

## Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Hancock K, Veguilla V, Lu X, et al. Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. *N Engl J Med* 2009;361:1945-52. DOI: [10.1056/NEJMoa0906453](https://doi.org/10.1056/NEJMoa0906453).

## **Supplementary Appendix**

**Supplement to Hancock K, Veguilla V, Lu X et al.,**

**Supplementary online Materials and Methods, Tables, and Figure**

### ***Study design***

Stored sera from 124 pediatric participants, ages 6 months to 9 years, 348 adult participants, 18 to 64 years, and 188 older adult participants,  $\geq 60$  years were received from different sources as outlined in the Supplementary Figure. Unless otherwise indicated, sera were from U.S. residents immunized with one dose of either trivalent, inactivated influenza vaccine (TIV) intramuscularly, with or without an adjuvant, or with live, attenuated influenza vaccine (LAIV) intranasally. In pediatric studies, children received two doses of vaccine if they had not received influenza vaccination in a previous year. The vaccine products received by the study participants included those from Sanofi-Pasteur, GlaxoSmithKline Biologicals (formulated with or without the proprietary adjuvant ASO3), Novartis (formulated with or without the proprietary adjuvant MF-59), CSL Biotherapies and MedImmune all of which were formulated for northern hemisphere influenza seasons according to WHO recommendations. The H1N1 components in the trivalent influenza vaccines were an A/New Caledonia/20/99-like virus for 2005-2006 and 2006-2007 seasons, an A/Solomon Islands/3/2006-like virus for the 2007-2008 season, and an A/Brisbane/59/2007-like virus for the 2008-2009 season. Paired sera from 83 adults who received one dose (400 chick cell-agglutinating units) intramuscularly of a monovalent, split A/New Jersey/76 virus vaccine (Wyeth Laboratories, Philadelphia, PA) in 1976 were also tested. All vaccinees were 25 years or

older at the time and thus had been naturally exposed to H1N1 viruses circulating prior to 1957 (reference 16 in main text).

A panel of 417 anonymous normal human sera (NHS) collected from U.S. residents either in 1971 (n=59) or between 2002 and February, 2009 (n=358) were also tested. The archived sera collected in 1971 were from residents of the metropolitan area of Atlanta, GA participating in an influenza vaccine study conducted at Emory University School of Medicine by Marine and Thomas (reference 8 in main text). Participants received either an H2N2 (A/Japan/305/57) or H3N2 (A/Aichi/2/68) vaccine. Of the 59 sera, 48 were pre-vaccination sera and 11 were post-vaccination sera. The titers for serum samples that were collected in 1971 are not included in the cumulative GMT for the decades 1980, 1990, and 2000 since the samples can not reflect exposure to influenza viruses in the subsequent decades. The sera collected between 2002 and February 2009 were from anonymous donors who had submitted specimens to CDC for diagnosis or research with consent for future testing against other pathogens (n=77), or were enrolled in either the CDC blood products program (n= 46), or baseline serum donation program (n=212), or were obtained from commercial sources (n=23). Only the age, and not the influenza vaccination status, is known for these donors.

The data were interpreted jointly by the study investigators. There were no agreements concerning confidentiality of the data.

### ***Influenza serology assays and virus growth***

Microneutralization (MN) and hemagglutination inhibition (HI) assays were performed according to procedures described previously (references 9 and 10 in main text). The HI assay used 0.5% turkey red blood cells. For both assays, serial two-fold

dilutions of serum were tested beginning with a 1:10 dilution. The seasonal influenza A H1N1 viruses and the swine influenza A/New Jersey/8/1976 virus were propagated in embryonated chicken eggs. The swine origin influenza A H1N1 virus, A/California/04/2009, was propagated in Madin Darby canine kidney (MDCK) cells. The following procedure was used to produce a virus stock with a high tissue culture infectious dose which is suitable for use in the MN assay. Cells were grown to 95 to 100% confluency in T162cm<sup>2</sup> cell culture flasks. Cell monolayers were washed 4 times in phosphate buffered saline and infected with a 1/100 to 1/5000 dilution of seed virus in a volume of 10 ml. After one hr incubation at 37° C in 5% CO<sub>2</sub>, 30 ml of virus growth medium (DMEM containing 0.25% bovine serum albumin, 25mM Hepes buffer, 100 U/ml Penicillin, 100µg/ml Streptomycin and 3µg/ml TPCK-trypsin) was added and flasks were incubated for 16 hr at 37° C in 5% CO<sub>2</sub>. The culture supernatant was then replaced with 15 ml of fresh virus growth medium and the cultures were incubated a further 16-27 hr or until cells exhibited approximately 50% CPE and supernatants had a hemagglutination activity of at least 32 hemagglutination units using a 0.5% suspension of turkey erythrocytes.

