Supplementary Information for

‘Serological imprinting and vaccination history shape adult influenza antibody landscapes and H5N1 cross-reactivity’

This Supplementary Information accompanies the manuscript ‘Serological imprinting and vaccination history shape adult influenza antibody landscapes and H5N1 cross-reactivity’ by Wilson K.K. et al.

Submitted to *Nature Communications* (2026)

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Supplemental Tables

Table S1. Influenza Vaccination cohort – Clinical and demographic information.

See Excel file: *Table S1_Vaccination cohort.xlsx*

Table S2. Influenza Infection cohort – Clinical and demographic information.

See Excel file: *Table S2_Infection cohort.xlsx*

Table S3. Antigen and bead information.

See Excel file: *Table S3_Antigen and bead information.xlsx*

Table S4. Egg-adapted mutations in hemagglutinin antigens.

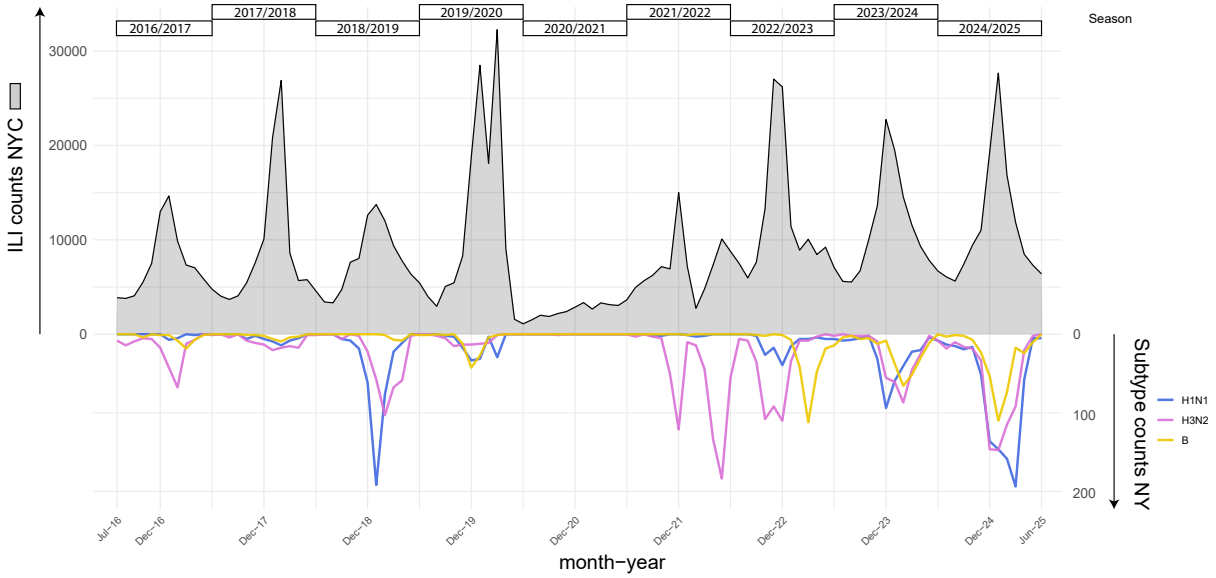
Antigens		Presence of egg-adapted mutations per (sub)type					
		H1: Q223R	H3: H156Q	H3: T160K	H3: L194P	B: G141R	B: N197D
A/bald/eagle/Florida/W22/134/OP/H5N1/HA|2022	A group 1	-					
A/Brevig/Mission1/H1N1/HA/i|1918	A group 1	-					
A/California/04/H1N1/HA/NonEggs|2009	A group 1	-					
A/Japan/305/H2N2/HA/Eggs|1957	A group 1	-					
A/Michigan/45/H1N1/HA|2015	A group 1	YES					
A/Victoria/4897/H1N1/HA|2022	A group 1	-					
A/Vietnam/1194/H5N1/HA/i|2004	A group 1	-					
A/Wisconsin/588/H1N1/HA|2019	A group 1	-					
A/Cambodia/e0826360/H3N2/HA|2020	A group 2		-	-	-		
A/Darwin/9/H3N2/HA|2021	A group 2		-	-	-		
A/HongKong/1/ACU79871/A/H3N2|1968	A group 2		-	-	-		
A/Shanghai/1/H7N9/HA|2013	A group 2		-	-	-		
B/Austria/1359417/Victoria/lineage/HA|2021	B					-	YES
B/Phuket/3073/Yamagata/lineage/HA|2013	B					-	-
B/Washington/02/Victoria/lineage/HA|2019	B					-	-

Table S5. Linear and logistic regression data on binding responses by year of birth.

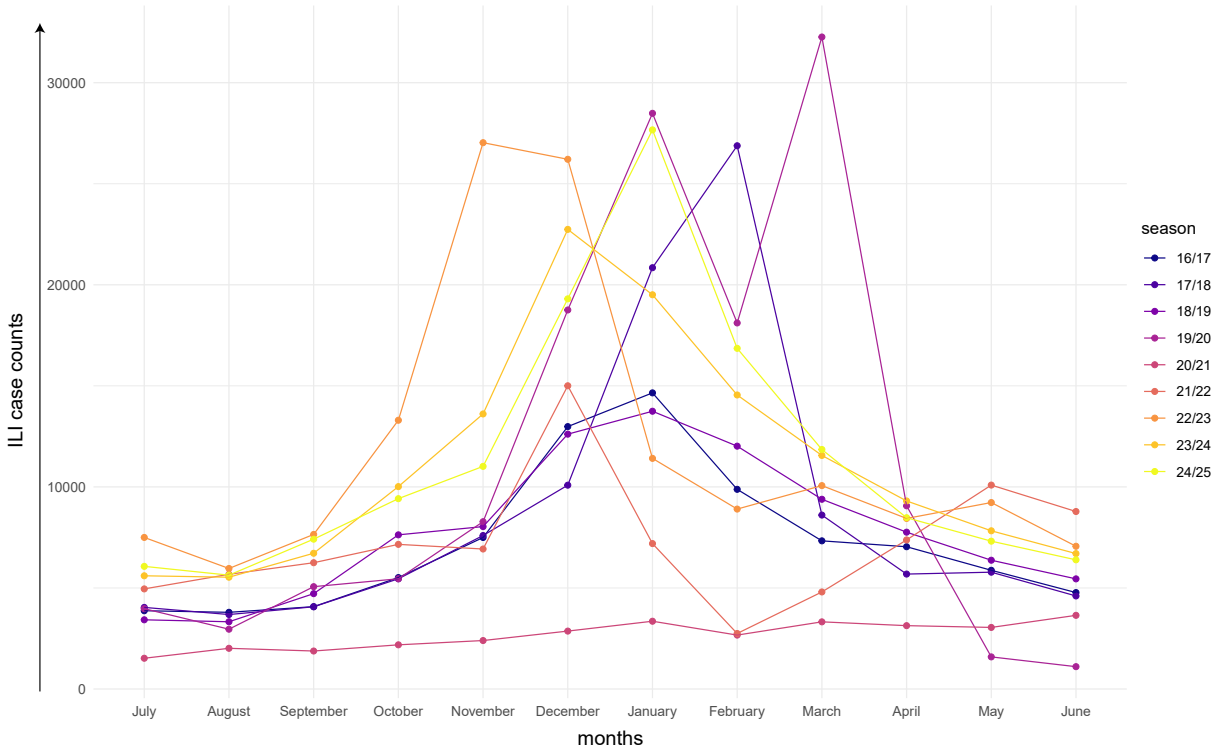
See Excel file: *Table S5_Linear and logistic regression data.xlsx*

Supplemental Figures

A Influenza−like illnesses (ILI) and Influenza subtypes in NY over the past 10 years



B ILI in NYC per season, overlay



C Vaccination strains per season over the past 10 years

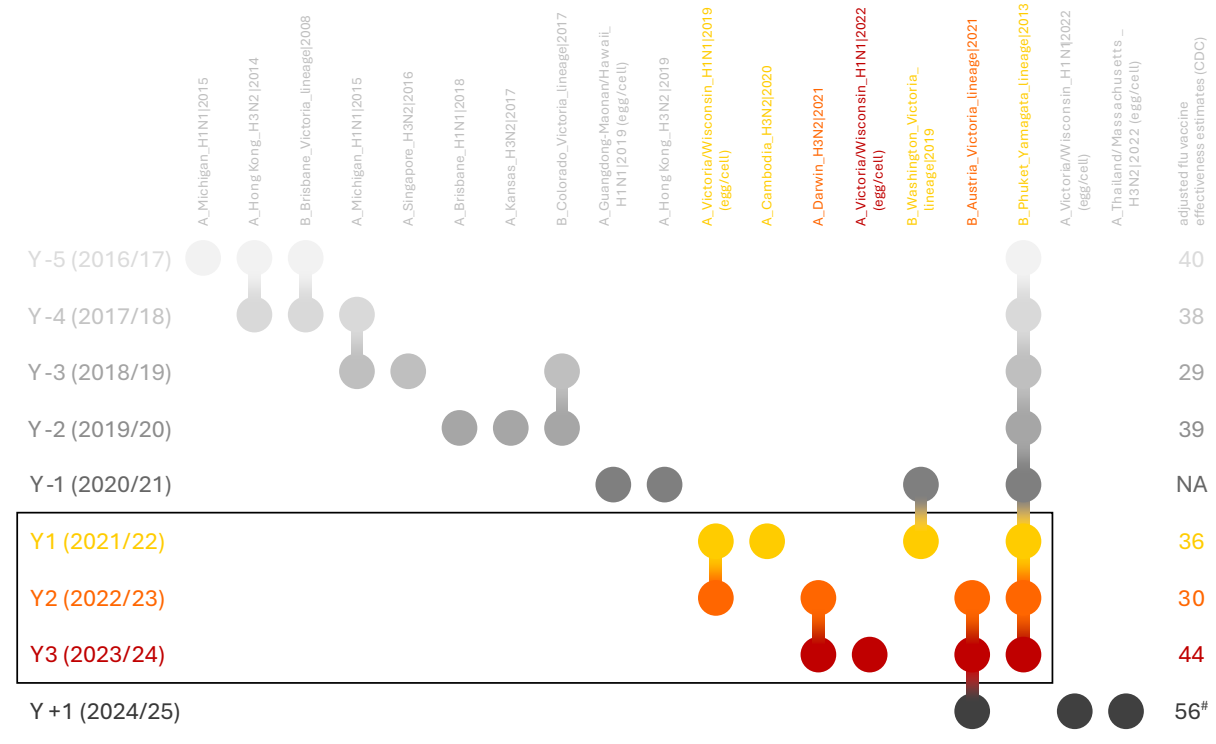


Figure S1. Influenza-like illnesses (ILI), subtypes, and vaccine composition.
A. Timeline of ILI cases in NYC (facing up; source: NYC.gov) and subtype counts in the state of New York (facing down; source: GISAID) over the past ten years. **B.** Overlay of yearly ILI cases (from A) to compare seasonality and monthly ILI counts across the past ten years. **C.** Strain composition of quadri/tri-valent vaccines and their adjusted overall effectiveness per year (%). Study years are highlighted. Source: CDC.gov/flu-vaccines-work, [#]preliminary data.

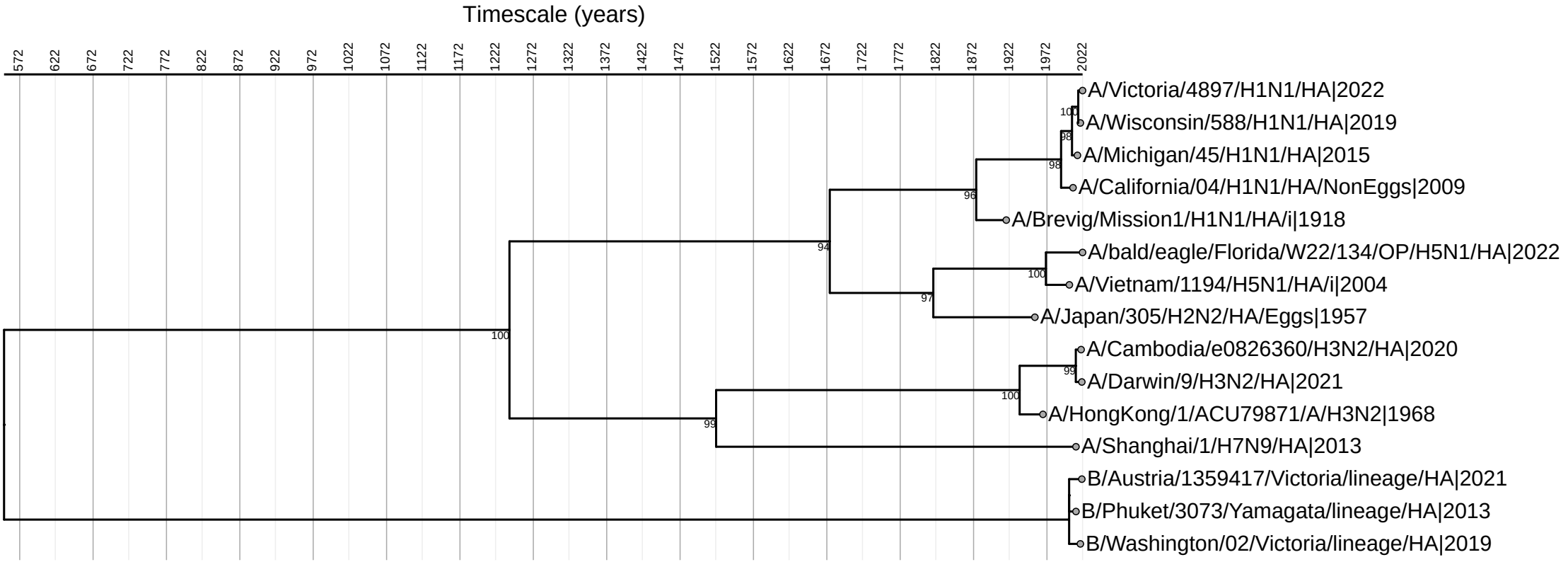

Timescale (years)
572
622
672
722
772
822
872
922
972
1022
1072
1122
1172
1222
1272
1322
1372
1422
1472
1522
1572
1622
1672
1722
1772
1822
1872
1922
1972
2022
A/Victoria/4897/H1N1/HA|2022
A/Wisconsin/588/H1N1/HA|2019
A/Michigan/45/H1N1/HA|2015
A/California/04/H1N1/HA/NonEggs|2009
A/Brevig/Mission1/H1N1/HA/i|1918
A/bald/eagle/Florida/W22/134/OP/H5N1/HA|2022
A/Vietnam/1194/H5N1/HA/i|2004
A/Japan/305/H2N2/HA/Eggs|1957
A/Cambodia/e0826360/H3N2/HA|2020
A/Darwin/9/H3N2/HA|2021
A/HongKong/1/ACU79871/A/H3N2|1968
A/Shanghai/1/H7N9/HA|2013
B/Austria/1359417/Victoria/lineage/HA|2021
B/Phuket/3073/Yamagata/lineage/HA|2013
B/Washington/02/Victoria/lineage/HA|2019
100
98
98
96
94
100
97
100
99
100
99

Figure S2. Phylogenetic relatedness of used hemagglutinin antigens.
B. Time-calibrated global tree using sequences of the studied HA antigens across (sub)types and groups (IQ tree, 1,000 bootstrap replicates). Bootstrap values > 70 are indicated.

