres against A(H3N2) viruses organized antigenically[21]. We used the GAM function from the R package mgcv, and accounted for repeated measurements on each individual through specification of a random effect[43]. Plots were generated with ggplot2[44]. The lowess model has been published online (https://github.com/acorg/ablandscapes).

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Author Contributions

MA assisted with virus propagation, performed serology, assisted with data analysis and co-drafted the manuscript.

HVMP co-management of the Ha Nam Cohort including sample collection and processing and diagnostic testing over the course of the vaccination study, and critically reviewed the manuscript.

LC assisted with virus propagation, and serology, sequenced virus HA and NA genes and plaqued viruses, and critically reviewed the manuscript.

LTQM co-conceived and co-designed the study, co-managed the Ha Nam Cohort sample collection and processing and diagnostic testing over the course of 9-years of cohort investigation and over the vaccination study, and critically reviewed the manuscript.

RT performed components of the data analysis and critically reviewed the manuscript.

SW performed components of the data analysis and critically reviewed the manuscript.

PQT co-designed the study, co-managed Ha Nam Cohort field work and data collection over the course of 9-years of cohort investigation and over the vaccination study, and critically reviewed the manuscript.

DP assisted with data analysis and critically reviewed the manuscript.
NTD assisted with study design, managed all activities of the health care workers to collect samples and data, managed communication with participants, and critically reviewed the manuscript.
NLKH, LTT, NTHT, TTKH, NTND, and VTNB processed samples, performed influenza diagnostic testing and virus isolation over 9-years of cohort investigation between 2007 and 2016, and also over the course of vaccination and subsequent follow-up, assisted with data cleaning, and critically reviewed the manuscript.
AK assisted with data analysis and critically reviewed the manuscript.
LH assisted with virus propagation and critically reviewed the manuscript.
TND and DDA co-management of the Ha Nam Cohort over the course of 9-years of cohort investigation and over the vaccination study, and critically reviewed the manuscript.
KK contributed to data interpretation and critical review of the manuscript.
SDB, KG-J, DS, IB, HW co-designed the study and critically reviewed the manuscript.
SS assisted with data analysis and critically reviewed the manuscript.
HRvD co-conceived and co-designed the study, co-managed the Ha Nam Cohort over the course of the vaccination study, and critically reviewed the manuscript.
AF conceived the study, co-managed the Ha Nam Cohort sample collection and processing and diagnostic testing over the course of 9-years of cohort investigation and over the vaccination study, assisted with sample processing, virus propagation, and serology, managed data and data analysis, and co-drafted the manuscript.

Declaration of interests

HRvD was funded by Sanofi (travel and consultancy fee) to present at, and attend a meeting about the potential role of influenza vaccination in AMR in 2019.

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589

590 **Fig. 1: Participant selection and investigation of previously circulating A(H3N2) viruses. a,** Study design and
591 timeline. **b**, Phylogenetic tree of the HA genes of viruses recovered from Ha Nam Cohort ILI cases (coloured
592 by season), and viruses used to construct antibody landscapes (coloured black if cell grown or red if egg
593 grown). Viruses from participants of the vaccine study are indicated by the suffix "Vax". Clades (cl.) and sub-
594 clades are delineated using parentheses. **c**, Model of the globular head of HK14e HA (SWISS-MODEL:
595 A0A0K0YAS1), showing amino acid positions within antigenic sites A to E that differed from at least one of
596 the prior infecting strains, and receptor binding site (RBS) residues. **d**, Antigenic site positions that varied
597 between HK14e and at least one prior infecting strain are tabulated and shaded according to amino acid
598 properties. Substitutions that result in gain (+) or loss (-) of glycosylation are coloured in pink. Egg adapted
599 substitutions are indicated by a superscript ^{e}.

600 **Fig. 2: Kinetic and strain coverage of the A(H3N2) virus-reactive antibody response to vaccination. a**, Titres
601 against the HK14e vaccine strain are shown for each participant (n=100) by time-point and prior A(H3N2)
602 infection status. Symbols indicate whether titres were at least 4-fold higher than pre-vaccine titres
603 (Seroconverted, legend). Bars and error bars show geometric means and 95% confidence intervals. **b**, Pre-
604 vaccination titre landscapes across strains spanning 1968 to 2018 were estimated using GAMs. Participants
605 are grouped by year of birth (YoB) with dashed and colour-matched vertical lines representing the earliest
606 strain that participants could have been exposed to. Shading indicates 95% confidence intervals (CI) for the
607 model, and dots show individual participant titres against antigens representing each year. **c**, Antibody titre
608 landscapes for pre-vaccination (grey-shaded area) and post-vaccination (coloured lines) time-points are
609 compared for all vaccinees. **d**, Fitted landscapes of post-vaccination titre rise are shown for all vaccinees.
610 Dotted horizontal lines indicate thresholds for seropositivity or seroconversion. Dotted vertical lines in **b**-**d**
611 indicate the position of the vaccine antigen.

612 **Fig. 3: Recent A(H3N2) virus infection enhances the titre and strain-coverage of A(H3N2)-reactive**
613 **antibodies induced by vaccination.** Titres and titre ratios are compared for vaccinees without recent
614 A(H3N2) infection or with infection confirmed by seroconversion or RT PCR, coloured as per legends. **a**, Pre-
615 vaccination titres presented as GAM estimates against year of A(H3N2) virus circulation or as GMTs for

individual viruses. **b**, GMTs on d14 and d280 post-vaccination. **c**, GMTs averaged against 2008-2018 strains by time-point. **d**, Post-vaccination titres ratios presented as in A. **e**, GMRs averaged across 2014-2018 viruses by time-point. 95% confidence intervals are indicated by shading or error bars. Horizontal lines indicate thresholds for seropositivity or seroconversion. Dotted vertical lines or red panels indicate the position of the vaccine antigen. GMT or GMR values are indicated for select groups in **c** and **e**.