### ***Statistical analysis***

To estimate the MN titer that corresponds to an HI titer of 40, a correlation analysis of these serological assays was performed using linear regression models. MN and HI titers, ranging from 5 to 320, were transformed to log<sub>2</sub>. To create and validate the model the data were randomly divided into two sets. Variables in the linear regression model included: log<sub>2</sub> HI titer, log<sub>2</sub> HI titer<sup>2</sup>, log<sub>2</sub> HI titer<sup>3</sup>, age category (pediatric, adult or older adult), and interactions between log<sub>2</sub> HI titer and age. Variables with P-values

$\leq 0.05$  were kept in the model. The correlation between MN and HI titers was determined using analysis of variance and the final regression model was validated using Goodness of Fit Tests. The final model with regression coefficients is:

$$\log_2 \text{ MN titer} = 0.305 + 0.788 \log_2 \text{ HI titer} + 0.031 \log_2 \text{ HI titer}^2 + 1.341 \text{ adult} + 1.723 \text{ older adults} + 0.054 \log_2 \text{ HI titer} * \text{adults} - 0.182 \log_2 \text{ HI titer} * \text{older adults}$$

SAS V9.1 software was used to fit linear regression and multivariable models, perform t-tests, estimate geometric mean titers (GMT) with confidence intervals (CI) and corresponding P-values. In the statistical models, study participants were treated as random effects sampled from a larger population of study participants, and duplicate samples were treated as random effects nested within each study participant. The pre- and post-vaccination GMT values were calculated separate from one another.

### ***HI and MN assay correlation and criteria for titer achievement***

A comparison between the HI and MN assays was made for panels of pediatric (n=104), adult (n=82), or older adult (n=134) sera from seasonal vaccine studies (where n=number of sets of HI and MN data used in the analysis). Although the correlation between these serologic methods was good when estimated for the seasonal vaccine strain ( $r = 0.70$ ), the MN assay generally yielded higher titers and/or detected more 4-fold or greater rises to the pandemic H1N1 strain, A/California/04/2009, compared with the HI assay. Therefore, we used the MN assay to assess the extent of cross-reactive antibody to A/California/04/2009. It is generally accepted that serum HI antibody titers of 40 are associated with at least a 50% reduction in risk of infection or disease with influenza viruses in human populations (references 11-13 in main text). However, no such correlate of protection exists for neutralizing antibody titers. Therefore, we used a

regression model to predict the MN titer for seasonal H1N1 viruses that corresponded to an HI titer of 40 and used this as a measurement for titer achievement against the seasonal vaccine strain and the pandemic H1N1 virus. This analysis revealed that in the pediatric population, an HI titer of 40 corresponded to a MN titer of 40, whereas in the adult and older adult populations the corresponding MN titers were 160 and 80, respectively. One possible reason for the observed age-related difference is that children generally have a more strain-specific, and presumably higher avidity, antibody response to exposure to influenza that may be detected equally by the HI and MN assays. The antibody response in adults, on the other hand, may be comprised of a greater proportion of cross-reactive antibody which may have lower avidity for which the MN assay may offer greater detection ability than the HI assay.

### **Supplemental Figure Legend**

**Supplemental Figure.** Flow chart identifying the stored serum samples from recent seasonal vaccine trials contributed by academic, government, and industry partners. In some cases, data from trials in the same population; pediatric, adult, and older adult; were combined for presentation in the tables. Solid lines connect boxes to identify how the data were combined. Dashed lines identify the adjuvanted and nonadjuvanted arms of the same study. The dose for the LAIV (FluMist) was the same for all studies. It contains  $10^{6.5-7.5}$  fluorescent focal units of each of the three live attenuated influenza virus reassortants for the influenza season indicated.