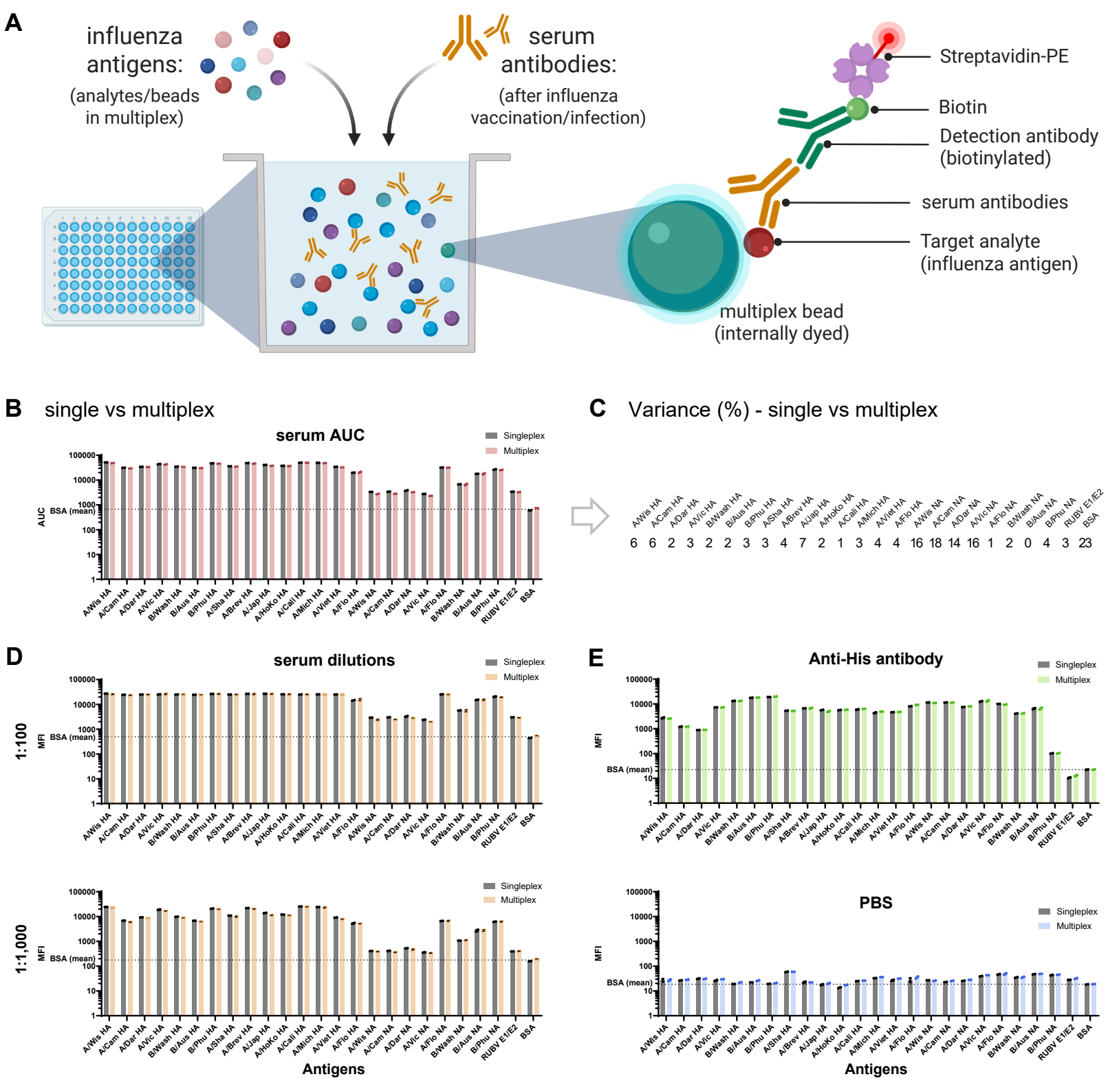

A
influenza antigens:
(analytes/beads in multiplex)
serum antibodies:
(after influenza vaccination/infection)
Streptavidin-PE
Biotin
Detection antibody (biotinylated)
serum antibodies
Target analyte (influenza antigen)
multiplex bead (internally dyed)
B single vs multiplex
serum AUC
Singleplex
Multiplex
AUC
BSA (mean)
C Variance (%) - single vs multiplex
A/Wis HA A/Cam HA A/Dar HA A/Vic HA B/Wash HA B/Aus HA B/Phu HA A/Sha HA A/Brev HA A/Jap HA A/HoKo HA A/Call HA A/Mich HA A/Viet HA A/Flo HA A/Wis NA A/Cam NA A/Dar NA A/Vic NA A/Flo NA B/Wash NA B/Aus NA B/Phu NA RUBV E1/E2 BSA
6 6 2 3 2 2 3 3 4 7 2 1 3 4 4 16 18 14 16 1 2 0 4 3 23
D
serum dilutions
1:100
1:1,000
MFI
BSA (mean)
Antigens
E
Anti-His antibody
PBS

Figure S3. Comparability of signals obtained in multiplex versus singleplex.
To verify the absence of significant cross-competition among antigens in the multiplex format, we compared total Ig signals obtained with the positive control serum in the multiplex (multiple Influenza antigens run simultaneously) with those in the singleplex format (only one Influenza antigen measured at a time). Each Influenza singleplex was run with BSA and IC45 controls included, and was technically a triplex. **A**. Schematic setup of multiplex bead-based binding assay (MBA). **B**. Signal comparison for area under the curve (AUC) measurements. **C**. Variance between multiplex and singleplex in B: ([multiplex – singleplex]/singleplex)*100%. **D**. Comparison as in B, but with individual dilutions measured in median fluorescence intensities (MFI; raw values). **D**. Additional controls, i.e., using PBS (bottom) instead of the positive control serum, and using anti-His antibody (1:2,000, top) instead of the positive control serum and Ig detection antibody. The dashed line indicates the averaged binding responses against BSA as a reference.

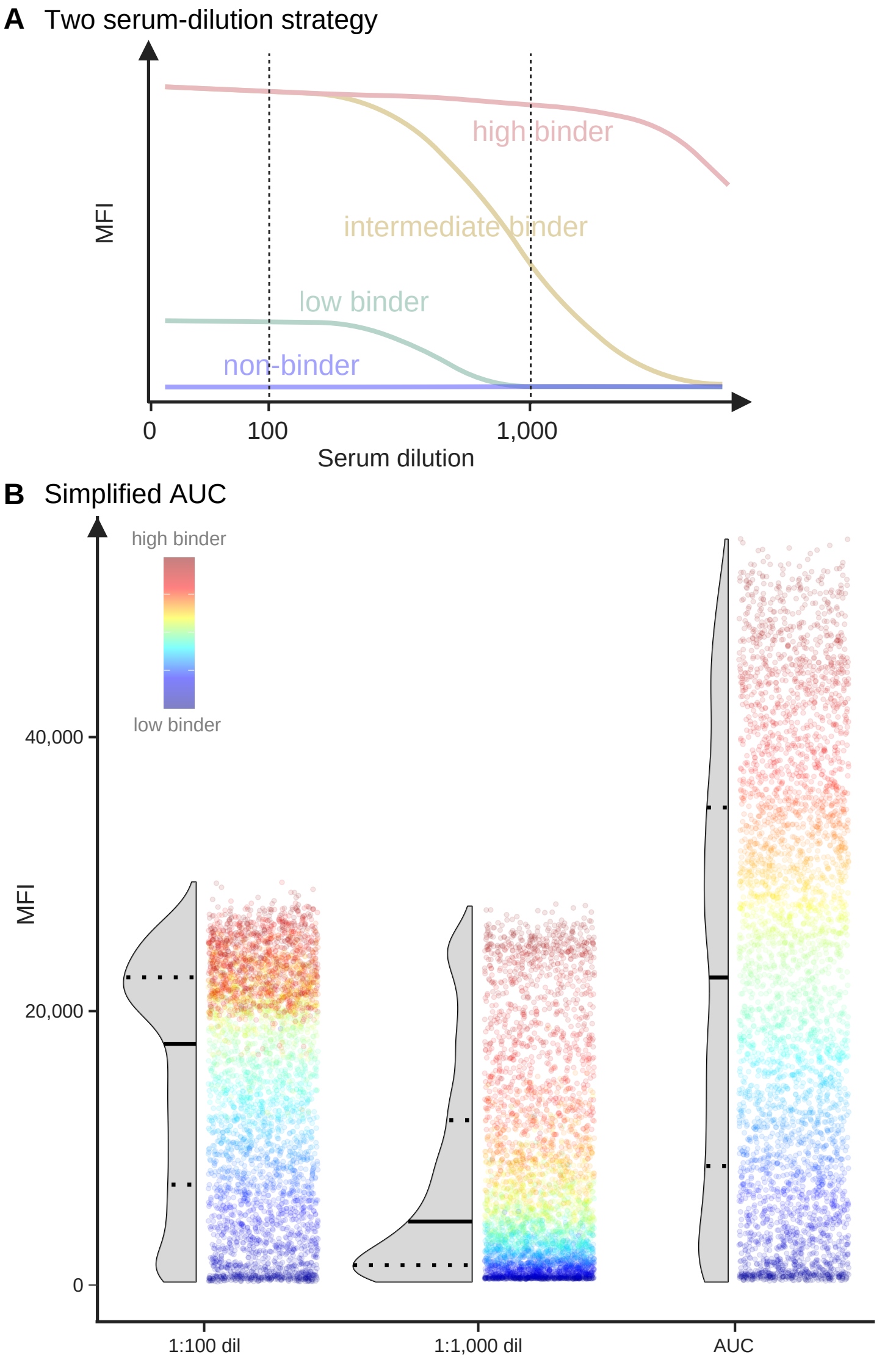

A Two serum-dilution strategy
MFI
high binder
intermediate binder
low binder
non-binder
0
100
1,000
Serum dilution
B Simplified AUC
high binder
low binder
40,000
20,000
0
MFI
1:100 dil
1:1,000 dil
AUC

Figure S4. Two-Serum Dilution Strategy for high-throughput immunoprofiling with high resolution.
A. Schematic representation of antigen-specific IgG titration curves observed in a human vaccination or infection study cohort. Examples include participants categorized as non-binders, low binders, intermediate binders, and high binders. **B**. Distribution of antigen-specific IgG levels measured at two distinct serum dilutions or represented as a simplified AUC (area under the curve), calculated by summing both measurements per data point (n=3956 data points, vaccination cohort of 30 participants: 172 visits, 23 Influenza antigens).

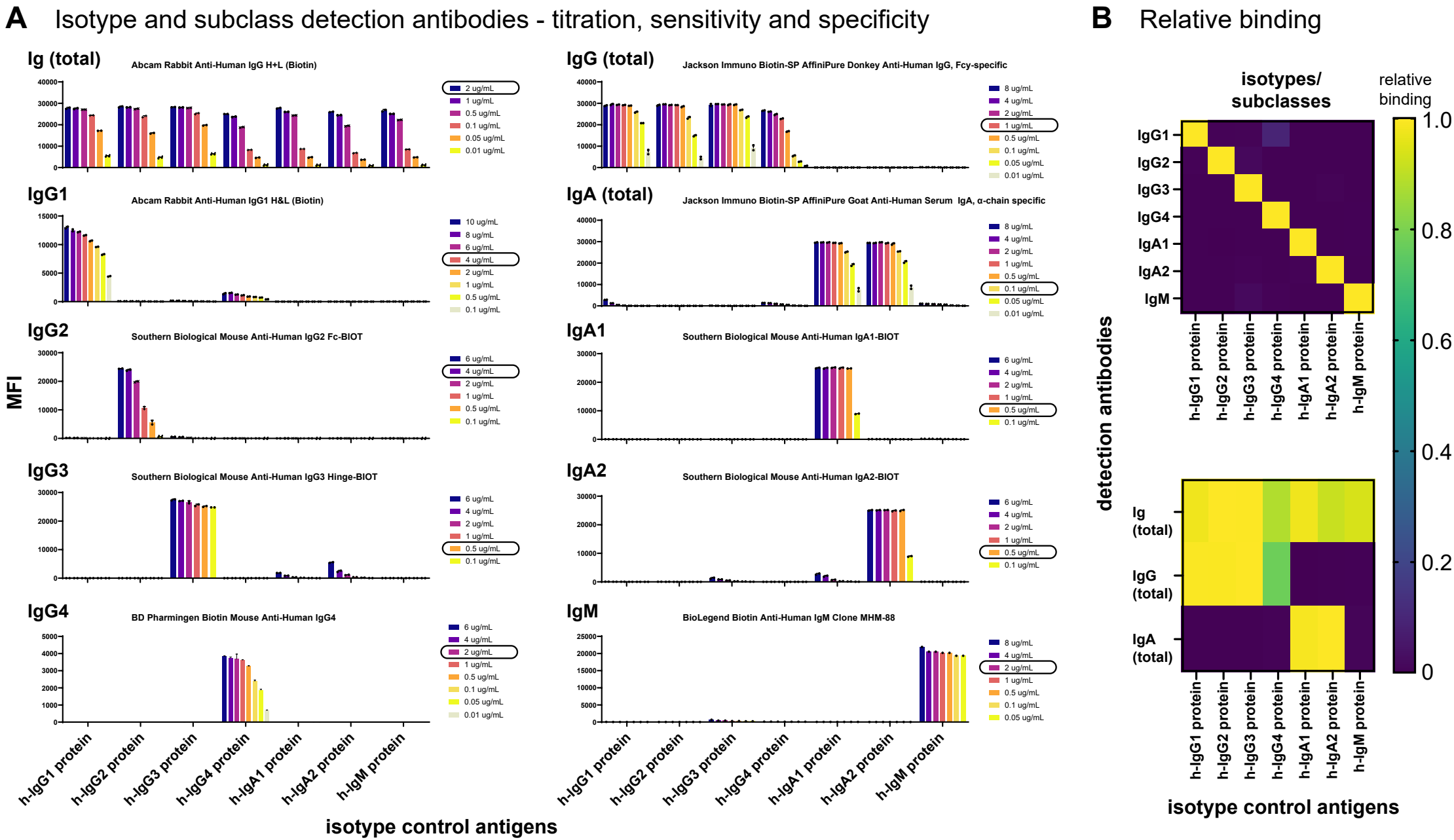

A
Isotype and subclass detection antibodies - titration, sensitivity and specificity
Ig (total)
Abcam Rabbit Anti-Human IgG H+L (Biotin)
IgG (total)
Jackson Immuno Biotin-SP AffiniPure Donkey Anti-Human IgG, Fcγ-specific
IgG1
Abcam Rabbit Anti-Human IgG1 H&L (Biotin)
IgA (total)
Jackson Immuno Biotin-SP AffiniPure Goat Anti-Human Serum IgA, α-chain specific
IgG2
Southern Biological Mouse Anti-Human IgG2 Fc-BIOT
IgA1
Southern Biological Mouse Anti-Human IgA1-BIOT
IgG3
Southern Biological Mouse Anti-Human IgG3 Hinge-BIOT
IgA2
Southern Biological Mouse Anti-Human IgA2-BIOT
IgG4
BD Pharmingen Biotin Mouse Anti-Human IgG4
IgM
BioLegend Biotin Anti-Human IgM Clone MHM-88
MFI
h-IgG1 protein
h-IgG2 protein
h-IgG3 protein
h-IgG4 protein
h-IgA1 protein
h-IgA2 protein
h-IgM protein
isotype control antigens
B
Relative binding
isotypes/ subclasses
relative binding
IgG1
IgG2
IgG3
IgG4
IgA1
IgA2
IgM
Ig (total)
IgG (total)
IgA (total)
detection antibodies
isotype control antigens

Figure S5. Development and optimization of an Ig isotype and subclass-specific binding assay.
A. Titration of indicated Ig, IgG, IgA, and IgM isotype and subclass antibodies against isotype/subclass control antigens. The antibody concentrations selected for the main experiments are encircled. **B.** Heatmaps to highlight the specificity of the antibodies.