Fig. 4: The strain-coverage of antibodies induced by vaccination is influenced by the A(H3N2) strain that caused prior infection. **a, b** GAMs were used to fit titres against a two-dimensional map of virus antigenic distances for participants grouped by prior A(H3N2) infection status. Estimated titres are represented as contours. Viruses are coloured by (sub)clade, and circles are filled if sera were titrated against that virus. Abbreviated virus designations are shown in the top left panel, and only for clades that caused prior infection in the other panels. **c**, Sera were titrated against reverse engineered viruses bearing wild-type HK14e HA or HA containing a Y159S substitution in site B, which was antigenically distinct based on titres of HK14e antisera and a site B directed mAb (Q12C9). **d**, representative microneutralization (MN) assay data, showing d14 post-vaccination sera titrated against wild-type versus Y159S virus, and comparing participants with prior HN12 and HN14 infection (graph legend). **e**, correlation of MN versus HI antibody titres of pre and d14 sera from 27 vaccinees. **f-i**, MN and HI titres, and ratios of titres against Y159S versus wild-type virus. Results for each individual (symbols) are presented with geometric means and 95% confidence intervals. Paired t-test values are shown for within group comparisons across viruses, and non-paired t-test values are shown for across group comparisons within viruses.

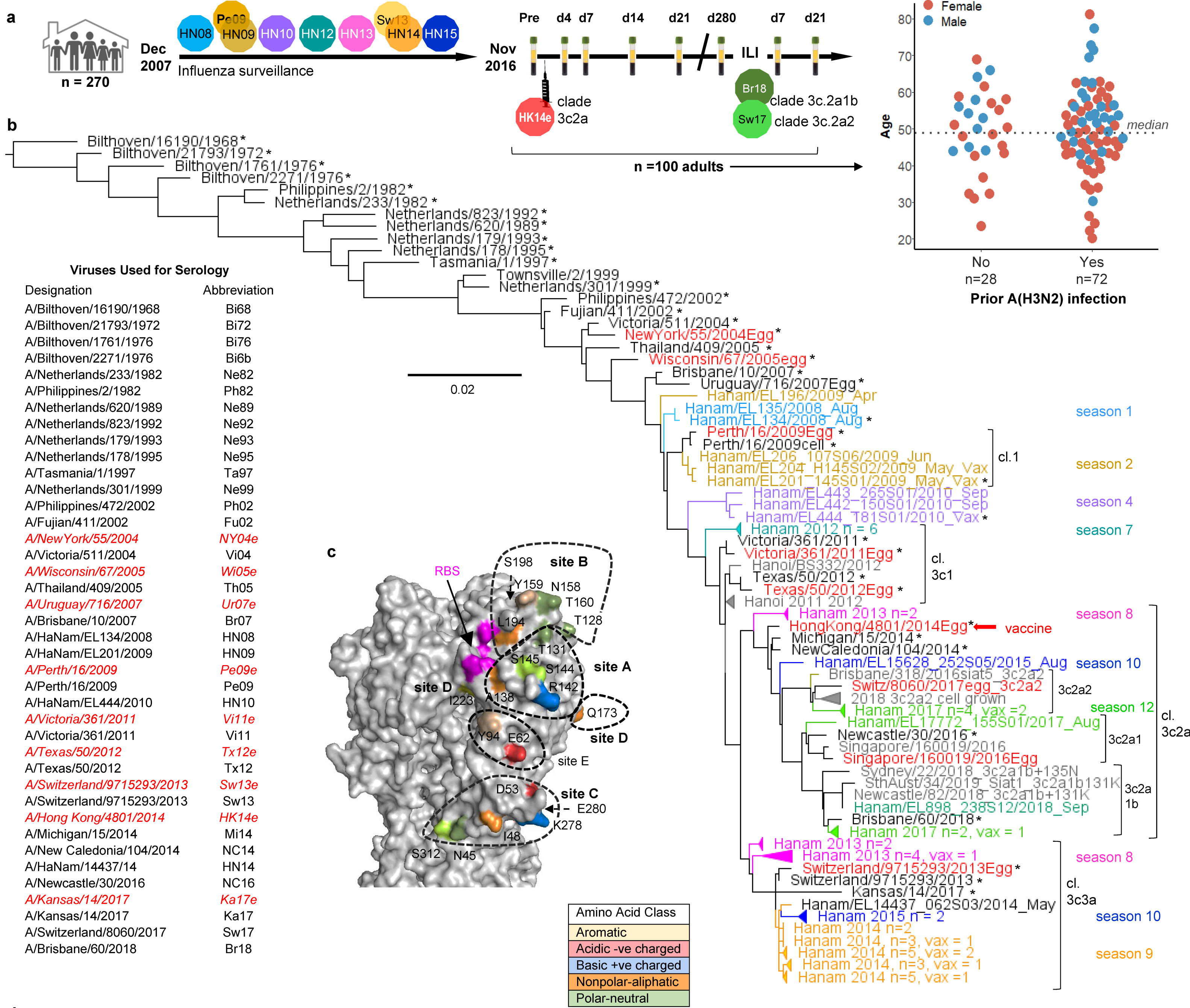
Fig. 5: Antibody titre landscapes associated with infection in the season after vaccination. **a-d**, GAMs were used to fit titres and titre ratios across A(H3N2) viruses arranged by circulation year. 95 vaccinees who did not develop ILI are compared with 5 who developed A(H3N2) ILI. Shading indicates 95% confidence intervals. **e, f**, GMTs for vaccinees who developed A(H3N2) ILI and who lacked A(H3N2) infection during 9 years prior to vaccination (n = 4) are compared to titres of one vaccinee who had prior infection. Error bars indicate 95% confidence intervals. Dotted horizontal lines indicate thresholds for seropositivity or seroconversion. Dotted vertical lines or red panels indicate the position of the vaccine antigen.