**Supplemental Table 1. Cross-reactive hemagglutination-inhibition (HI) antibody response to pandemic influenza A (H1N1) virus in recipients of seasonal influenza vaccines\***

Vaccine	Influenza Season	Influenza Virus <sup>†</sup>	Population (Age Group)	No.	% with fourfold or greater increase in antibody titer	Geometric mean titer (GMT) <sup>¶</sup>			% with HI ≥ 40	
						Pre-vaccination (95%CI <sup>**</sup> )	Post-vaccination (95% CI)	Post-vaccination to pre-vaccination ratio	Pre-vacc	Post-vacc
TIV <sup>††</sup>	2005-2007	A/New Caledonia/20/1999	Pediatric (6m-9y)	33	55	12 (8-19)	85 (54-134)	7	27	76
		A/California/04/2009			0	5 (-)	5 (-)	1	0	0
	2008-2009	A/Brisbane/59/2007	Pediatric (6-23m)	9	89	5 (3-8)	80 (52-122)	16	0	89
		A/California/04/2009			0	5 (-)	5 (-)	1	0	0
LAIV <sup>¶¶</sup>	2005-2007	A/New Caledonia/20/1999	Pediatric (6m-9y)	24	13	14 (8-25)	27 (15-47)	2	33	46
		A/California/04/2009			0	5 (-)	5 (-)	1	0	0
TIV	2007-2008	A/Solomon Is/3/2006	Adult (18-64y)	59	71	13 (9-19)	133 (92-192)	10	25	81
		A/California/04/2009			7	5 (5-6)	7 (6-8)	1	0	7

Vaccine	Influenza Season	Influenza Virus <sup>†</sup>	Population (Age Group)	No.	% with fourfold or greater increase in antibody titer	Geometric mean titer (GMT) <sup>¶</sup>			% with HI ≥ 40	
						Pre-vaccination (95%CI <sup>**</sup> )	Post-vaccination (95% CI)	Post-vaccination to pre-vaccination ratio	Pre-vacc	Post-vacc
TIV	2007-2008	A/Solomon Is/3/2006	Older Adult (>60y)	63	44	11 (8-15)	40 (30-53)	4	22	57
		A/California/04/2009			5	8 (6-10)	11 (9-13)	1	5	13
TIV + adjuvant	2007-2008	A/Solomon Is/3/2006	Older Adult (>64y)	25	92	15 (11-21)	211 (149-298)	14	24	100
		A/California/04/2009			4	8 (6-12)	10 (7-14)	1	8	12

\* All pediatric subjects received 2 doses of vaccine unless they had received influenza vaccine in a prior year; those aged 6-35 months received two, ½ doses of vaccine (7.5 µg HA).

<sup>†</sup> Pandemic H1N1 virus used was A/California/04/2009; seasonal H1N1 viruses used were: 2005-2007, A/New Caledonia/20/1999; 2007-2008, A/Solomon Islands/3/2006; 2008-2009, A/Brisbane/59/2007.

<sup>¶</sup> A titer of 1280 was used for all samples with a titer of ≥1280.

\*\* Confidence interval.

<sup>††</sup> Trivalent, inactivated influenza vaccine.

<sup>¶¶</sup> Live, attenuated influenza vaccine.

**Supplemental Table 2. Cross-reactive microneutralization (MN) antibody response to pandemic influenza A (H1N1) virus in pediatric recipients (aged 6 months – 9 years) of seasonal influenza vaccines \***

Vaccine	Influenza Season	Influenza Virus <sup>†</sup>	Age Group	No.	% with fourfold or greater increase in antibody titer	Geometric mean titer (GMT) <sup>‡</sup>			% with MN ≥ 40 <sup>§</sup>	
						Pre-vaccination (95% CI <sup>**</sup> )	Post-vaccination (95% CI)	Post- vaccination to pre-vaccination ratio	Pre-vacc	Post-vacc
TIV <sup>††</sup>	2005-2007 <sup>§§</sup>	A/New Caledonia/20/1999	6m-9y	33	67	26 (16-40)	267 (171-418)	10	45	94
		A/California/04/2009			0	5 (5-6)	6 (5-6)	1	0	0
	2007-2008	A/Solomon Is/3/2006	5-9y	13	85	42 (22-80)	575 (303-1093)	14	54	100
		A/California/04/2009			0	10 (7-15)	12 (8-17)	1	8	15
	2008-2009	A/Brisbane/59/2007	6-23m	9	100	5 (4-7)	285 (202-402)	57	0	100
		A/California/04/2009			0	5 (-)	5 (-)	1	0	0
TIV- adjuvanted	2008-2009	A/Brisbane/59/2007	6-59m	45 <sup>&amp;</sup>	96	12 (8-18)	193 (134-280)	16	24	100
		A/California/04/2009			2	6 (5-7)	8 (7-9)	1	0	4

Vaccine	Influenza Season	Influenza Virus <sup>†</sup>	Age Group	No.	% with fourfold or greater increase in antibody titer	Geometric mean titer (GMT) <sup>¶</sup>			% with MN ≥ 40 <sup>§</sup>	
						Pre-vaccination (95% CI <sup>**</sup> )	Post-vaccination (95% CI)	Post- vaccination to pre-vaccination ratio	Pre-vacc	Post-vacc
LAIV <sup>¶¶</sup>	2005-2007 <sup>§§</sup>	A/New Caledonia/20/1999	6m-9y	24	29	32 (16-63)	74 (38-148)	2	46	79
		A/California/04/2009			0	5 (5-6)	6 (5-7)	1	0	4

\* All subjects received 2 doses of vaccine unless they had received influenza vaccine in a prior year; those aged 6-35 months received two, ½ doses of vaccine (7.5 µg HA).