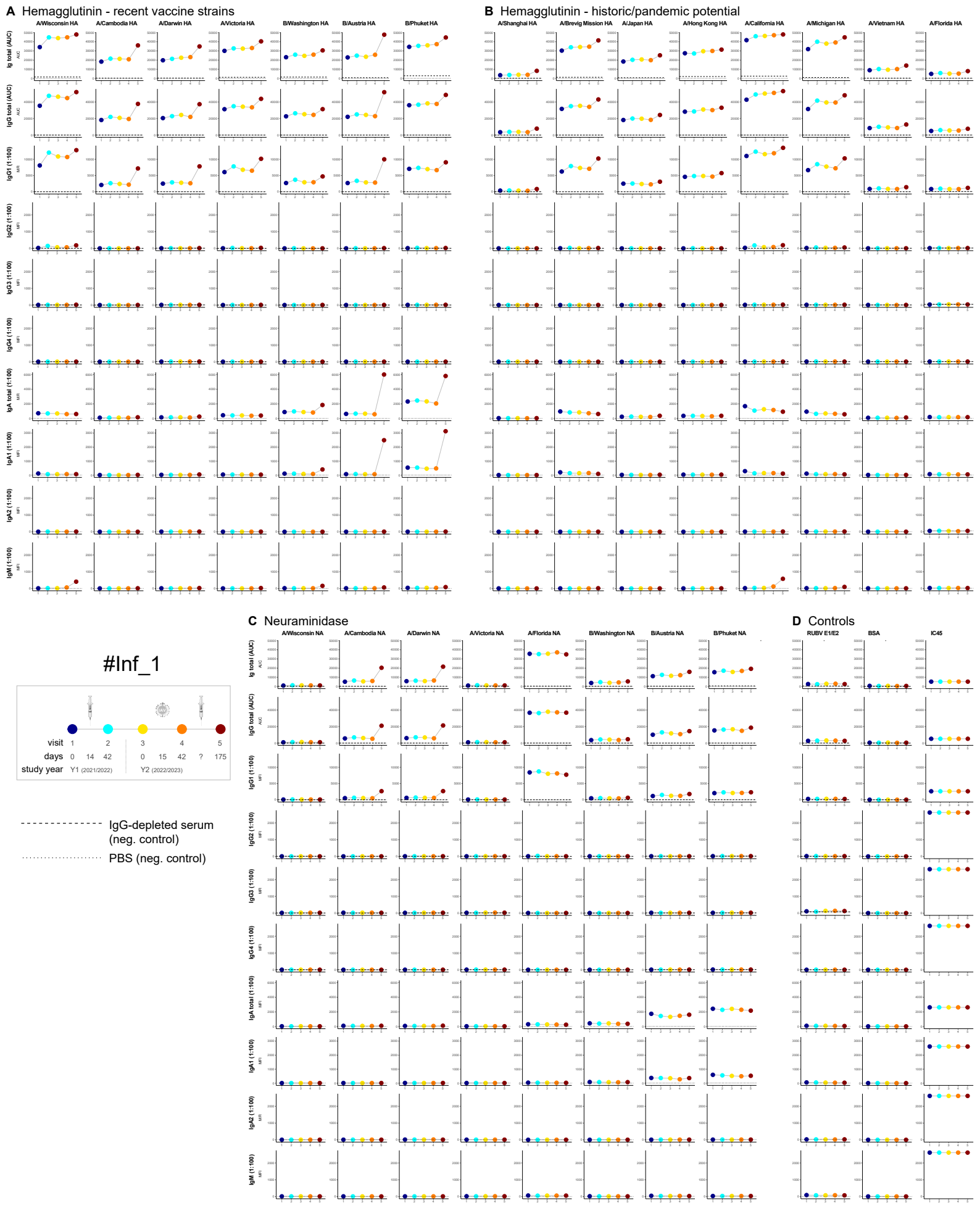

A Hemagglutinin - recent vaccine strains
A/Wisconsin HA
A/Cambodia HA
A/Darwin HA
A/Victoria HA
B/Washington HA
B/Austria HA
B/Phuket HA
B Hemagglutinin - historic/pandemic potential
A/Shanghai HA
A/Brevig Mission HA
A/Japan HA
A/Hong Kong HA
A/California HA
A/Michigan HA
A/Vietnam HA
A/Florida HA
Ig total (AUC)
IgG total (AUC)
IgG1 (1:100)
IgG2 (1:100)
IgG3 (1:100)
IgG4 (1:100)
IgA total (1:100)
IgA1 (1:100)
IgA2 (1:100)
IgM (1:100)
#Inf_1
visit 1 2 3 4 5
days 0 14 42 0 15 42 ? 175
study year Y1 (2021/2022) Y2 (2022/2023)
IgG-depleted serum (neg. control)
PBS (neg. control)
C Neuraminidase
A/Wisconsin NA
A/Cambodia NA
A/Darwin NA
A/Victoria NA
A/Florida NA
B/Washington NA
B/Austria NA
B/Phuket NA
D Controls
RUBV E1/E2
BSA
IC45

Figure S6. Antibody subclass-specific binding profiles in infection cohort participant #1. Longitudinal multi-isotype and -subclass anti-Influenza profiling against the 26-plex antigens/beads, including 15 hemagglutinins (**A**, **B**), 8 neuraminidases (**C**), and three controls (**D**). The timeline of longitudinal samplings, vaccinations, and infection is shown in the box. Antibody binding levels in MFI using 1:100 serum dilution or AUC (for total Ig and IgG) are shown where the dashed line indicates IgG-depleted serum control and the dotted line PBS (run in single measurements). AUC: area under the curve, MFI: median fluorescence intensity.

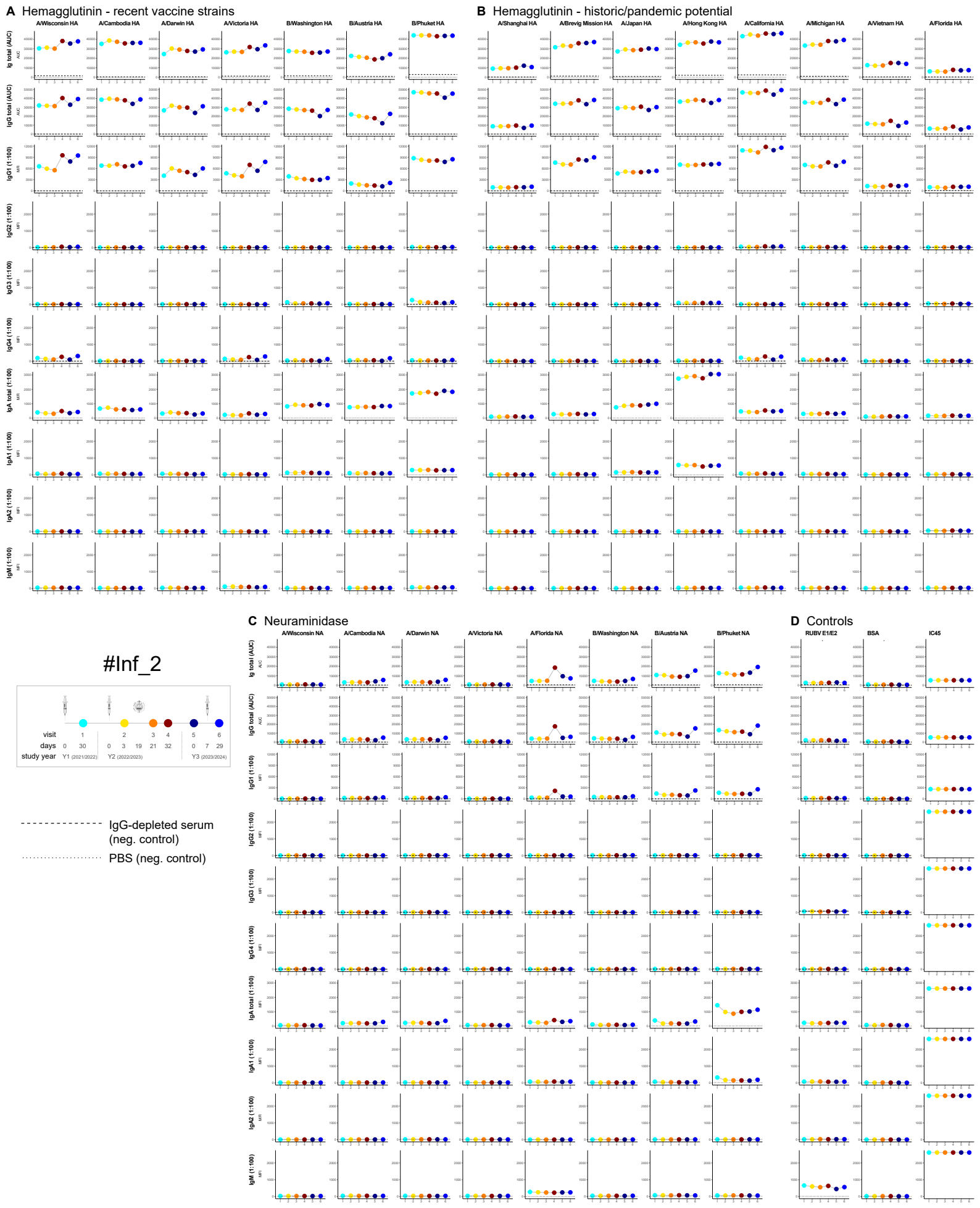

A Hemagglutinin - recent vaccine strains
A/Wisconsin HA
A/Cambodia HA
A/Darwin HA
A/Victoria HA
B/Washington HA
B/Austria HA
B/Phuket HA
B Hemagglutinin - historic/pandemic potential
A/Shanghai HA
A/Brevig Mission HA
A/Japan HA
A/Hong Kong HA
A/California HA
A/Michigan HA
A/Vietnam HA
A/Florida HA
Ig total (AUC)
IgG total (AUC)
IgG1 (1:100)
IgG2 (1:100)
IgG3 (1:100)
IgG4 (1:100)
IgA total (1:100)
IgA1 (1:100)
IgA2 (1:100)
IgM (1:100)
C Neuraminidase
A/Wisconsin NA
A/Cambodia NA
A/Darwin NA
A/Victoria NA
A/Florida NA
B/Washington NA
B/Austria NA
B/Phuket NA
D Controls
RUBV E1/E2
BSA
IC45
#Inf_2
visit 1 2 3 4 5 6
days 0 30 0 3 19 21 32 0 7 29
study year Y1 (2021/2022) Y2 (2022/2023) Y3 (2023/2024)
IgG-depleted serum (neg. control)
PBS (neg. control)

Figure S7. Antibody subclass-specific binding profiles in infection cohort participant #2. Longitudinal multi-isotype and -subclass anti-Influenza profiling against the 26-plex antigens/beads, including 15 hemagglutinins (**A**, **B**), 8 neuraminidases (**C**), and three controls (**D**). The timeline of longitudinal samplings, vaccinations, and infection is shown in the box. Antibody binding levels in MFI using 1:100 serum dilution or AUC (for total Ig and IgG) are shown where the dashed line indicates IgG-depleted serum control and the dotted line PBS (run in single measurements). AUC: area under the curve, MFI: median fluorescence intensity.

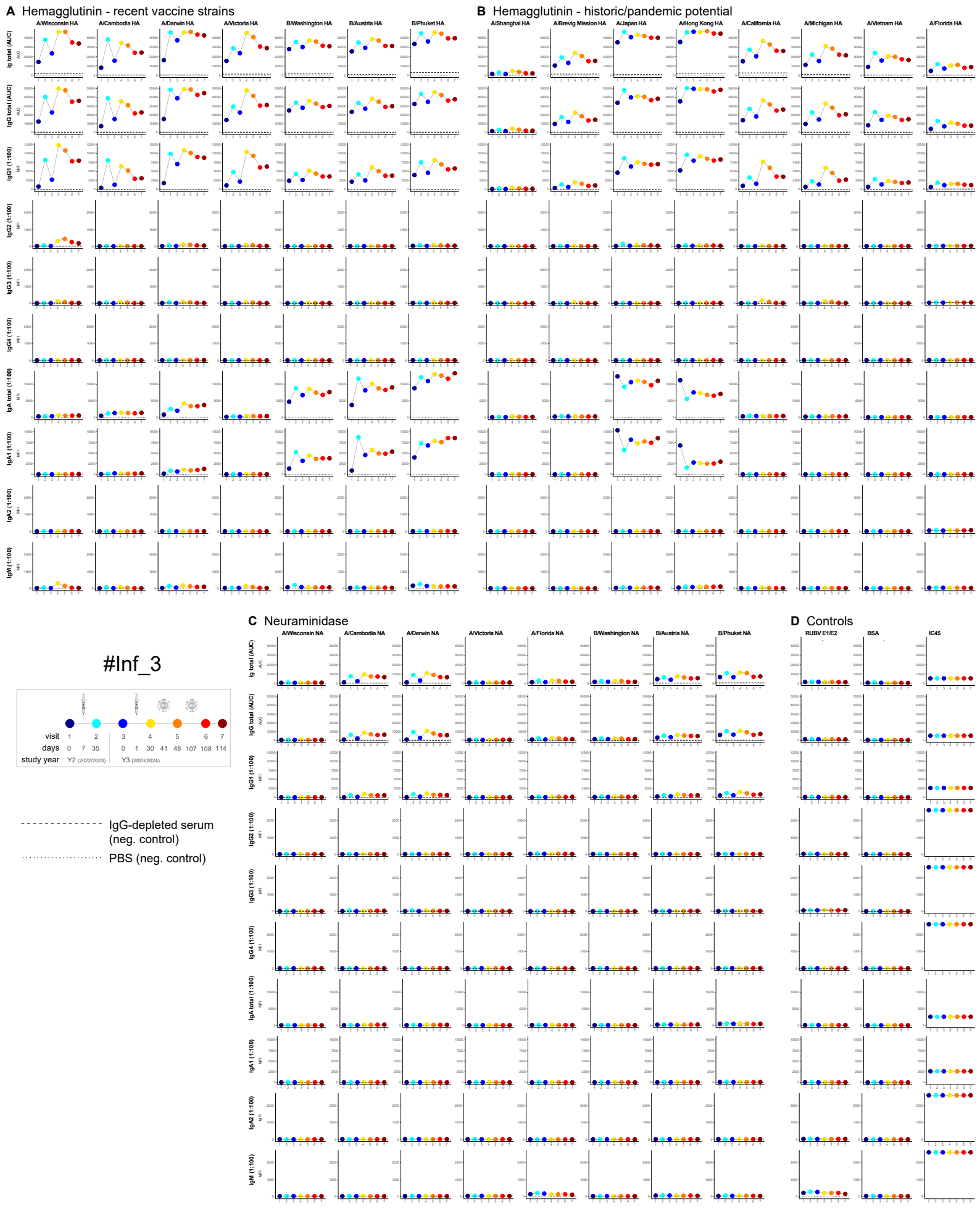

A Hemagglutinin - recent vaccine strains
A/Wisconsin HA
A/Cambodia HA
A/Darwin HA
A/Victoria HA
B/Washington HA
B/Austria HA
B/Phuket HA
B Hemagglutinin - historic/pandemic potential
A/Shanghai HA
A/Brevig Mission HA
A/Japan HA
A/Hong Kong HA
A/California HA
A/Michigan HA
A/Vietnam HA
A/Florida HA
Ig total (AUC)
IgG total (AUC)
IgG1 (1:100)
IgG2 (1:100)
IgG3 (1:100)
IgG4 (1:100)
IgA total (1:100)
IgA1 (1:100)
IgA2 (1:100)
IgM (1:100)
#Inf_3
visit 1 2 3 4 5 6 7
days 0 7 35 0 1 30 41 48 107 108 114
study year Y2 (2022/2023) Y3 (2023/2024)
IgG-depleted serum (neg. control)
PBS (neg. control)
C Neuraminidase
A/Wisconsin NA
A/Cambodia NA
A/Darwin NA
A/Victoria NA
A/Florida NA
B/Washington NA
B/Austria NA
B/Phuket NA
D Controls
RUBV E1/E2
BSA
IC45

Figure S8. Antibody subclass-specific binding profiles in infection cohort participant #3. Longitudinal multi-isotype and -subclass anti-Influenza profiling against the 26-plex antigens/beads, including 15 hemagglutinins (**A**, **B**), 8 neuraminidases (**C**), and three controls (**D**). The timeline of longitudinal samplings, vaccinations, and infections is shown in the box. Antibody binding levels in MFI using 1:100 serum dilution or AUC (for total Ig and IgG) are shown where the dashed line indicates IgG-depleted serum control and the dotted line PBS (run in single measurements). AUC: area under the curve, MFI: median fluorescence intensity.