642

643 **Table 1.** Proportions of participants with and without recent A(H3N2) virus infection who were seropositive or seroconverted against vaccine and
644 subsequently circulating strains

Prior H3N2	Test antigen clade	Seropositive (HI ≥ 40)										Seroconvert			
		Pre		d7		d14		d21		d280 [a]		d14		d280 [a]	
		n (%)	p	n (%)	p	n (%)	p	n (%)	p	n (%)	p	n (%)	p	n (%)	p
No	HK14e 3c2a	8 (29)	0.000	25 (89)	0.065	26 (93)	0.076	23 (82)	0.006	21 (78)	0.026	18 (64)	0.058	12 (44)	0.180
Yes		51 (71)		71 (99)		72 (100)		71 (99)		66 (94)		60 (83)		42 (60)	
No	Mi14 3c2a	1 (4)	0.001	16 (57)	0.001	20 (71)	0.000	19 (68)	0.000	17 (63)	0.114	20 (71)	0.064	12 (44)	0.656
Yes		25 (35)		64 (89)		71 (99)		69 (96)		56 (80)		64 (89)		36 (51)	
No	NC16 3c2a1	3 (11)	0.002	17 (61)	0.003	21 (75)	0.000	19 (68)	0.000	15 (56)	0.008	17 (61)	0.140	11 (41)	0.652
Yes		31 (43)		65 (90)		72 (100)		69 (96)		58 (83)		55 (76)		33 (47)	
No	Br18 3c2a1b	5 (18)	0.020	20 (71)	0.003	25 (89)	0.065	25 (89)	0.683	15 (56)	0.008	13 (46)	0.066	7 (26)	0.805
Yes		32 (44)		68 (94)		71 (99)		67 (93)		58 (83)		49 (68)		22 (31)	
No	Ka17 3c3a	0 (0)	0.017	9 (32)	0.014	11 (39)	0.000	9 (32)	0.000	3 (11)	0.004	12 (43)	0.005	3 (11)	0.024
Yes		13 (18)		44 (61)		58 (81)		53 (74)		30 (43)		53 (74)		24 (34)	
No	Sw17 3c2a2	0 (0)	1.000	1 (4)	0.035	4 (14)	0.082	2 (7)	0.019	1 (4)	0.170	6 (21)	0.235	2 (7)	0.722
Yes		1 (1)		16 (22)		24 (33)		21 (29)		11 (16)		25 (35)		9 (13)	
No	*H1N1pdm09*	*5 (18)*	*1.000*					*23 (82)*	*0.527*			*23 (82)*	*0.756*		
Yes		*14 (19)*						*63 (88)*				*62 (86)*			

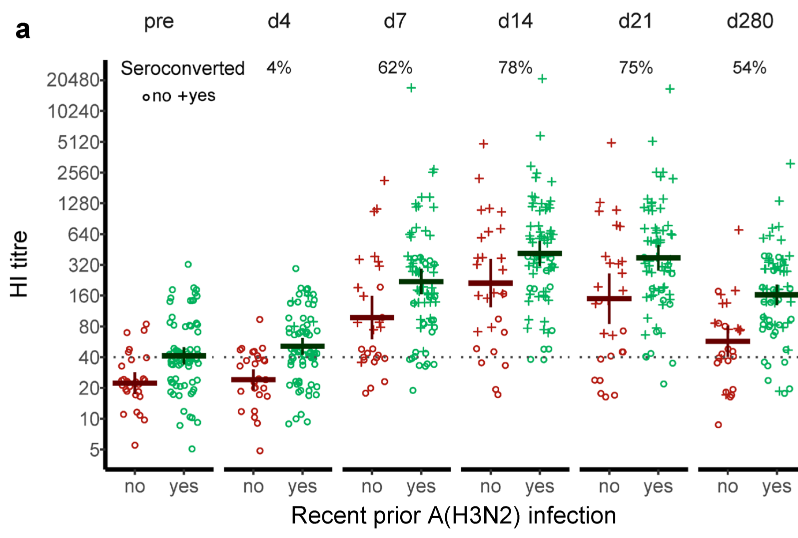
645 a = 27/28 participants without prior H3N2 and 70/72 participants with prior H3N2 provided samples on d280
646 p = Fishers Exact test