† Pandemic H1N1 virus used was A/California/04/2009; seasonal H1N1 viruses used were: 2005-2007, A/New Caledonia/20/1999; 2007-2008, A/Solomon Islands/3/2006; 2008-2009, A/Brisbane/59/2007.

¶ A titer of 1280 was used for all samples with a titer of ≥1280.

\*\* Confidence interval.

§ For pediatric populations, an HI titer of 40 corresponded to a MN titer of 40.

†† Trivalent, inactivated influenza vaccine.

§§ 2005-06 and 2006-07 influenza seasons.

& Sera obtained from participants residing in Central America.

¶¶ Live, attenuated influenza vaccine.

**Supplemental Table 3. Cross-reactive microneutralization (MN) antibody response to pandemic influenza A (H1N1) virus in adult recipients of seasonal influenza vaccines**

Vaccine	Influenza Season	Influenza Virus <sup>†</sup>	Population (Age Group)	No.	% with fourfold or greater increase in antibody titer	Geometric mean titer (GMT) <sup>‡</sup>			% with MN ≥ 80		% with MN ≥ 160	
						Pre-vaccination (95%CI)**	Post-vaccination (95% CI)	Post-vaccination to pre-vaccination ratio	Pre-vacc	Post-vacc	Pre-vacc	Post-vacc
TIV <sup>††</sup>	2007-2008	A/Solomon Is/3/2006	Adult (18-64y)	148 <sup>^</sup>	75	48 (40-58)	598 (497-720)	13	41	97	29	93
		A/California/04/2009			22	25 (21-31)	54 (44-65)	2	26	45	7	25
	2008-2009	A/Brisbane/59/2007	Adult (18-40y)	83	78	29 (22-38)	546 (418-713)	19	28	94	20	88
		A/California/04/2009			12	11 (9-14)	21 (16-26)	2	7	13	6	7
	2007-2008	A/Solomon Is/3/2006	Older Adult (≥60y)	63	54	31 (22-42)	143 (105-194)	5	29	71	14	54
		A/California/04/2009			5	92 (71-121)	97 (74-127)	1	60	65	33	43
	2008-2009	A/Brisbane/59/2007	Older Adult (>60y)	49 <sup>&amp;</sup>	18	22 (17-28)	51 (39-66)	2	16	45	6	14
		A/California/04/2009			50 <sup>&amp;</sup>	0	47 (36-61)	51 (39-65)	1	16	18	8

Vaccine	Influenza Season	Influenza Virus <sup>†</sup>	Population (Age Group)	No.	% with fourfold or greater increase in antibody titer	Geometric mean titer (GMT) <sup>¶</sup>			% with MN ≥ 80		% with MN ≥ 160	
						Pre-vaccination (95%CI <sup>**</sup> )	Post-vaccination (95% CI)	Post-vaccination to pre-vaccination ratio	Pre-vacc	Post-vacc	Pre-vacc	Post-vacc
LAIV <sup>¶¶</sup>	2007-2008	A/Solomon Is/3/2006	Adult (18-49y)	50	0	71 (47-108)	78 (51-119)	1	58	62	46	50
		A/California/04/2009			0	13 (11-18)	13 (10-17)	1	4	12	4	4
	2008-2009	A/Brisbane/59/2007	Adult (18-40y)	17	12	54 (24-121)	89 (40-198)	2	47	65	29	35
		A/California/04/2009			0	10 (7-14)	10 (7-14)	1	0	0	0	0

<sup>†</sup> Pandemic H1N1 virus used was A/California/04/2009; seasonal H1N1 viruses used were: 2007-2008, A/Solomon Islands/3/2006; 2008-2009, A/Brisbane/59/2007.

<sup>¶</sup> A titer of 1280 was used for all samples with a titer of ≥1280.

<sup>\*\*</sup> Confidence interval.

<sup>††</sup> Trivalent, inactivated influenza vaccine.

<sup>¶¶</sup> Live, attenuated influenza vaccine.

<sup>^</sup> Fifty sera obtained from participants residing in Europe.