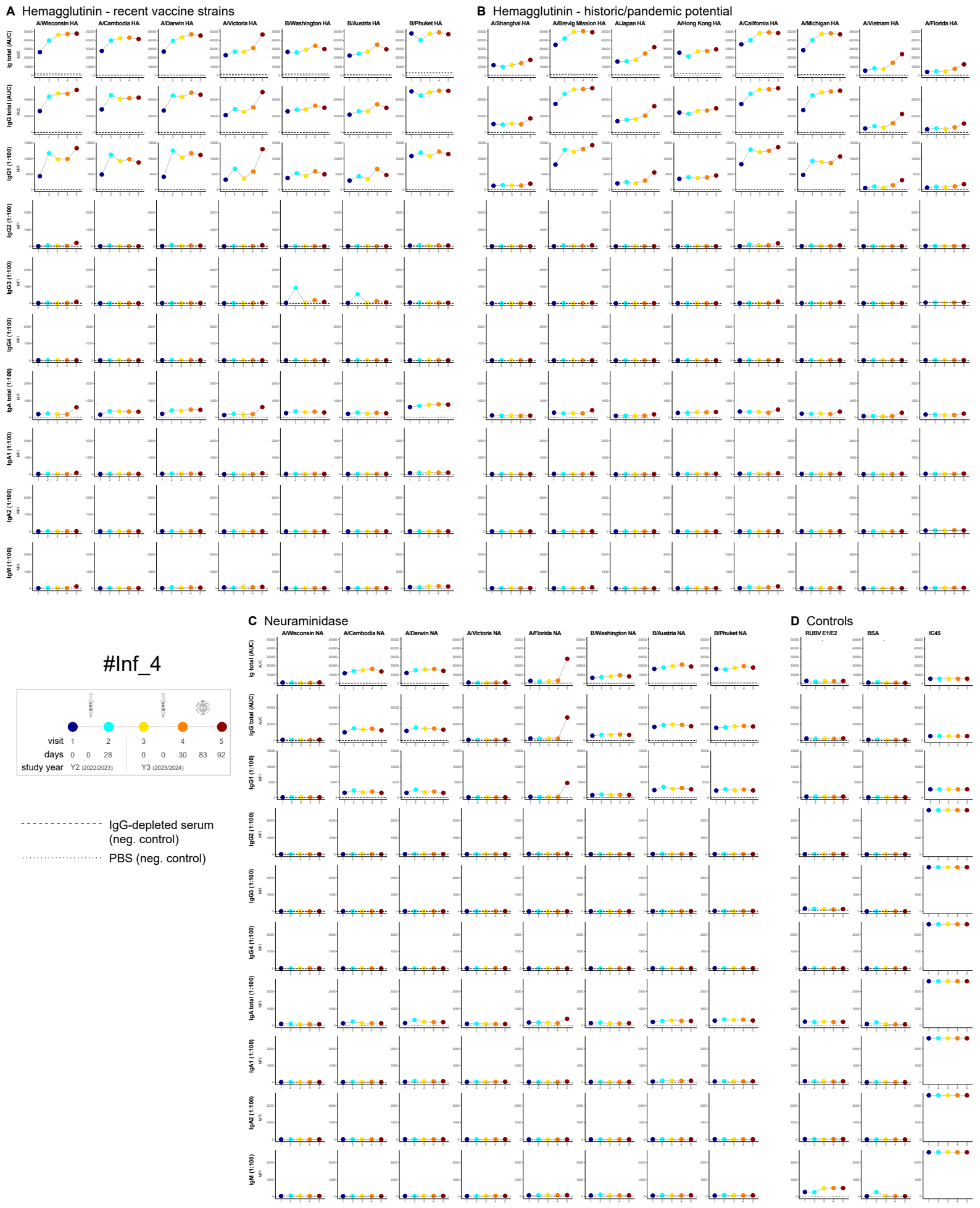

A Hemagglutinin - recent vaccine strains
A/Wisconsin HA
A/Cambodia HA
A/Darwin HA
A/Victoria HA
B/Washington HA
B/Austria HA
B/Phuket HA
B Hemagglutinin - historic/pandemic potential
A/Shanghai HA
A/Brevig Mission HA
A/Japan HA
A/Hong Kong HA
A/California HA
A/Michigan HA
A/Vietnam HA
A/Florida HA
Ig total (AUC)
IgG total (AUC)
IgG1 (1:100)
IgG2 (1:100)
IgG3 (1:100)
IgG4 (1:100)
IgA total (1:100)
IgA1 (1:100)
IgA2 (1:100)
IgM (1:100)
C Neuraminidase
A/Wisconsin NA
A/Cambodia NA
A/Darwin NA
A/Victoria NA
A/Florida NA
B/Washington NA
B/Austria NA
B/Phuket NA
D Controls
RUBV E1/E2
BSA
IC45
#Inf_4
visit 1 2 3 4 5
days 0 0 28 0 0 30 83 92
study year Y2 (2022/2023) Y3 (2023/2024)
IgG-depleted serum (neg. control)
PBS (neg. control)

Figure S9. Antibody subclass-specific binding profiles in infection cohort participant #4. Longitudinal multi-isotype and -subclass anti-Influenza profiling against the 26-plex antigens/beads, including 15 hemagglutinins (**A**, **B**), 8 neuraminidases (**C**), and three controls (**D**). The timeline of longitudinal samplings, vaccinations, and infection is shown in the box. Antibody binding levels in MFI using 1:100 serum dilution or AUC (for total Ig and IgG) are shown where the dashed line indicates IgG-depleted serum control and the dotted line PBS (run in single measurements). AUC: area under the curve, MFI: median fluorescence intensity.

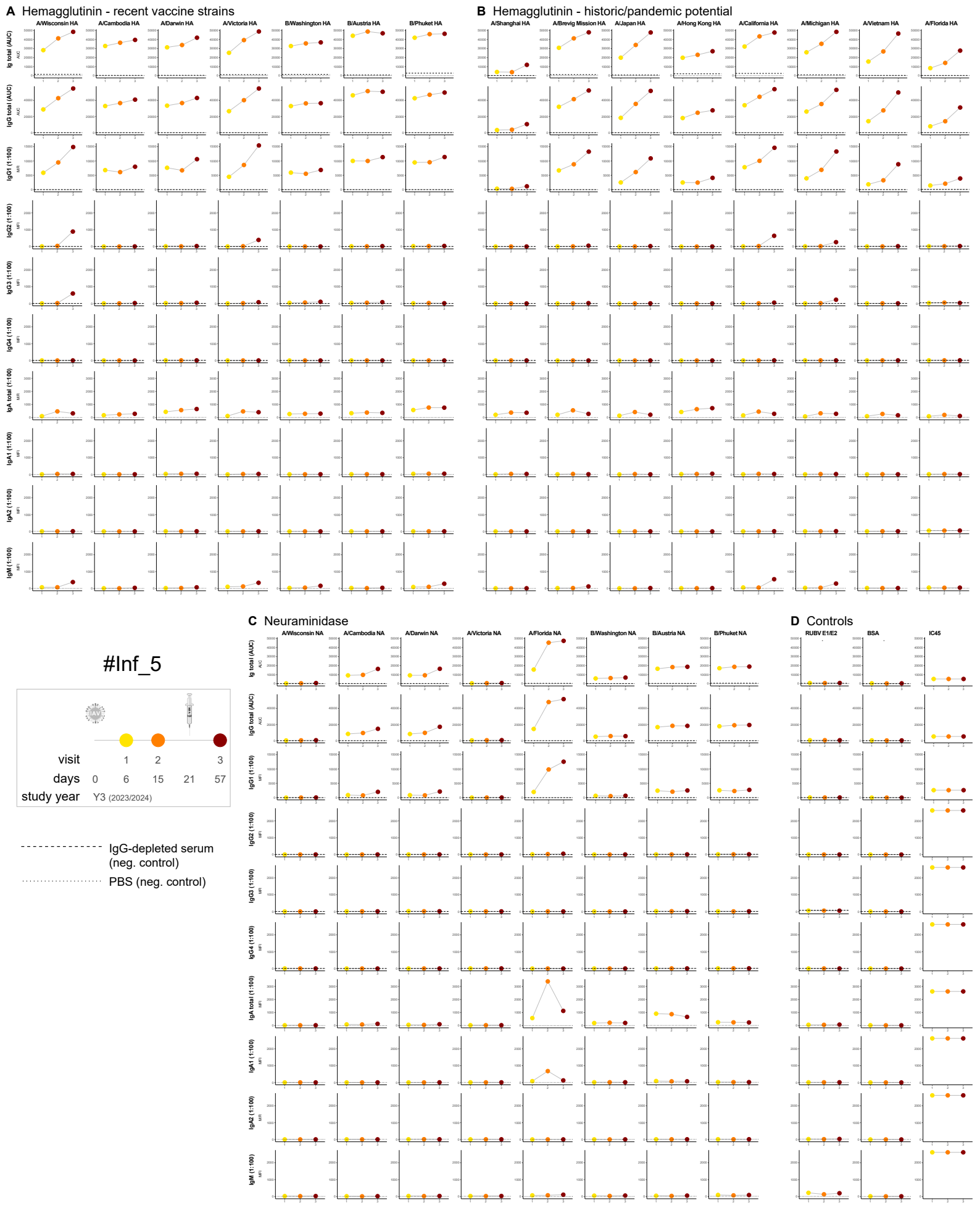

A Hemagglutinin - recent vaccine strains
A/Wisconsin HA
A/Cambodia HA
A/Darwin HA
A/Victoria HA
B/Washington HA
B/Austria HA
B/Phuket HA
B Hemagglutinin - historic/pandemic potential
A/Shanghai HA
A/Brevig Mission HA
A/Japan HA
A/Hong Kong HA
A/California HA
A/Michigan HA
A/Vietnam HA
A/Florida HA
Ig total (AUC)
IgG total (AUC)
IgG1 (1:100)
IgG2 (1:100)
IgG3 (1:100)
IgG4 (1:100)
IgA total (1:100)
IgA1 (1:100)
IgA2 (1:100)
IgM (1:100)
#Inf_5
visit 1 2 3
days 0 6 15 21 57
study year Y3 (2023/2024)
IgG-depleted serum (neg. control)
PBS (neg. control)
C Neuraminidase
A/Wisconsin NA
A/Cambodia NA
A/Darwin NA
A/Victoria NA
A/Florida NA
B/Washington NA
B/Austria NA
B/Phuket NA
D Controls
RUBV E1/E2
BSA
IC45

Figure S10. Antibody subclass-specific binding profiles in infection cohort participant #5. Longitudinal multi-isotype and -subclass anti-Influenza profiling against the 26-plex antigens/beads, including 15 hemagglutinins (**A**, **B**), 8 neuraminidases (**C**), and three controls (**D**). The timeline of longitudinal samplings, vaccinations, and infection is shown in the box. Antibody binding levels in MFI using 1:100 serum dilution or AUC (for total Ig and IgG) are shown where the dashed line indicates IgG-depleted serum control and the dotted line PBS (run in single measurements). AUC: area under the curve, MFI: median fluorescence intensity.

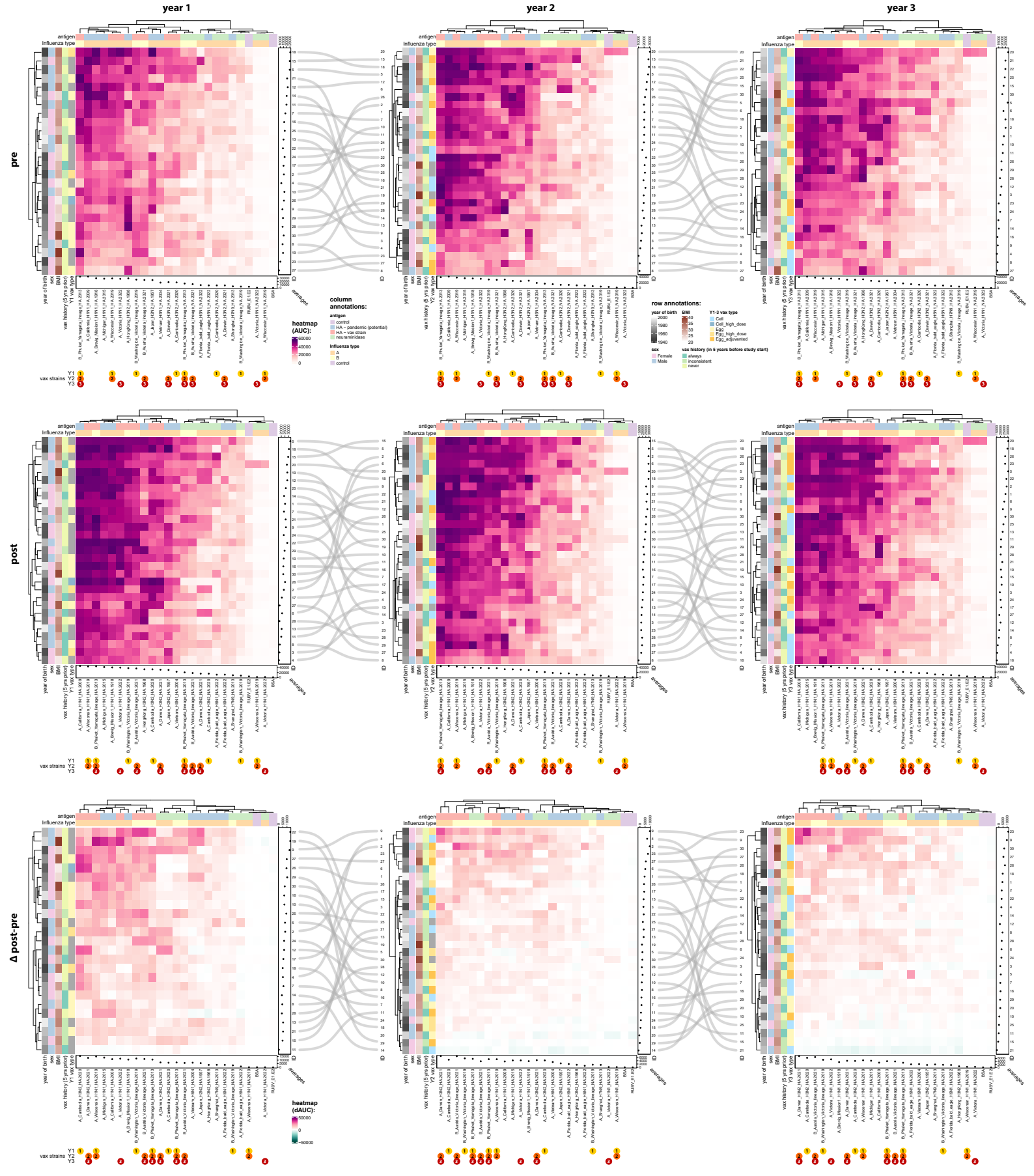

year 1
year 2
year 3
pre
post
Δ post-pre
antigen
Influenza type
year of birth
sex
BMI
vax history (5 yrs prior)
Y2 vax type
Y3 vax type
ID
BSA
averages
vax strains
Y1
Y2
Y3
column annotations:
antigen
control
HA – pandemic (potential)
HA – vax strain
neuraminidase
Influenza type
A
B
control
heatmap (AUC):
60000
40000
20000
0
heatmap (dAUC):
50000
0
−50000
row annotations:
year of birth
2000
1980
1960
1940
BMI
40
35
30
25
20
Y1-3 vax type
Cell
Cell_high_dose
Egg
Egg_high_dose
Egg_adjuvanted
sex
Female
Male
vax history (in 5 years before study start)
always
inconsistent
never

Figure S11. Multi-variable longitudinal profiling of Ig binding responses in response to seasonal Influenza vaccination.
Heatmaps of Ig binding profiles for 30 study participants against the 26-plex antigen/bead panel. Antibody levels are shown before (top row), approximately one month after vaccination (middle row), and for the vaccine-induced response (difference between pre- and post-vaccination; bottom row) across the three study years (left to right). The curved lines connecting the heatmaps enable track participants across years. Additional antigen and participant information are annotated above and to the left of each heatmap. Details about which antigens were included in the seasonal vaccines for the three study years are provided at the bottom of each heatmap.