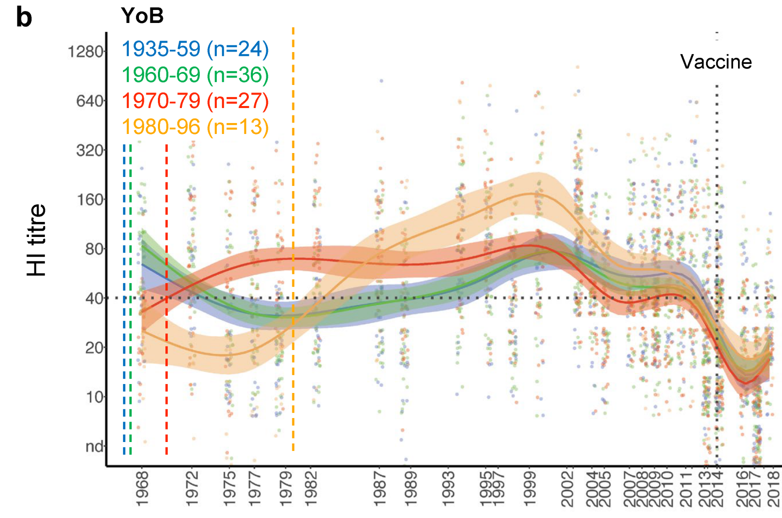


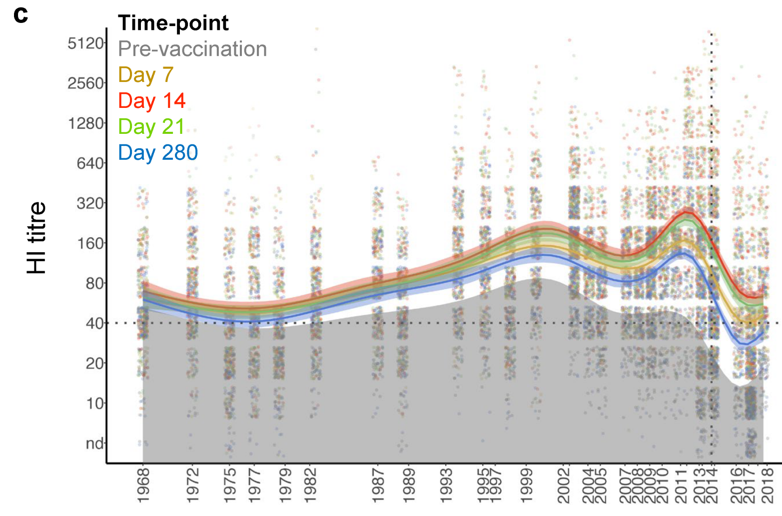
d

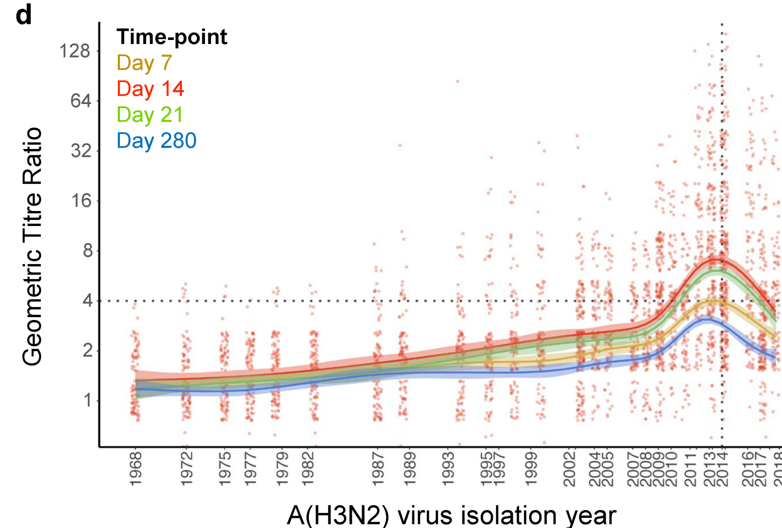
Antigenic Site and Amino Acid (aa) Position (H3 numbering)