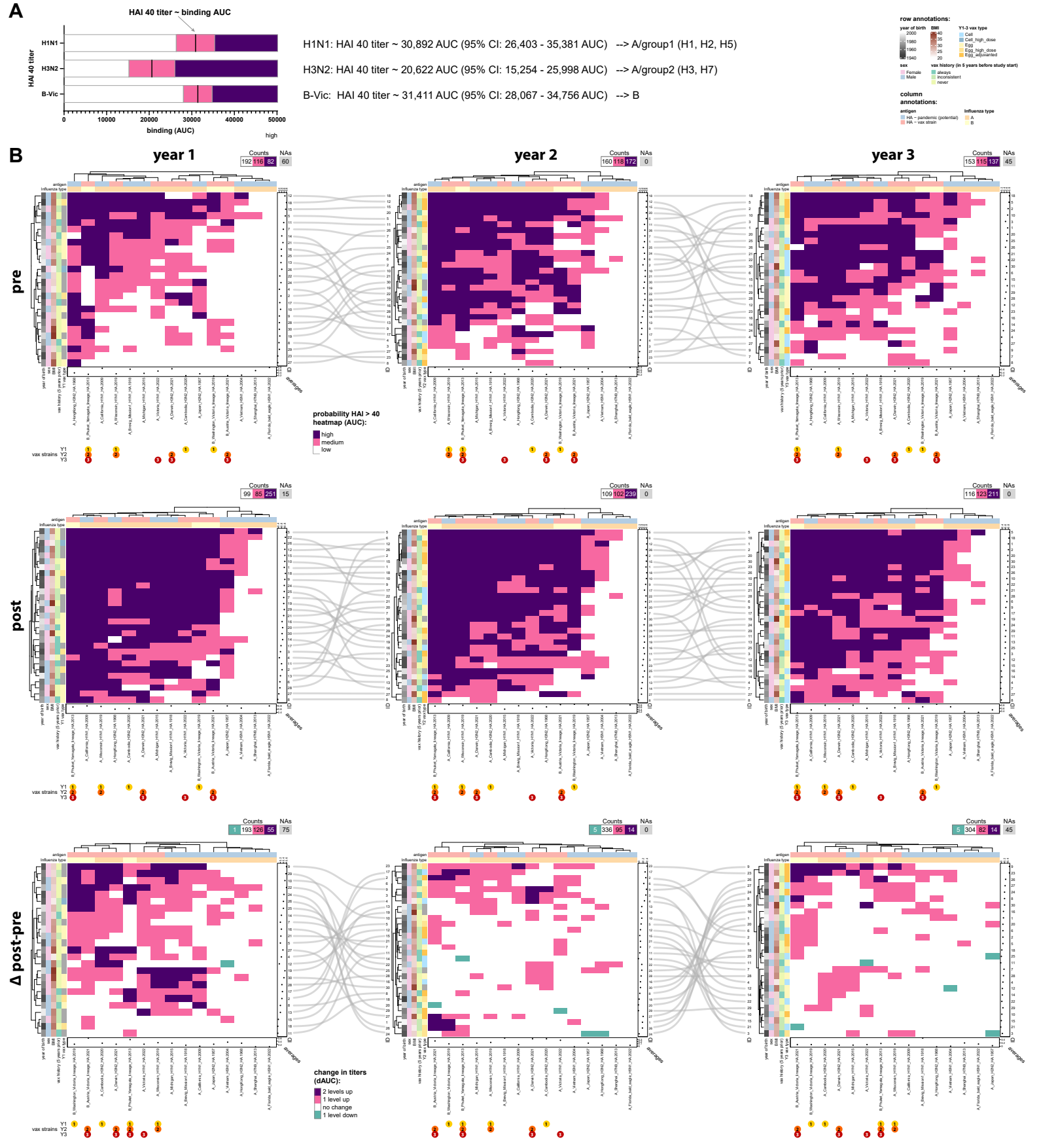

A
HAI 40 titer ~ binding AUC
H1N1
H3N2
B-Vic
HAI 40 titer
0
10000
20000
30000
40000
50000
binding (AUC)
high
H1N1: HAI 40 titer ~ 30,892 AUC (95% CI: 26,403 - 35,381 AUC) --> A/group1 (H1, H2, H5)
H3N2: HAI 40 titer ~ 20,622 AUC (95% CI: 15,254 - 25,998 AUC) --> A/group2 (H3, H7)
B-Vic: HAI 40 titer ~ 31,411 AUC (95% CI: 28,067 - 34,756 AUC) --> B
row annotations:
year of birth
BMI
Y1-3 vax type
Cell
Cell_high_dose
Egg
Egg_high_dose
Egg_adjuvanted
sex
Female
Male
vax history (in 5 years before study start)
always
inconsistent
never
column annotations:
antigen
HA ~ pandemic (potential)
HA ~ vax strain
Influenza type
A
B
B
year 1
year 2
year 3
Counts
NAs
192 116 82
60
160 118 172
0
153 115 137
45
pre
99 85 251
15
109 102 239
0
116 123 211
0
post
1 193 126 55
75
5 336 95 14
0
5 304 82 14
45
Δ post-pre
antigen
Influenza type
year of birth
sex
BMI
vax history (5 years prior)
Y1 vax type
averages
vax strains
Y1
Y2
Y3
probability HAI > 40 heatmap (AUC):
high
medium
low
change in titers (dAUC):
2 levels up
1 level up
no change
1 level down

Figure S12. Model for estimating protective Influenza responses according to HAI correlations.
A. Linear mixed models using data from Figure 3 to interpolate binding levels correlating with HAI 40 titers including 95% confidence intervals (CI), for Influenza type A group 1, 2, and type B hemagglutinin responses. Values >95% CI are considered highly probable to have HAI > 40 titers and protective against related viruses. **B.** Heatmaps as in **Figure S12** applying color-coding as in **A** per Influenza type and group and focusing on hemagglutinin responses only. Counts of responses in the respective categories are shown on the top right for each heatmap. Unavailable data points (NAs), i.e., missed participant visits, are counted but not shown in heatmaps (empty rows).

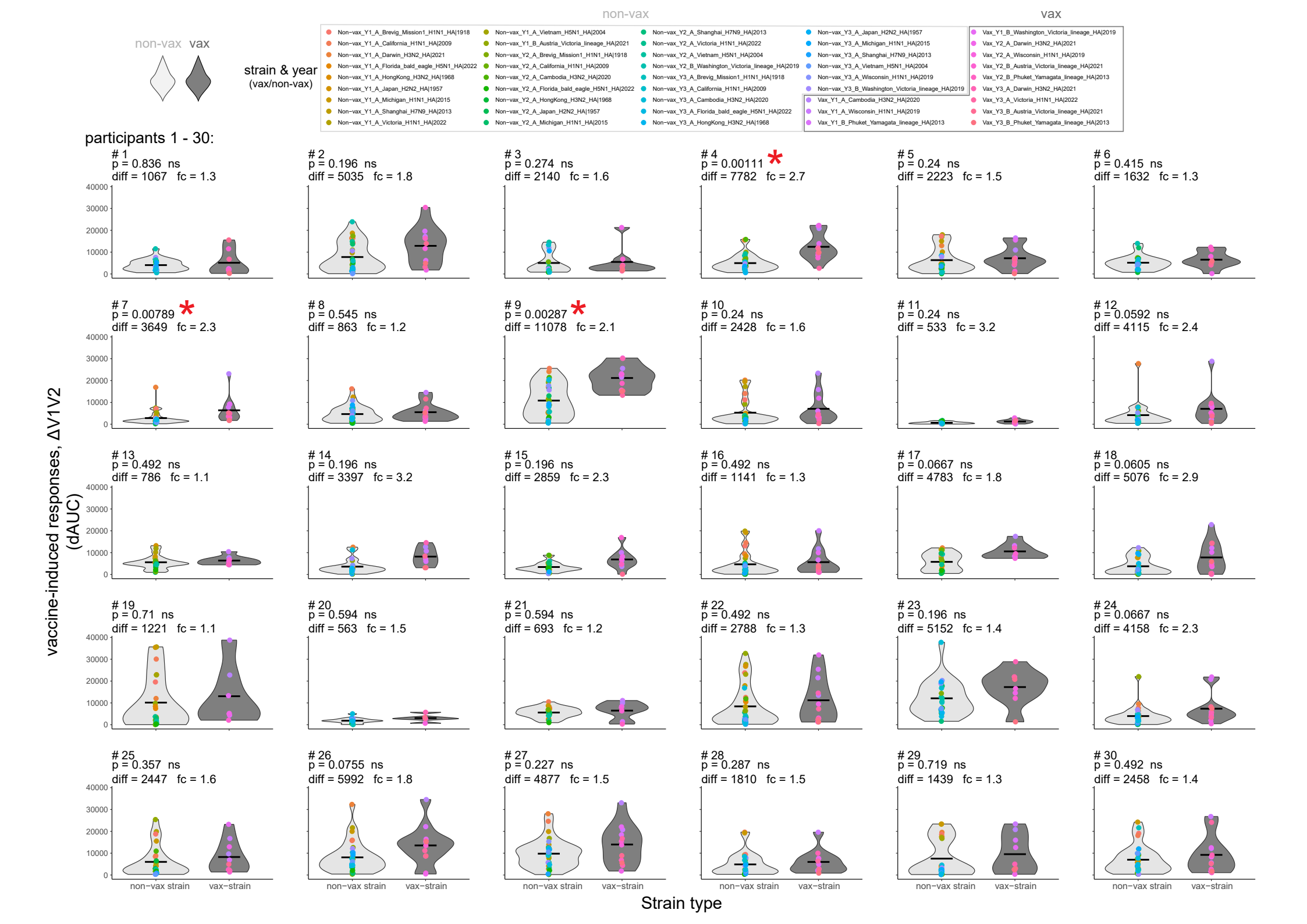

non-vax vax
strain & year
(vax/non-vax)
non-vax
vax
Non−vax_Y1_A_Brevig_Mission1_H1N1_HA|1918
Non−vax_Y1_A_California_H1N1_HA|2009
Non−vax_Y1_A_Darwin_H3N2_HA|2021
Non−vax_Y1_A_Florida_bald_eagle_H5N1_HA|2022
Non−vax_Y1_A_HongKong_H3N2_HA|1968
Non−vax_Y1_A_Japan_H2N2_HA|1957
Non−vax_Y1_A_Michigan_H1N1_HA|2015
Non−vax_Y1_A_Shanghai_H7N9_HA|2013
Non−vax_Y1_A_Victoria_H1N1_HA|2022
Non−vax_Y1_A_Vietnam_H5N1_HA|2004
Non−vax_Y1_B_Austria_Victoria_lineage_HA|2021
Non−vax_Y2_A_Brevig_Mission1_H1N1_HA|1918
Non−vax_Y2_A_California_H1N1_HA|2009
Non−vax_Y2_A_Cambodia_H3N2_HA|2020
Non−vax_Y2_A_Florida_bald_eagle_H5N1_HA|2022
Non−vax_Y2_A_HongKong_H3N2_HA|1968
Non−vax_Y2_A_Japan_H2N2_HA|1957
Non−vax_Y2_A_Michigan_H1N1_HA|2015
Non−vax_Y2_A_Shanghai_H7N9_HA|2013
Non−vax_Y2_A_Victoria_H1N1_HA|2022
Non−vax_Y2_A_Vietnam_H5N1_HA|2004
Non−vax_Y2_B_Washington_Victoria_lineage_HA|2019
Non−vax_Y3_A_Brevig_Mission1_H1N1_HA|1918
Non−vax_Y3_A_California_H1N1_HA|2009
Non−vax_Y3_A_Cambodia_H3N2_HA|2020
Non−vax_Y3_A_Florida_bald_eagle_H5N1_HA|2022
Non−vax_Y3_A_HongKong_H3N2_HA|1968
Non−vax_Y3_A_Japan_H2N2_HA|1957
Non−vax_Y3_A_Michigan_H1N1_HA|2015
Non−vax_Y3_A_Shanghai_H7N9_HA|2013
Non−vax_Y3_A_Vietnam_H5N1_HA|2004
Non−vax_Y3_A_Wisconsin_H1N1_HA|2019
Non−vax_Y3_B_Washington_Victoria_lineage_HA|2019
Vax_Y1_A_Cambodia_H3N2_HA|2020
Vax_Y1_A_Wisconsin_H1N1_HA|2019
Vax_Y1_B_Phuket_Yamagata_lineage_HA|2013
Vax_Y1_B_Washington_Victoria_lineage_HA|2019
Vax_Y2_A_Darwin_H3N2_HA|2021
Vax_Y2_A_Wisconsin_H1N1_HA|2019
Vax_Y2_B_Austria_Victoria_lineage_HA|2021
Vax_Y2_B_Phuket_Yamagata_lineage_HA|2013
Vax_Y3_A_Darwin_H3N2_HA|2021
Vax_Y3_A_Victoria_H1N1_HA|2022
Vax_Y3_B_Austria_Victoria_lineage_HA|2021
Vax_Y3_B_Phuket_Yamagata_lineage_HA|2013
participants 1 - 30:
# 1
p = 0.836 ns
diff = 1067 fc = 1.3
# 2
p = 0.196 ns
diff = 5035 fc = 1.8
# 3
p = 0.274 ns
diff = 2140 fc = 1.6
# 4
p = 0.00111 *
diff = 7782 fc = 2.7
# 5
p = 0.24 ns
diff = 2223 fc = 1.5
# 6
p = 0.415 ns
diff = 1632 fc = 1.3
# 7
p = 0.00789 *
diff = 3649 fc = 2.3
# 8
p = 0.545 ns
diff = 863 fc = 1.2
# 9
p = 0.00287 *
diff = 11078 fc = 2.1
# 10
p = 0.24 ns
diff = 2428 fc = 1.6
# 11
p = 0.24 ns
diff = 533 fc = 3.2
# 12
p = 0.0592 ns
diff = 4115 fc = 2.4
# 13
p = 0.492 ns
diff = 786 fc = 1.1
# 14
p = 0.196 ns
diff = 3397 fc = 3.2
# 15
p = 0.196 ns
diff = 2859 fc = 2.3
# 16
p = 0.492 ns
diff = 1141 fc = 1.3
# 17
p = 0.0667 ns
diff = 4783 fc = 1.8
# 18
p = 0.0605 ns
diff = 5076 fc = 2.9
# 19
p = 0.71 ns
diff = 1221 fc = 1.1
# 20
p = 0.594 ns
diff = 563 fc = 1.5
# 21
p = 0.594 ns
diff = 693 fc = 1.2
# 22
p = 0.492 ns
diff = 2788 fc = 1.3
# 23
p = 0.196 ns
diff = 5152 fc = 1.4
# 24
p = 0.0667 ns
diff = 4158 fc = 2.3
# 25
p = 0.357 ns
diff = 2447 fc = 1.6
# 26
p = 0.0755 ns
diff = 5992 fc = 1.8
# 27
p = 0.227 ns
diff = 4877 fc = 1.5
# 28
p = 0.287 ns
diff = 1810 fc = 1.5
# 29
p = 0.719 ns
diff = 1439 fc = 1.3
# 30
p = 0.492 ns
diff = 2458 fc = 1.4
0
10000
20000
30000
40000
non−vax strain
vax−strain
vaccine-induced responses, ΔV1V2 (dAUC)
Strain type

Figure S13. Comparison of Ig binding responses against Influenza hemagglutinins included vs not included in the seasonal vaccine.
Multiple Mann-Whitney tests to compare vaccine-induced responses (differences between pre- and post-vaccination) against Influenza hemagglutinins included in the seasonal vaccine of the respective study year (right, vax) vs others that were not included (left, non-vax). Both groups are plotted next to each other in each panel, shown both as violin plots and dot plots, colored by antigen and year. Horizontal black lines indicate the means. Corrections for multiple comparisons were done using the Benjamin-Hochberg method, and adjusted p values are indicated (* $p<0.01$), as well as absolute differences (diff) and fold changes of mean dAUCs between vax and non-vax strains. dAUC: difference in area under the curve between pre- and post-vaccination time points.

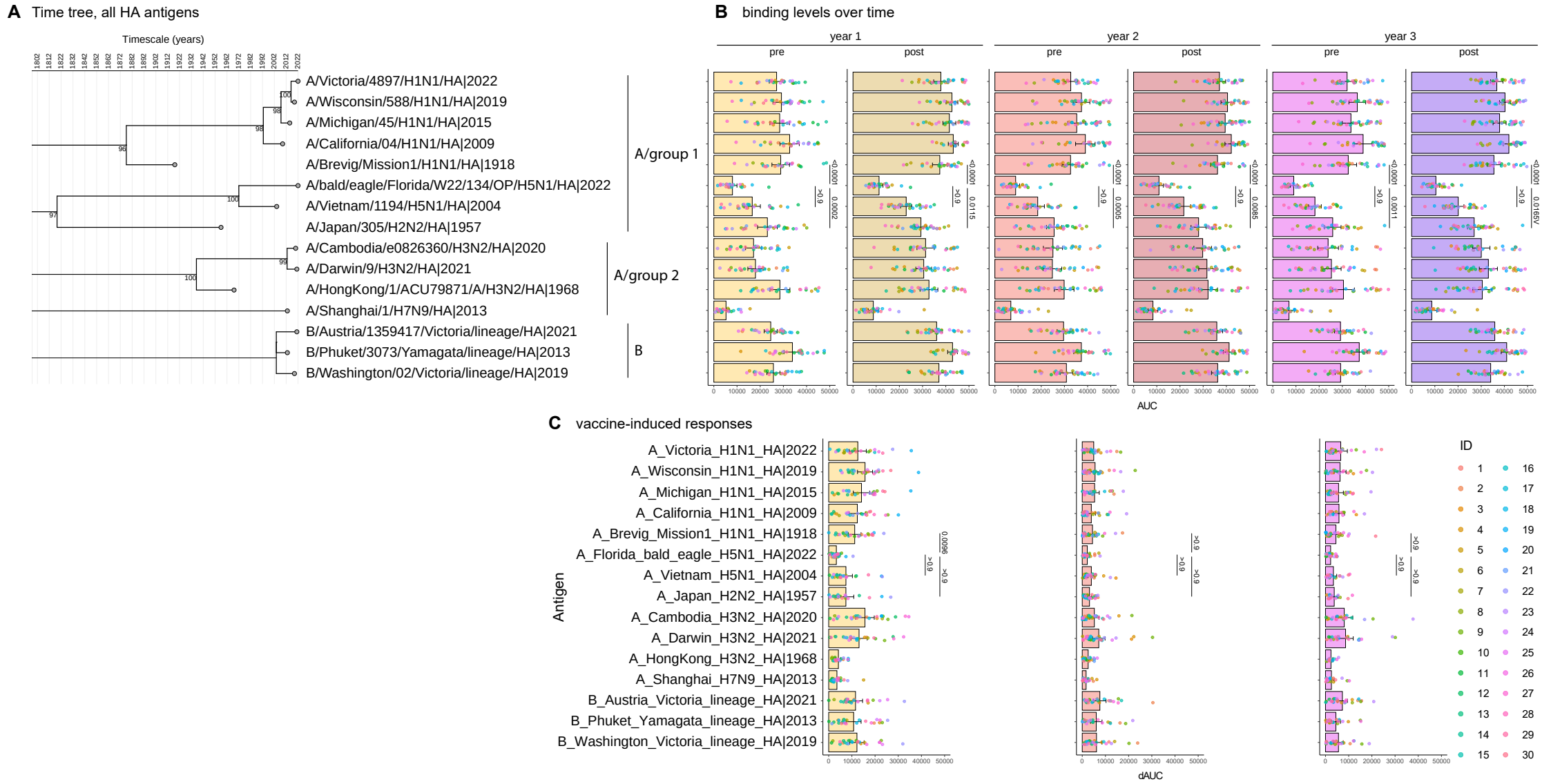

A Time tree, all HA antigens
Timescale (years)
A/Victoria/4897/H1N1/HA|2022
A/Wisconsin/588/H1N1/HA|2019
A/Michigan/45/H1N1/HA|2015
A/California/04/H1N1/HA|2009
A/Brevig/Mission1/H1N1/HA|1918
A/bald/eagle/Florida/W22/134/OP/H5N1/HA|2022
A/Vietnam/1194/H5N1/HA|2004
A/Japan/305/H2N2/HA|1957
A/Cambodia/e0826360/H3N2/HA|2020
A/Darwin/9/H3N2/HA|2021
A/HongKong/1/ACU79871/A/H3N2/HA|1968
A/Shanghai/1/H7N9/HA|2013
B/Austria/1359417/Victoria/lineage/HA|2021
B/Phuket/3073/Yamagata/lineage/HA|2013
B/Washington/02/Victoria/lineage/HA|2019
A/group 1
A/group 2
B
B binding levels over time
year 1
year 2
year 3
pre
post
AUC
C vaccine-induced responses
Antigen
A_Victoria_H1N1_HA|2022
A_Wisconsin_H1N1_HA|2019
A_Michigan_H1N1_HA|2015
A_California_H1N1_HA|2009
A_Brevig_Mission1_H1N1_HA|1918
A_Florida_bald_eagle_H5N1_HA|2022
A_Vietnam_H5N1_HA|2004
A_Japan_H2N2_HA|1957
A_Cambodia_H3N2_HA|2020
A_Darwin_H3N2_HA|2021
A_HongKong_H3N2_HA|1968
A_Shanghai_H7N9_HA|2013
B_Austria_Victoria_lineage_HA|2021
B_Phuket_Yamagata_lineage_HA|2013
B_Washington_Victoria_lineage_HA|2019
dAUC
ID

Figure S14. Anti-hemagglutinin Influenza responses by phylogenetic relatedness.
A. Time-calibrated maximum-likelihood tree as in **Figure 2B** using sequences of the studied HA antigens across (sub)types and groups. **B.** Ig binding levels shown as horizontal bar graph (means with standard deviation), aligned with the tree sequences in A. Graphs are shown for pre- and post-vaccination levels in all three study years. The individual data points are shown as dots colored by participant. **C.** Vaccine-induced responses (differences between pre- and post-vaccination visit) are plotted in the same manner, aligned below each year's visit-specific responses in **B**. Selected non-parametric Kruskal-Wallis tests with Dunn's multiplicity correction are shown, i.e., for A/Florida/H5/2022 (clade 2.3.4.4b) against neighboring H1, H2, and H5 antigens (adjusted p values).

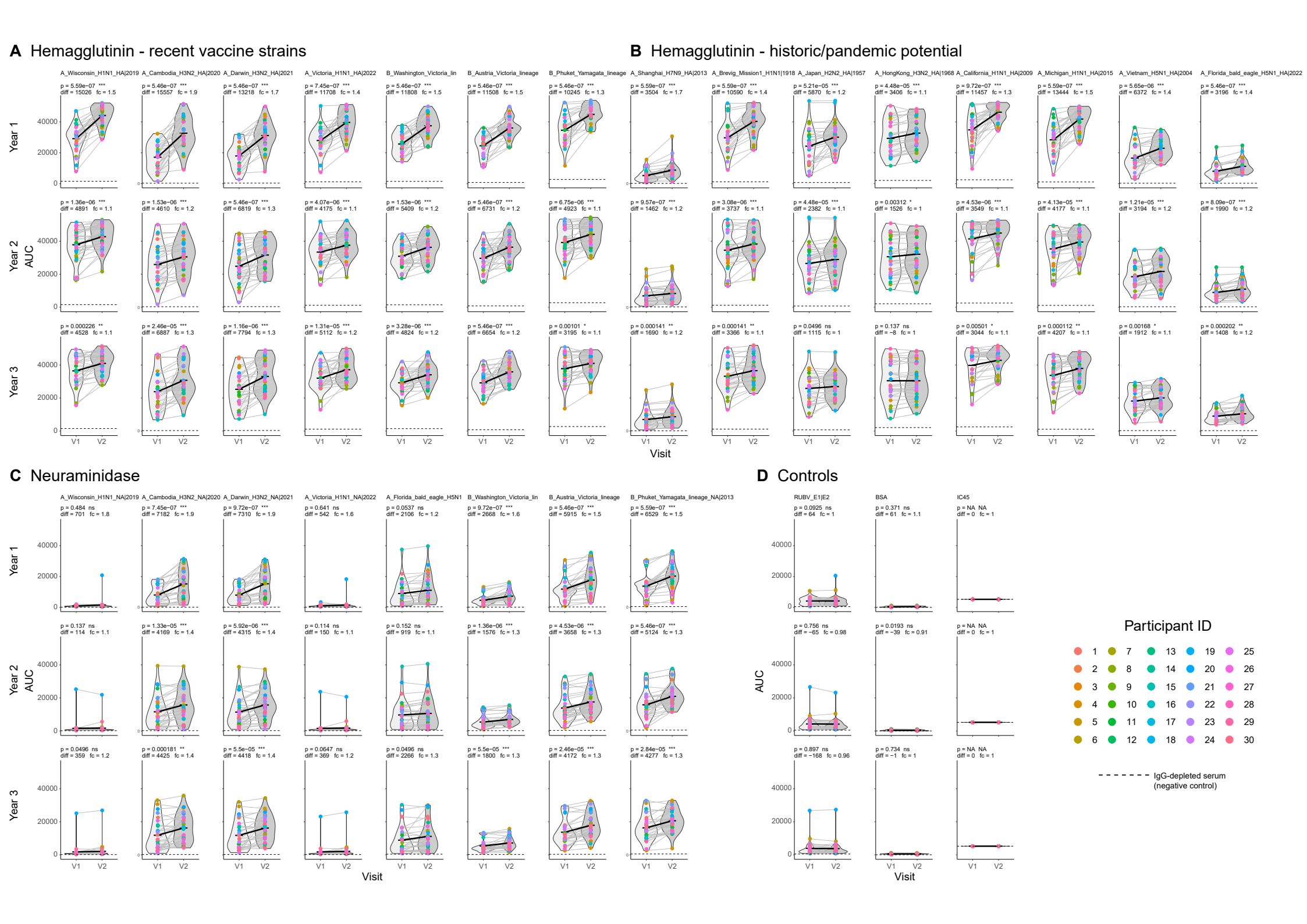

A Hemagglutinin - recent vaccine strains
B Hemagglutinin - historic/pandemic potential
C Neuraminidase
D Controls
A_Wisconsin_H1N1_HA|2019
A_Cambodia_H3N2_HA|2020
A_Darwin_H3N2_HA|2021
A_Victoria_H1N1_HA|2022
B_Washington_Victoria_lin
B_Austria_Victoria_lineage
B_Phuket_Yamagata_lineage
A_Shanghai_H7N9_HA|2013
A_Brevig_Mission1_H1N1|1918
A_Japan_H2N2_HA|1957
A_HongKong_H3N2_HA|1968
A_California_H1N1_HA|2009
A_Michigan_H1N1_HA|2015
A_Vietnam_H5N1_HA|2004
A_Florida_bald_eagle_H5N1_HA|2022
A_Wisconsin_H1N1_NA|2019
A_Cambodia_H3N2_NA|2020
A_Darwin_H3N2_NA|2021
A_Victoria_H1N1_NA|2022
A_Florida_bald_eagle_H5N1
B_Washington_Victoria_lin
B_Austria_Victoria_lineage
B_Phuket_Yamagata_lineage_NA|2013
RUBV_E1|E2
BSA
IC45
Year 1
Year 2
Year 3
AUC
Visit
V1
V2
Participant ID
IgG-depleted serum
(negative control)

Figure S15. Vaccine-induced responses by antigen and study year.
Multiple Wilcoxon paired tests were done to study vaccine-induced responses against each antigen in each year. The pre-vaccination/baseline (V1) and approximately 1-month post-vaccination time points (V2) are plotted next to each other in each panel, shown both as violin plots and dot plots, colored by participant. Linked data points (longitudinal samples of same participant) are connected by gray lines, and the average responses are connected by a bold black line. Corrections for multiple comparisons were done using the Benjamin-Hochberg method, and adjusted p values (* $p<0.01$, ** $p<0.001$, *** $p<0.0001$), absolute differences (diff) in AUC (ΔV1V2), and fold changes (fc) are indicated. The dashed line represents the response obtained with IgG-depleted serum as reference. AUC: area under the curve.

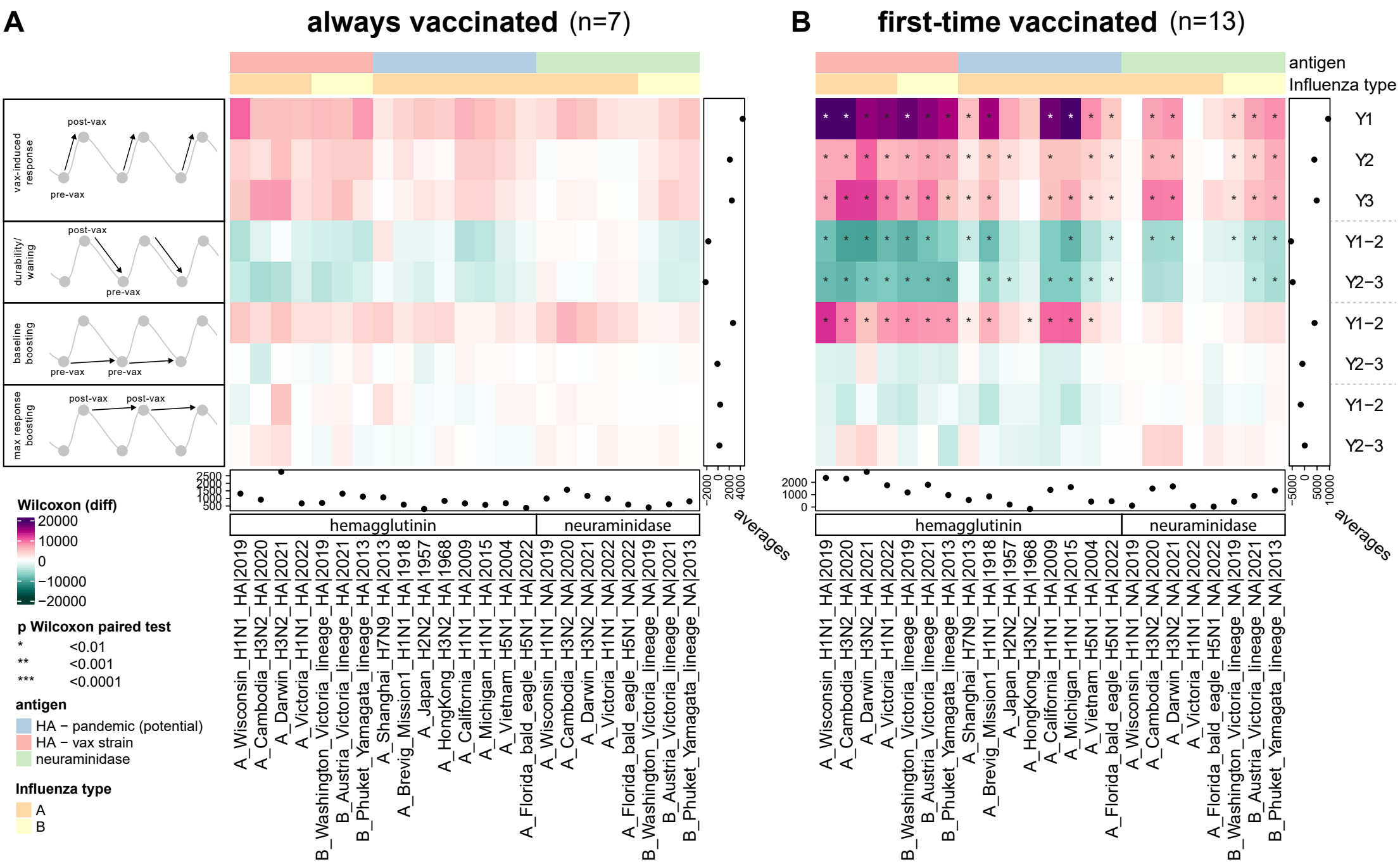

A
always vaccinated (n=7)
B
first-time vaccinated (n=13)
vax-induced response
durability/ waning
baseline boosting
max response boosting
post-vax
pre-vax
antigen
Influenza type
Y1
Y2
Y3
Y1−2
Y2−3
Wilcoxon (diff)
20000
10000
0
−10000
−20000
p Wilcoxon paired test
* <0.01
** <0.001
*** <0.0001
antigen
HA − pandemic (potential)
HA − vax strain
neuraminidase
Influenza type
A
B
hemagglutinin
neuraminidase
averages
A_Wisconsin_H1N1_HA|2019
A_Cambodia_H3N2_HA|2020
A_Darwin_H3N2_HA|2021
A_Victoria_H1N1_HA|2022
B_Washington_Victoria_lineage_HA|2019
B_Austria_Victoria_lineage_HA|2021
B_Phuket_Yamagata_lineage_HA|2013
A_Shanghai_H7N9_HA|2013
A_Brevig_Mission1_H1N1_HA|1918
A_Japan_H2N2_HA|1957
A_HongKong_H3N2_HA|1968
A_California_H1N1_HA|2009
A_Michigan_H1N1_HA|2015
A_Vietnam_H5N1_HA|2004
A_Florida_bald_eagle_H5N1_HA|2022
A_Wisconsin_H1N1_NA|2019
A_Cambodia_H3N2_NA|2020
A_Darwin_H3N2_NA|2021
A_Victoria_H1N1_NA|2022
A_Florida_bald_eagle_H5N1_NA|2022
B_Washington_Victoria_lineage_NA|2019
B_Austria_Victoria_lineage_NA|2021
B_Phuket_Yamagata_lineage_NA|2013

Figure S16. Vaccination response dynamics by vaccination history. Multiple Wilcoxon paired tests as in **Figure 4A** but split according to vaccination history in the five years before study start into participants who were always vaccinated (all five years) (**A**) and first-time vaccinated (**B**). Studied responses from top to bottom are: 1) vaccine-induced responses in each year (top), 2) waning to the following year, 3) boosting of baseline levels to the next year, and 4) changes in maximum (1-month post vax) responses to the next year (bottom). Corrections for multiple comparisons were done separately for 1) – 4) using the Benjamin-Hochberg method. Average responses per heatmap row and column are shown as dot plot to the right and bottom, respectively. The analyses were done by antigen, with Ig responses averaged across participants. Resp: response; vax: vaccination; Y: year.