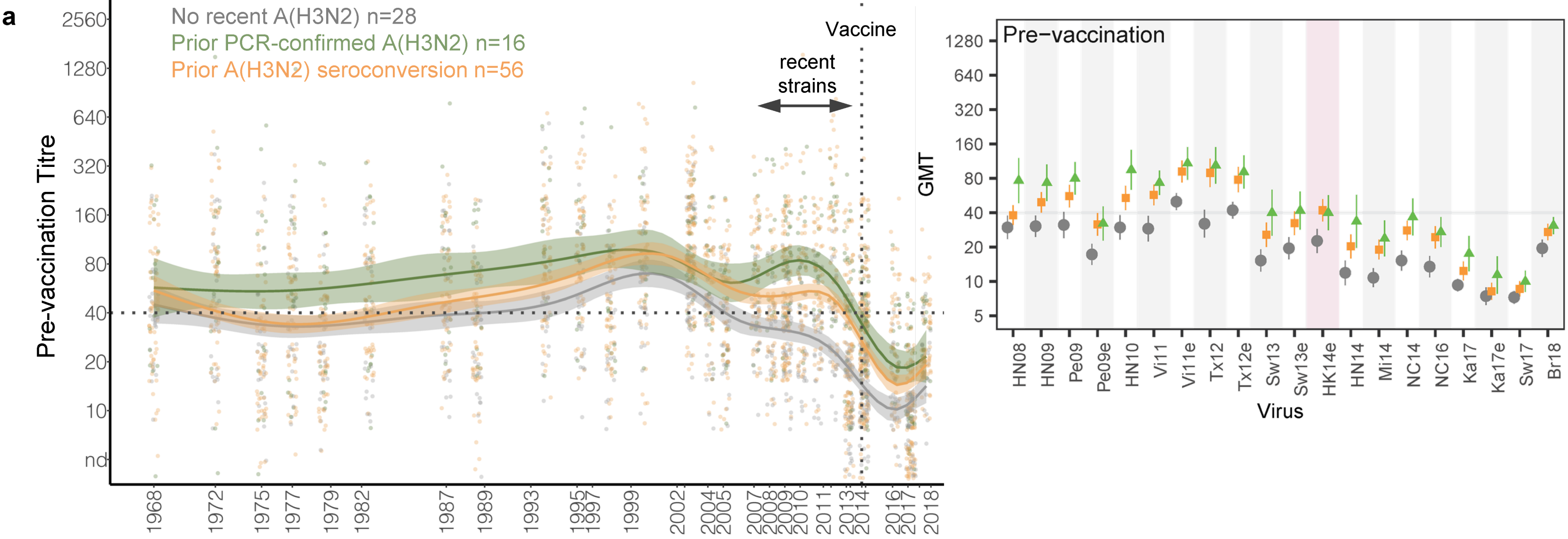
		Site E		Site A					Site B						Site C							Site D					
Virus season	clade	62	94	131	138	142	144	145	128	158	159	160	194	198	45	48	53	278	280	311	312	96	173	212	214	223	230
HK14e vaccine	**3c2a**	**E**	**Y**	**T**	**A**	**R**	**S** -	**S**	**T** +	**N**	**Y**	**K** -	**P** e	**S**	**N** +	**I**	**D**	**K**	**E**	**H**	**S**	**S** e	**Q**	**A**	**I**	**I**	**I**
HK14	3c2a	E	Y	T	A	R	S -	S	T +	N	Y	T +	L	S	N +	I	D	K	E	Q	S	N	Q	A	I	I	I
HN14/Sw13 s9	3c3a	E	Y	T	S	G	N +	S	A -	N	S	K -	L	S	N +	I	D	K	E	Q	S	N	Q	A	I	I	I
HN13 s8		E	Y	T	A	G	N +	S	A -	N	F	K -	L	S	N +	I	D	K	E	Q	S	N	Q	A	I	I	I
Vic11	3c1	E	Y	T	A	R	N +	N	T +	N	F	K -	L	S	N +	I	D	N	E	Q	S	N	Q	A	I	I	I
HN12 s7	3c1	E	Y	K	A	R	N +	S	T +	N	F	K -	L	S	S -	T	N	N	E	Q	S	N	Q	A	I	I	I
HN10 s4		E	H	T	A	R	N +	N	T +	N	F	K -	L	A	S -	T	N	N	A	Q	N	N	Q	A	I	V	V
HN09 s2	1	K	H	T	A	R	K -	N	T +	N	F	K -	L	A	S -	T	D	N	E	Q	N	N	Q	T	I	V	I
Pe09	1	K	Y	T	A	R	K -	N	T +	N	F	K -	L	A	S -	T	D	N	E	Q	N	N	Q	T	S	V	I
HN08 s1		E	Y	T	A	R	N +	N	T +	K	F	K -	L	A	S -	T	D	N	E	Q	N	N	Q	T	I	V	I
Br07		E	Y	T	A	R	N +	N	T +	K	F	K -	L	A	S -	T	D	N	E	Q	N	N	K	T	I	V	I

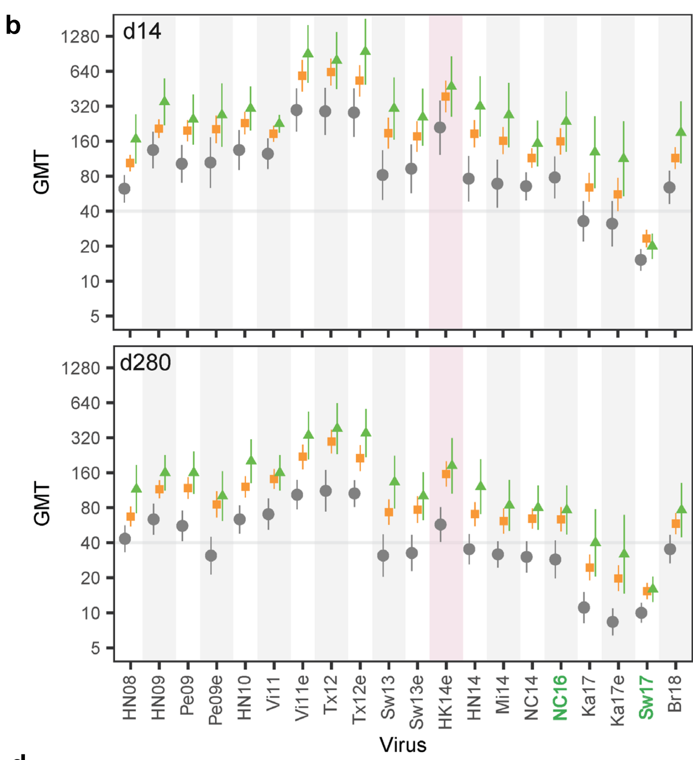

a
pre
d4
d7
d14
d21
d280
Seroconverted
4%
62%
78%
75%
54%
∘no +yes
HI titre
20480
10240
5120
2560
1280
640
320
160
80
40
20
10
5
no
yes
Recent prior A(H3N2) infection

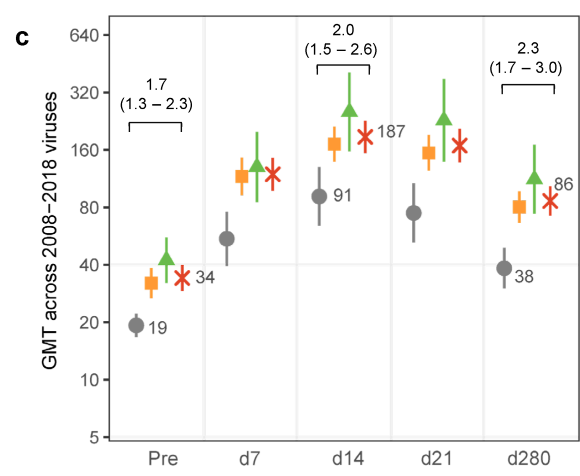

b
YoB
1935-59 (n=24)
1960-69 (n=36)
1970-79 (n=27)
1980-96 (n=13)
Vaccine
HI titre
1280
640
320
160
80
40
20
10
nd

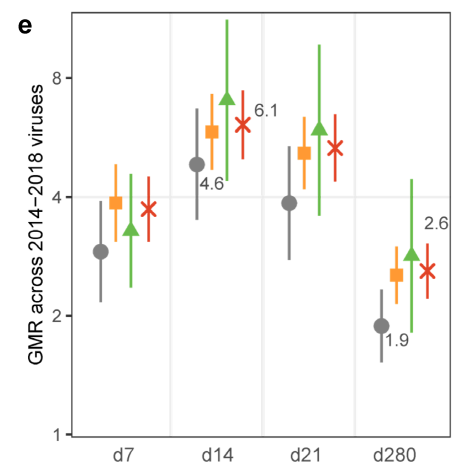

c
Time-point
Pre-vaccination
Day 7
Day 14
Day 21
Day 280
HI titre
5120
2560
1280
640
320
160
80
40
20
10
nd

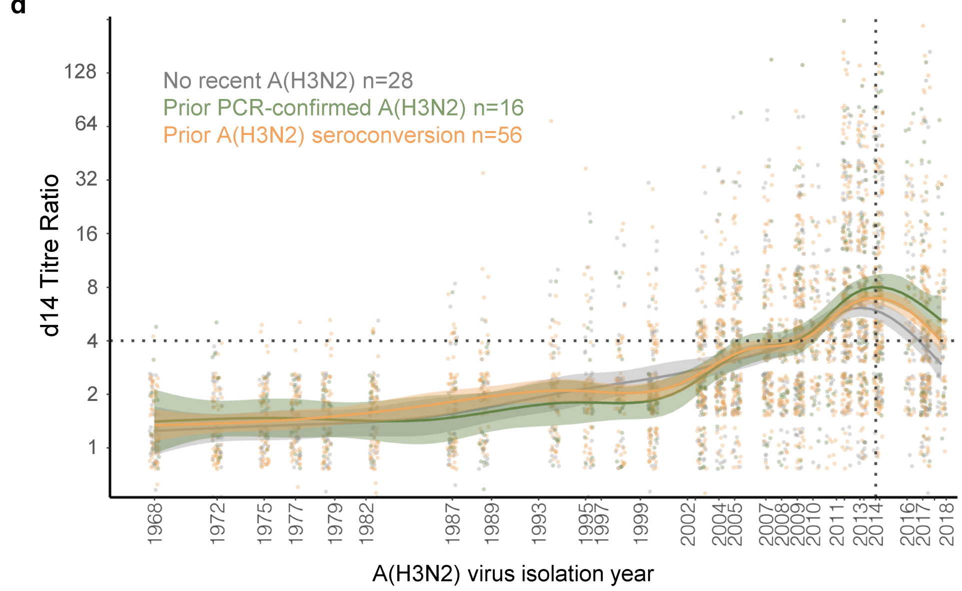

d
Time-point
Day 7
Day 14
Day 21
Day 280
Geometric Titre Ratio
128
64
32
16
8
4
2
1
1968
1972
1975
1977
1979
1982
1987
1989
1993
1995
1997
1999
2002
2004
2005
2007
2008
2009
2010
2011
2013
2014
2016
2017
2018
A(H3N2) virus isolation year

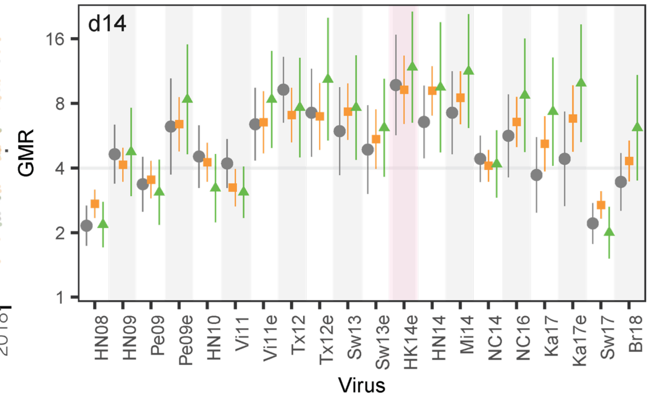

a
No recent A(H3N2) n=28
Prior PCR-confirmed A(H3N2) n=16
Prior A(H3N2) seroconversion n=56
Vaccine
recent strains
Pre-vaccination Titre
Pre−vaccination
GMT
Virus


b
d14
d280
GMT
Virus


c
GMT across 2008−2018 viruses
1.7 (1.3 – 2.3)
2.0 (1.5 – 2.6)
2.3 (1.7 – 3.0)
Pre
d7
d14
d21
d280


e
no prior
prior seroconversion
prior PCR
prior combined
GMR across 2014−2018 viruses
d7
d14
d21
d280


d
No recent A(H3N2) n=28
Prior PCR-confirmed A(H3N2) n=16
Prior A(H3N2) seroconversion n=56
d14 Titre Ratio
A(H3N2) virus isolation year


d14
GMR
Virus

No recent A(H3N2), n = 28
Prior HN09/HN12-like (1/3c1), n = 24
Prior HN14-like (3c3a), n = 14