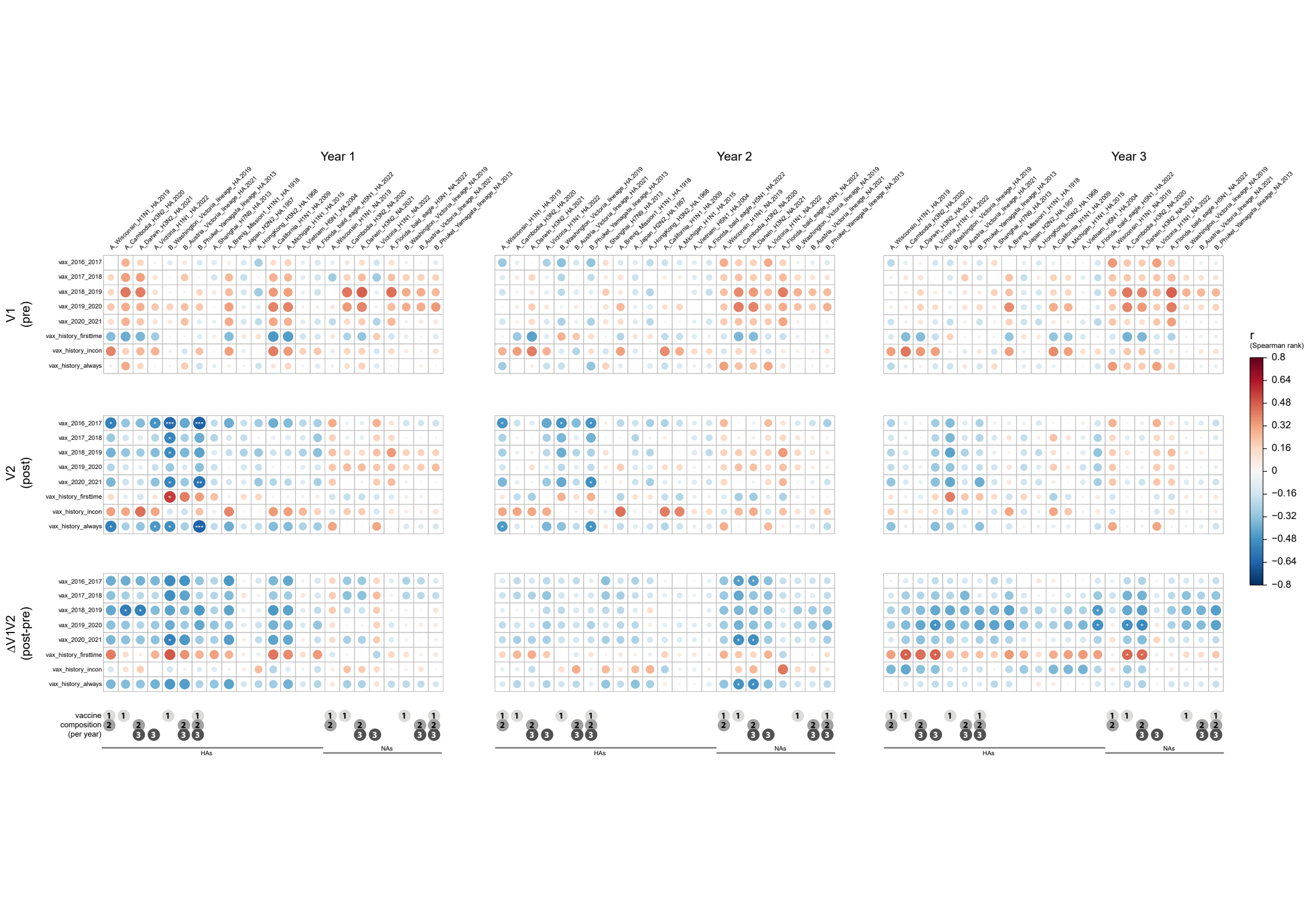

Year 1
Year 2
Year 3
V1 (pre)
V2 (post)
ΔV1V2 (post-pre)
vax_2016_2017
vax_2017_2018
vax_2018_2019
vax_2019_2020
vax_2020_2021
vax_history_firsttime
vax_history_incon
vax_history_always
A_Wisconsin_H1N1_HA.2019
A_Cambodia_H3N2_HA.2020
A_Darwin_H3N2_HA.2021
A_Victoria_H1N1_HA.2022
B_Washington_Victoria_lineage_HA.2019
B_Phuket_Yamagata_lineage_HA.2021
A_Shanghai_H7N9_HA.2013
A_Brevig_Mission1_H1N1_HA.1918
A_Japan_H2N2_HA.1957
A_Hongkong_H3N2_HA.1968
A_California_H1N1_HA.2009
A_Michigan_H1N1_HA.2015
A_Vietnam_H5N1_HA.2004
A_Florida_bald_eagle_H5N1_HA.2022
A_Wisconsin_H1N1_NA.2019
A_Cambodia_H3N2_NA.2020
A_Darwin_H3N2_NA.2021
A_Victoria_H1N1_NA.2022
A_Florida_bald_eagle_H5N1_NA.2022
B_Washington_Victoria_lineage_NA.2019
B_Austria_Victoria_lineage_NA.2021
B_Phuket_Yamagata_lineage_NA.2013
r
(Spearman rank)
0.8
0.64
0.48
0.32
0.16
0
−0.16
−0.32
−0.48
−0.64
−0.8
vaccine composition (per year)
HAs
NAs

Figure S17. Impact of vaccination history on antigen-specific Ig binding responses. The impact of previous vaccination was analyzed in all study years at time points pre-vaccination (top, V1), 1-month post-vaccination (middle, V2), and for vaccine-induced responses (bottom, dV1V2). Vaccination history referred to the five years before study start (y-axis; individual years or summed up as first-time, inconsistent, or always vaccinated), analyzed across all antigens (x-axis). Spearman rank correlations are summarized in correlograms, where red and blue colors indicate positive and negative correlations, respectively. The color intensity and size of circles indicate correlation strength. White asterisks indicate significance (* $p<0.05$, ** $p<0.01$, *** $p<0.005$). The gray spheres at the bottom indicate which antigens were included in the seasonal vaccine for the respective year.


A
HA vaccine strains
HA - pandemic (potential) strains
NA - vaccine strains & H5N1
binding levels (AUC)
Year 1
Year 2
Year 3
pre
post
AUC
year_of_birth
A_Wisconsin_H1N1_HA|2019
A_Cambodia_H3N2_HA|2020
A_Darwin_H3N2_HA|2021
A_Victoria_H1N1_HA|2022
B_Washington_Victoria_lineage_HA|2019
B_Austria_Victoria_lineage_HA|2021
B_Phuket_Yam_lineage_HA|2013
p=0.0245, adj.R2=0.16
A_Shanghai_H7N9_HA|2013
A_Brevig_Mission1_H1N1_HA|1918
A_Japan_H2N2_HA|1957
p=0.000345, adj.R2=0.396
A_HongKong_H3N2_HA|1968
p=0.00286, adj.R2=0.286
A_California_H1N1_HA|2009
A_Michigan_H1N1_HA|2015
A_Vietnam_H5N1_HA|2004
p=0.0167, adj.R2=0.183
A_Florida_bald_eagle_H5N1_HA|2022
p=0.0126, adj.R2=0.201
A_Wisconsin_H1N1_NA|2019
A_Cambodia_H3N2_NA|2020
A_Darwin_H3N2_NA|2021
A_Victoria_H1N1_NA|2022
A_Florida_bald_eagle_H5N1_NA|2022
p=0.00346, adj.R2=0.276
B_Washington_Victoria_lineage_NA|2019
B_Austria_Victoria_lineage_NA|2021
B_Phuket_Yamagata_lineage_NA|2013
p=0.0107, adj.R2=0.189
p=0.00677, adj.R2=0.213
p=0.0015, adj.R2=0.291
p=0.0148, adj.R2=0.165
p=0.00105, adj.R2=0.299
p=0.00862, adj.R2=0.194
p=0.00147, adj.R2=0.283
p=0.00141, adj.R2=0.285
p=0.0145, adj.R2=0.166
p=0.0333, adj.R2=0.121
p=0.0253, adj.R2=0.136
p=0.00776, adj.R2=0.2
p=0.00904, adj.R2=0.212
p=0.0222, adj.R2=0.16
p=0.00143, adj.R2=0.313
p=0.000851, adj.R2=0.309
p=0.0162, adj.R2=0.161
p=0.013, adj.R2=0.172
p=0.0222, adj.R2=0.144
B
HA vaccine strains
HA - pandemic (potential) strains
NA - vaccine strains & H5N1
vaccine response (dAUC)
Year 1
Year 2
Year 3
dAUC
year_of_birth
A_Wisconsin_H1N1_HA|2019
A_Cambodia_H3N2_HA|2020
A_Darwin_H3N2_HA|2021
A_Victoria_H1N1_HA|2022
B_Washington_Victoria_lineage_HA|2019
B_Austria_Victoria_lineage_HA|2021
B_Phuket_Yamagata_lineage_HA|2013
p=0.0243, adj.R2=0.167
A_Shanghai_H7N9_HA|2013
A_Brevig_Mission1_H1N1_HA|1918
A_Japan_H2N2_HA|1957
p=0.0126, adj.R2=0.307
A_HongKong_H3N2_HA|1968
A_California_H1N1_HA|2009
A_Michigan_H1N1_HA|2015
A_Vietnam_H5N1_HA|2004
p=0.0335, adj.R2=0.146
A_Florida_bald_eagle_H5N1_HA|2022
A_Wisconsin_H1N1_NA|2019
A_Cambodia_H3N2_NA|2020
A_Darwin_H3N2_NA|2021
A_Victoria_H1N1_NA|2022
A_Florida_bald_eagle_H5N1_NA|2022
B_Washington_Victoria_lineage_NA|2019
B_Austria_Victoria_lineage_NA|2021
B_Phuket_Yamagata_lineage_NA|2013
p=0.0177, adj.R2=0.156
p=0.0455, adj.R2=0.104
p=0.00396, adj.R2=0.234
p=0.0432, adj.R2=0.12
p=0.0319, adj.R2=0.138
p=0.00215, adj.R2=0.292
p=0.0103, adj.R2=0.205
p=0.0296, adj.R2=0.143
p=0.0226, adj.R2=0.159

Figure S18. Serological imprinting by year of birth across multiple antigens and study years, shown as linear regressions.
Multiple linear regression analysis of Ig binding levels (**A**) or vaccine-induced responses (differences between pre- and post-vaccination) (**B**) on the y-axis by year of birth on the x-axis. Individual data points are shown as dots, colored by participants. Corrections for multiple comparisons were done using the Benjamin-Hochberg method, and adjusted p values are indicated. Significant results ($p<0.05$) are shown with regression line, including 95% confidence interval and goodness-of-fit (R-squared). The dashed line represents the response obtained with IgG-depleted serum as reference. AUC: area under the curve. dAUC: difference in AUC between pre- and post-vaccination time points.


A
B
HA vaccine strains
HA - pandemic (potential) strains
NA - vaccine strains & H5N1
controls
binding levels (AUC)
vaccine response (dAUC)
Year 1
Year 2
Year 3
pre
post
Participant ID
IgG-depl. serum

Figure S19. Serological imprinting by year of birth, shown side-by-side comparing individuals born <1968 and ≥1968.

Multiple Mann-Whitney tests to compare binding levels (**A**) or vaccine-induced responses (differences between pre- and post-vaccination) (**B**) in participants born <1968 and ≥1968. Both groups are plotted next to each other in each panel, shown both as violin plots and dot plots, colored by participant. Corrections for multiple comparisons were done using the Benjamin-Hochberg method, and adjusted p values are indicated (* $p<0.05$). The dashed line represents the response obtained with IgG-depleted serum as reference. AUC: area under the curve. dAUC: difference in AUC between pre- and post-vaccination time points.

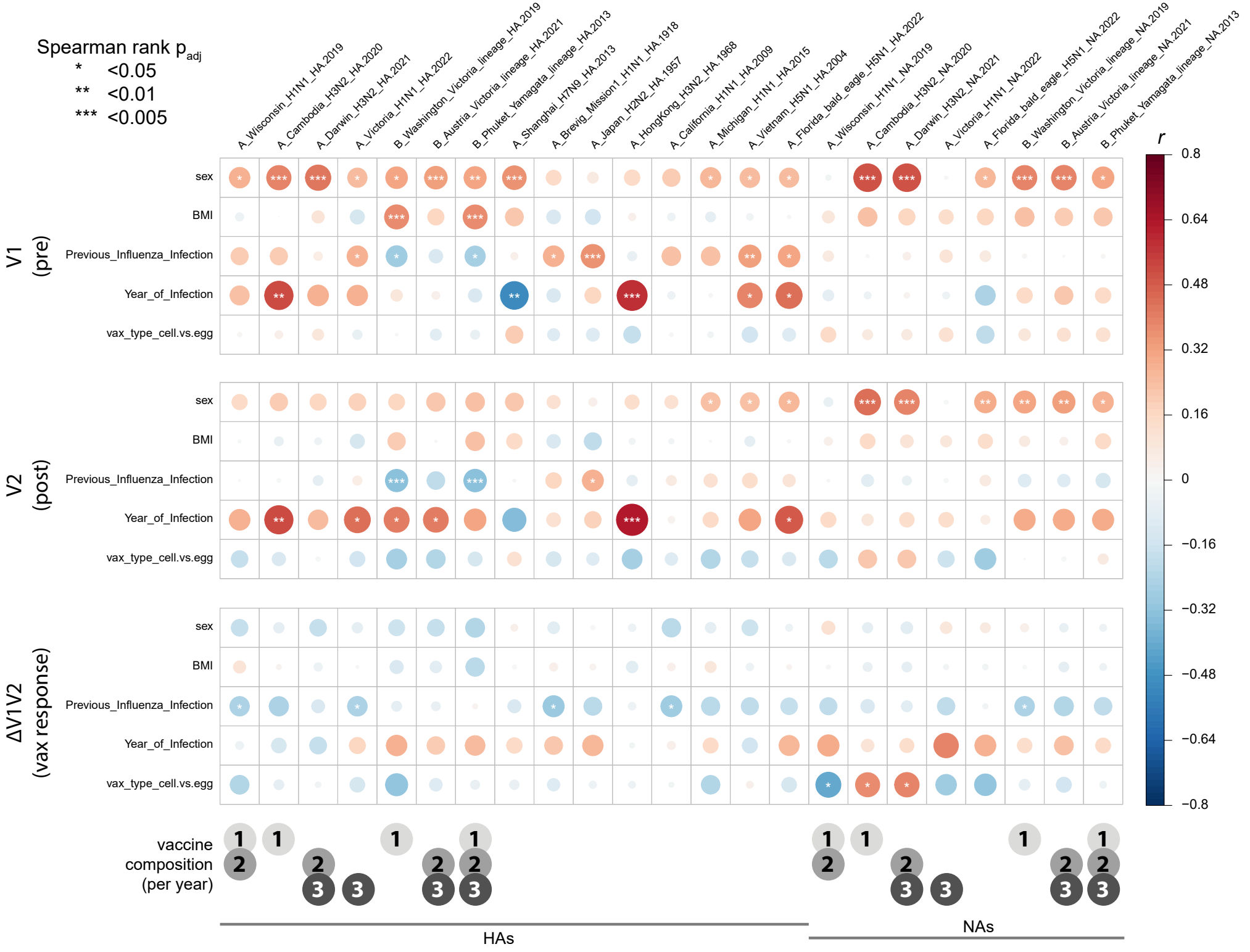

Spearman rank p_adj
* <0.05
** <0.01
*** <0.005
A_Wisconsin_H1N1_HA.2019
A_Cambodia_H3N2_HA.2020
A_Darwin_H3N2_HA.2021
A_Victoria_H1N1_HA.2022
B_Washington_Victoria_lineage_HA.2019
B_Austria_Victoria_lineage_HA.2021
B_Phuket_Yamagata_lineage_HA.2013
A_Shanghai_H7N9_HA.2013
A_Brevig_Mission1_H1N1_HA.1918
A_Japan_H2N2_HA.1957
A_HongKong_H3N2_HA.1968
A_California_H1N1_HA.2009
A_Michigan_H1N1_HA.2015
A_Vietnam_H5N1_HA.2004
A_Florida_bald_eagle_H5N1_HA.2022
A_Wisconsin_H1N1_NA.2019
A_Cambodia_H3N2_NA.2020
A_Darwin_H3N2_NA.2021
A_Victoria_H1N1_NA.2022
A_Florida_bald_eagle_H5N1_NA.2022
B_Washington_Victoria_lineage_NA.2019
B_Austria_Victoria_lineage_NA.2021
B_Phuket_Yamagata_lineage_NA.2013
V1 (pre)
V2 (post)
ΔV1V2 (vax response)
sex
BMI
Previous_Influenza_Infection
Year_of_Infection
vax_type_cell.vs.egg
r
0.8
0.64
0.48
0.32
0.16
0
−0.16
−0.32
−0.48
−0.64
−0.8
vaccine composition (per year)
HAs
NAs

Figure S20. Correlation analysis of antigen-specific binding responses, clinical, and demographic information.
Multivariable correlation analyses of anti-Influenza binding responses (x-axis) against selected clinical and demographic variables (y-axis) at baseline/pre-vaccination (top), approximately 1-month post-vaccination (middle), and for vaccine-induced responses (bottom, difference between pre- and post-vaccination levels). Spearman rank correlations are summarized in correlograms, where red and blue colors indicate positive and negative correlations, respectively. The color intensity and size of circles indicate correlation strength. White asterisks indicate adjusted significance after Benjamini-Hochberg multiplicity correction (* $p<0.05$, ** $p<0.01$, *** $p<0.005$). The gray spheres at the bottom indicate which antigens were included in the seasonal vaccine for the respective year. Sex: male vs female. Total of n=90 data points from 30 participants over 3 years.