a Pre-vaccination

HN08, Ur07e, Br07, Si16e, HK14e, Sw17e, Mi14, NC16, Br18², Br18¹, Sw17, Vi11, HN12, HN09, HN10, Pe09, Pe09e, Sw13, Sw13e, HN14, Ka17

b Post-vaccination d14

HK14e, Vi11, HN12, HN09, Pe09, Sw13, HN14

HI titre: 320, 160, 80, 40, 20, 10, 5, 2.5

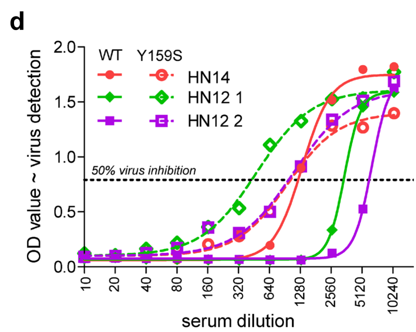
Clade: 1, 3c1, 3c2a, 3c2a1, 3c2a2, 3c3a, 3c2a1b

c

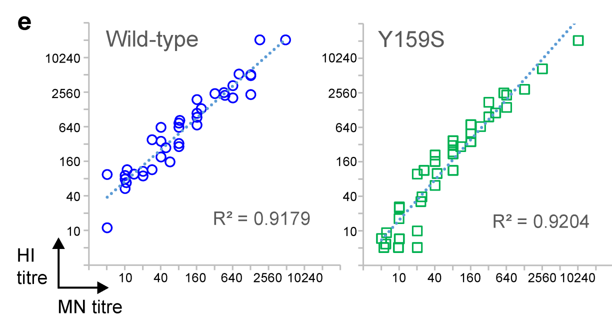
HI titre	wild-type (WT)	Y159S mutant
HK14e antisera	1280	320
Q12C9 mAb	80	20

RBS Y159S

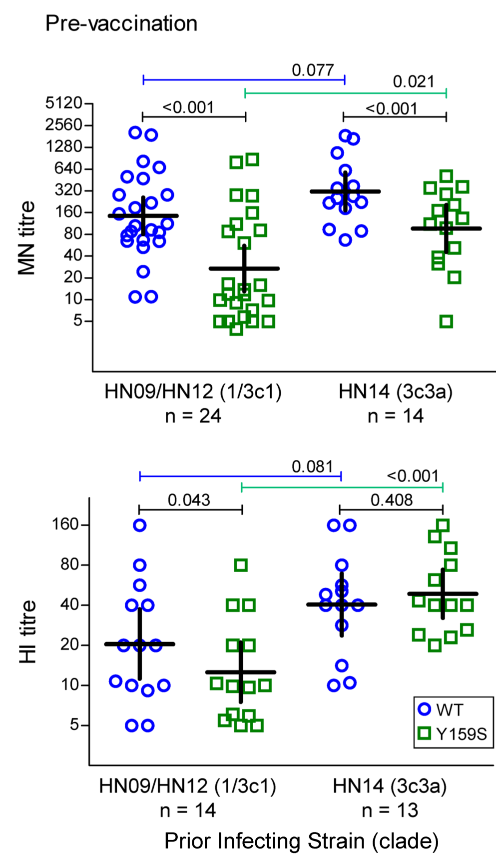
d



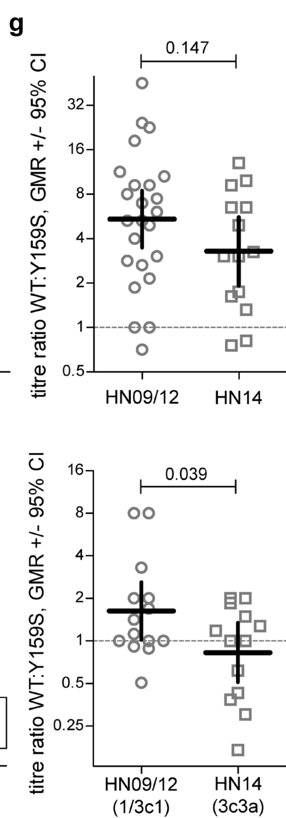
e



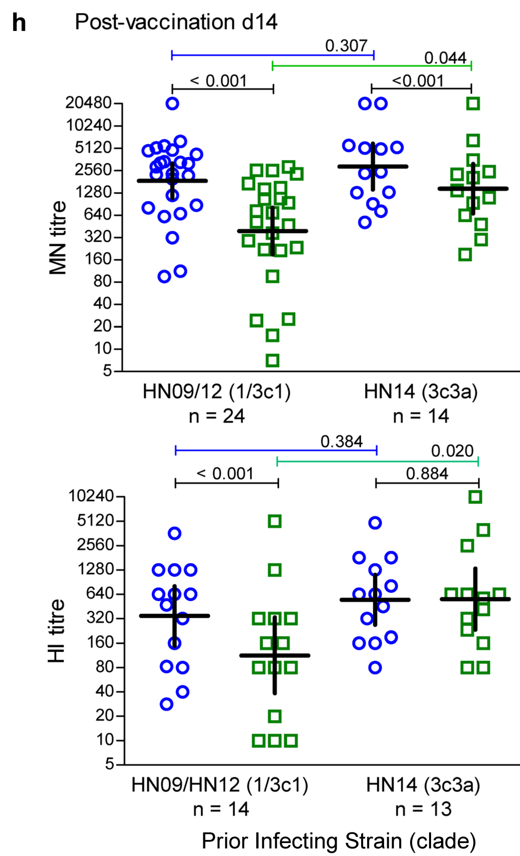
f Pre-vaccination



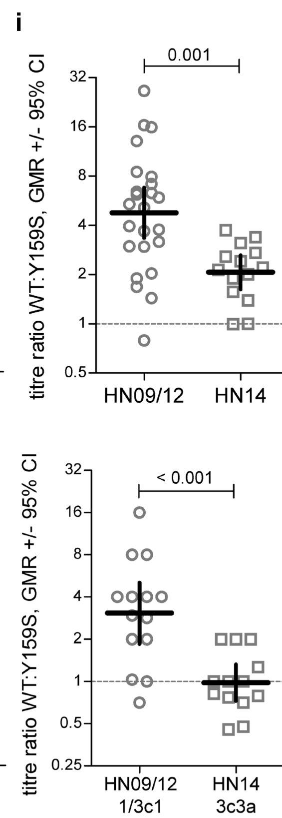
g

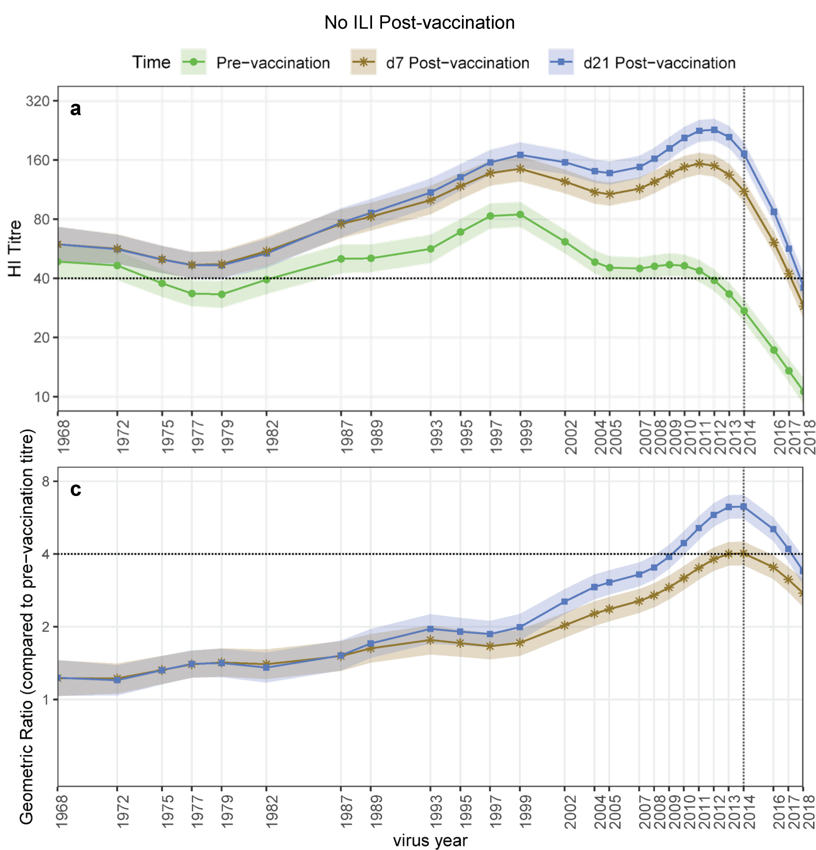


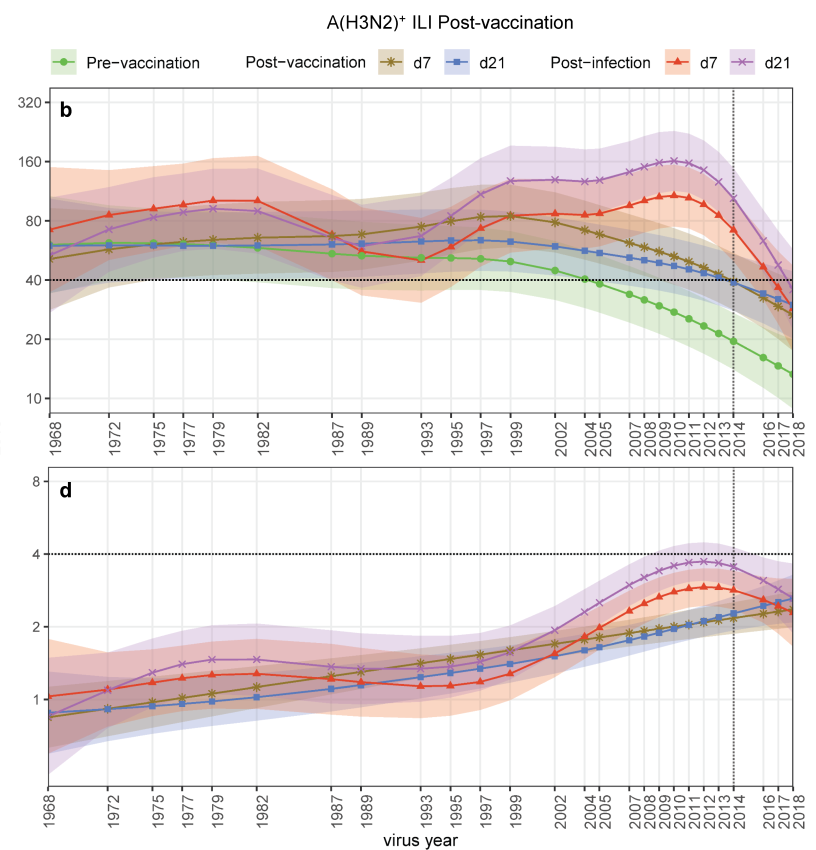
h Post-vaccination d14



i







A(H3N2)⁺ ILI Post-vaccination