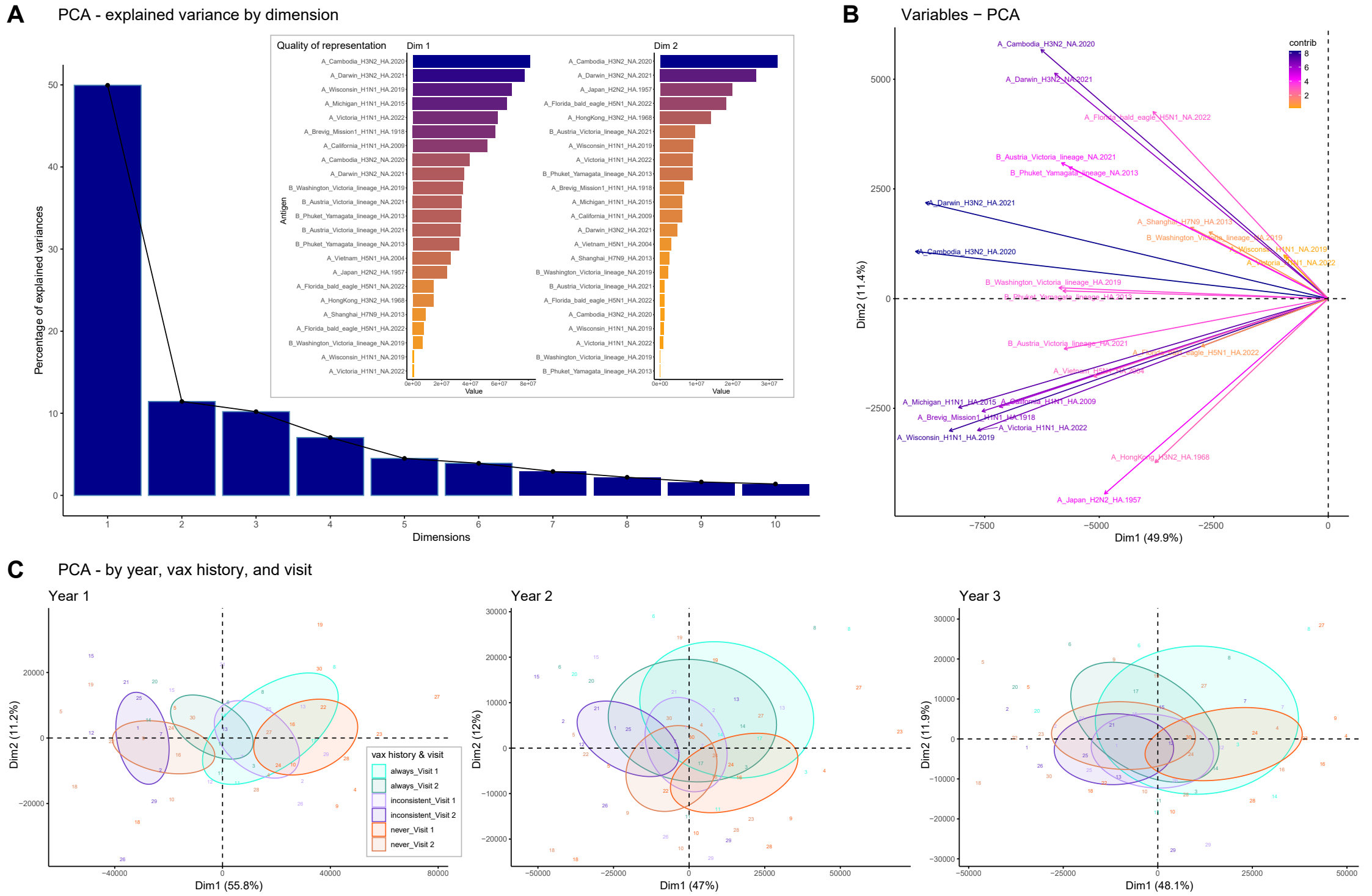
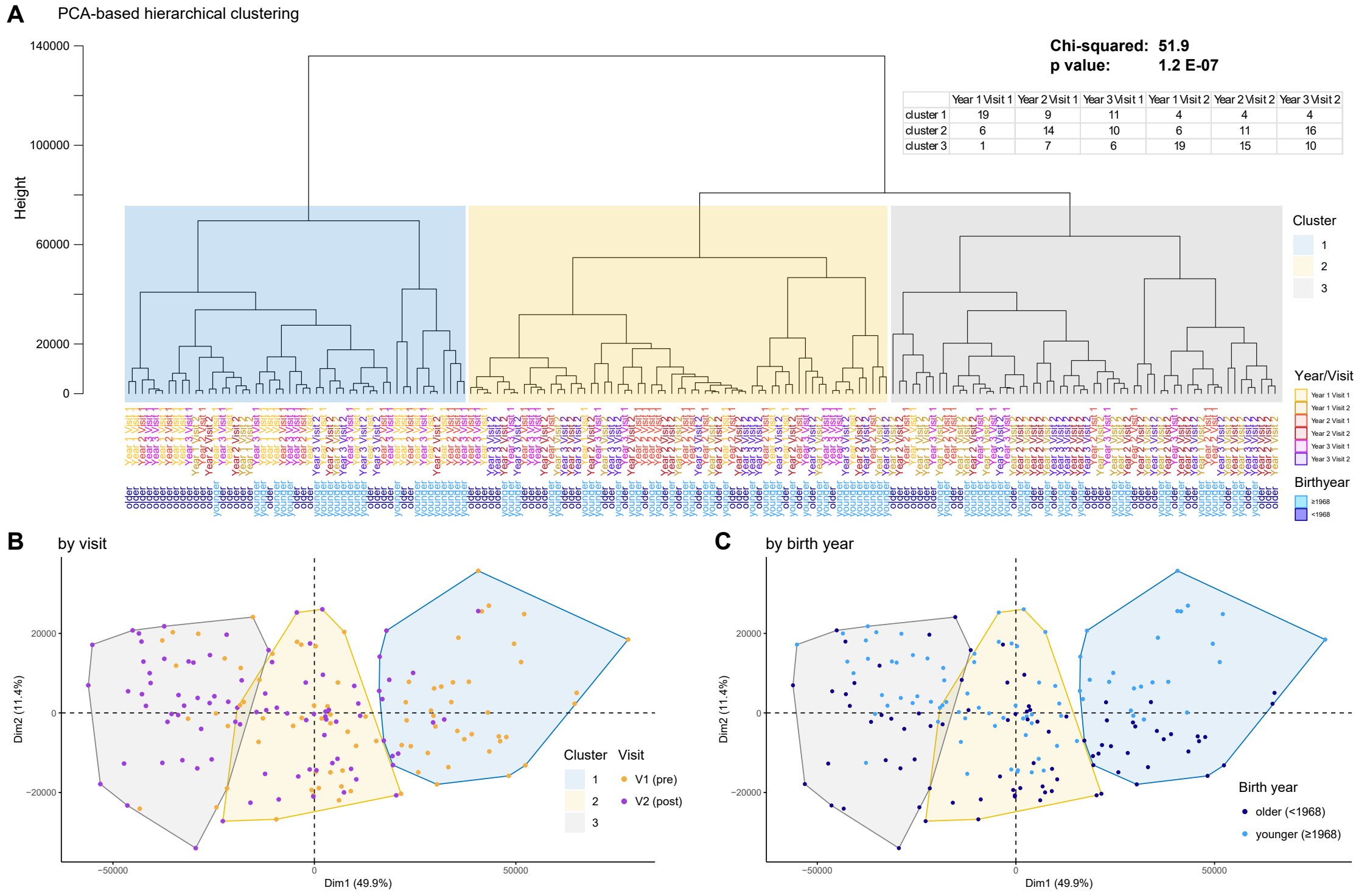

A
PCA - explained variance by dimension
Percentage of explained variances
Dimensions
Quality of representation
Dim 1
Dim 2
Antigen
Value
B
Variables − PCA
contrib
Dim2 (11.4%)
Dim1 (49.9%)
A_Cambodia_H3N2_NA.2020
A_Darwin_H3N2_NA.2021
A_Florida_bald_eagle_H5N1_NA.2022
B_Austria_Victoria_lineage_NA.2021
B_Phuket_Yamagata_lineage_NA.2013
A_Darwin_H3N2_HA.2021
A_Shanghai_H7N9_HA.2013
B_Washington_Victoria_lineage_HA.2019
A_Wisconsin_H1N1_NA.2019
A_Victoria_H1N1_NA.2022
A_Cambodia_H3N2_HA.2020
B_Washington_Victoria_lineage_HA.2019
B_Phuket_Yamagata_lineage_HA.2013
B_Austria_Victoria_lineage_HA.2021
A_Florida_bald_eagle_H5N1_HA.2022
A_Vietnam_H5N1_HA.2004
A_Michigan_H1N1_HA.2015
A_California_H1N1_HA.2009
A_Brevig_Mission1_H1N1_HA.1918
A_Victoria_H1N1_HA.2022
A_Wisconsin_H1N1_HA.2019
A_HongKong_H3N2_HA.1968
A_Japan_H2N2_HA.1957
C
PCA - by year, vax history, and visit
Year 1
Year 2
Year 3
Dim2 (11.2%)
Dim1 (55.8%)
Dim2 (12%)
Dim1 (47%)
Dim2 (11.9%)
Dim1 (48.1%)
vax history & visit
always_Visit 1
always_Visit 2
inconsistent_Visit 1
inconsistent_Visit 2
never_Visit 1
never_Visit 2

Figure S21. Principal component analysis (PCA) of multiplex binding responses.
A. PCA of 23 anti-Influenza Ig responses (against 15 HA and 8 NA antigens), with explained variance shown by dimension. Boxed areas indicate the quality of representation for dimensions 1 and 2, which were used in further analysis. The data include 30 participants from the NYU StopFlu vaccination cohort, with 172 total visits collected pre- and post-vaccination over three years. **B.** Variable plot illustrating the contribution strength and direction of the 23 variables in dimensions 1 and 2. **C.** PCA segmented by influenza vaccination history (based on 5 years before study start) and visit (visit 1: pre-vaccination; visit 2: approximately 1 month post-vaccination), displayed for all three study years from left to right. Ellipses depict 95% confidence intervals of the means.



	Year 1 Visit 1	Year 2 Visit 1	Year 3 Visit 1	Year 1 Visit 2	Year 2 Visit 2	Year 3 Visit 2
cluster 1	19	9	11	4	4	4
cluster 2	6	14	10	6	11	16
cluster 3	1	7	6	19	15	10

Figure S22. PCA-based hierarchical clustering.
A. PCA-based k-means hierarchical clustering of the first two principal components, using the same data set of 23 multiplex anti-Influenza Ig responses (against 15 HA and 8 NA antigens) on 172 data points as in **Fig. 21**. In the dendrogram, the clusters are highlighted with colored boxes; data points are labeled by year/visit (visits 1 and 2 staggered) and birth year, stratified into earlier than 1968 (older) or 1968 and later (younger). **B,C.** The 2-dimensional positioning of data points by visit (**B**) and birth year (**C**) in relation to the clustering.

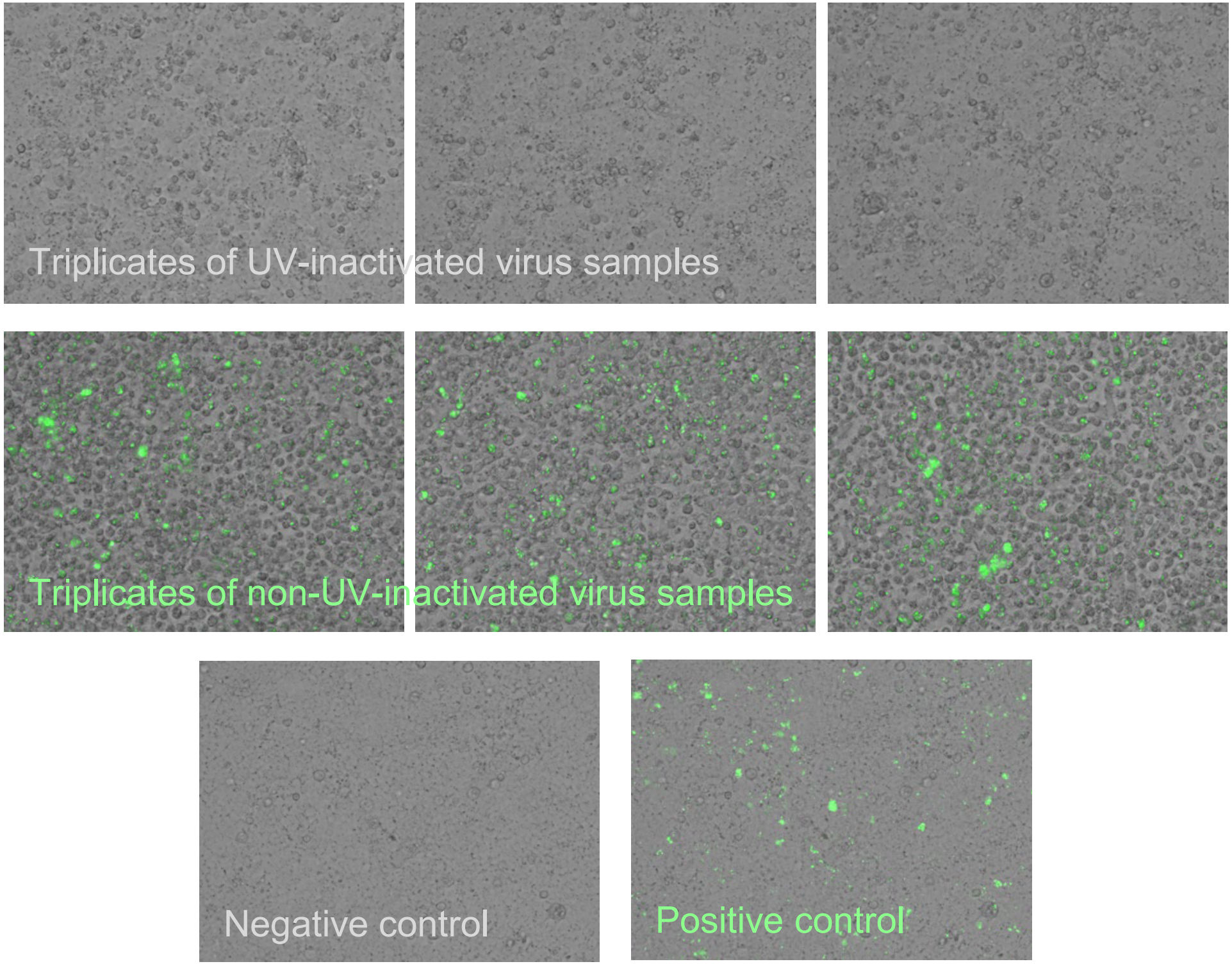

Triplicates of UV-inactivated virus samples
Triplicates of non-UV-inactivated virus samples
Negative control
Positive control

Figure S23. UV-inactivation of H5N1 mouse immune sera.
Immune serum obtained from mice challenged and vaccinated with H5N1 virus was UV-inactivated before conducting serological analyses. The inactivation process involved exposing the samples to UV light for 20 minutes (using a UV lamp with a 254 nm wavelength, 6 watts, positioned 3-5 cm above the samples). A positive control, PR8 GPF reporter virus, was included in the inactivation process, while the negative control contained no virus. After UV inactivation, the samples were inoculated onto MDCK cells, and the GFP signal was checked after 24 hours.