<sup>&</sup> Sera obtained from participants residing in Europe.

**Supplemental Table 4. Cross-reactive microneutralization (MN) antibody response to pandemic influenza A (H1N1) virus in adult recipients of seasonal trivalent inactivated influenza vaccines with and without adjuvant**

Influenza Season	Influenza Virus <sup>†</sup>	Population (Age Group)	No.	Adjuvanted	% with fourfold or greater increase in antibody titer	Geometric mean titer (GMT) <sup>¶</sup>			% with MN ≥ 80		% with MN ≥ 160	
						Pre-vaccination (95% CI <sup>**</sup> )	Post-vaccination (95% CI)	Post-vaccination to pre-vaccination ratio	Pre-vacc	Post-vacc	Pre-vacc	Post-vacc
2007-2008	A/Solomon Is/3/2006	Adult <sup>¶¶</sup> (18-64y)	50 <sup>&amp;, ^</sup>	Yes	86	28 (20-38)	449 (322-627)	16	34	92	18	92
	A/California/04/2009				24	16 (13-22)	39 (30-52)	2	8	32	6	14
2007-2008	A/Solomon Is/3/2006	Adult <sup>¶¶</sup> (18-64y)	50 <sup>&amp;</sup>	No	68	40 (29-56)	422 (302-590)	11	36	96	18	88
	A/California/04/2009				20	26 (18-36)	56 (40-79)	2	26	50	6	22
2007-2008	A/Solomon Is/3/2006	Older Adult <sup>&amp;&amp;</sup> (>64y)	25	Yes	92	25 (18-36)	465 (327-661)	18	24	100	4	96
	A/California/04/2009				12	75 (51-108)	128 (88-186)	2	56	72	24	52
2007-2008	A/Solomon Is/3/2006	Older Adult <sup>&amp;&amp;</sup> (>64y)	25	No	64	31 (18-54)	160 (91-281)	5	40	68	16	56
	A/California/04/2009				12	72 (54-96)	104 (78-139)	1	56	76	20	40

Influenza Season	Influenza Virus <sup>†</sup>	Population (Age Group)	No.	Adjuvanted	% with fourfold or greater increase in antibody titer	Geometric mean titer (GMT) <sup>‡</sup>			% with MN ≥ 80		% with MN ≥ 160	
						Pre-vaccination (95% CI <sup>**</sup> )	Post-vaccination (95% CI)	Post-vaccination to pre-vaccination ratio	Pre-vacc	Post-vacc	Pre-vacc	Post-vacc
2008-2009	A/Brisbane/59/2007	Older Adult (>60y)	50 <sup>&amp;</sup>	Yes	26	30 (22-40)	77 (58-104)	3	30	56	8	30
	A/California/04/2009				2	36 (27-47)	43 (33-56)	1	20	24	12	12
2008-2009	A/Brisbane/59/2007	Older Adult (>60y)	49 <sup>&amp;</sup>	No	18	22 (17-28)	51 (39-66)	2	16	45	6	14
	A/California/04/2009				50 <sup>&amp;</sup>	0	47 (36-61)	51 (39-65)	1	16	18	8

<sup>†</sup> Pandemic H1N1 virus used was A/California/04/2009; seasonal H1N1 viruses used were: 2007-2008, A/Solomon Islands/3/2006; 2008-2009, A/Brisbane/59/2007.

<sup>‡</sup> A titer of 1280 was used for all samples with a titer of ≥1280.

<sup>\*\*</sup> Confidence interval.

<sup>&</sup> Sera obtained from participants residing in Europe.

<sup>^</sup> Vaccine contained 5 µg HA per strain.

<sup>‡‡</sup> Participants from two arms of the same vaccine trial.

<sup>&&</sup> Participants from two arms of the same vaccine trial.

**Supplemental Table 5. Cross-reactive microneutralization (MN) antibody response to pandemic influenza A (H1N1) virus\* in adult recipients of 1976 influenza vaccine**

Influenza Season	Influenza Virus	Age Group	No.	% with fourfold or greater increase in antibody titer	Geometric mean titer (GMT) <sup>¶</sup>			% with MN ≥ 80		% with MN ≥ 160	
					Pre-vaccination (95% CI <sup>**</sup> )	Post-vaccination (95% CI)	Post-vaccination to pre-vaccination ratio	Pre-vacc	Post-vacc	Pre-vacc	Post-vacc
1976-1977	A/New Jersey/8/1976	≥25y	83	81	29 (22-37)	260 (198-341)	9	12	82	5	71
	A/California/04/2009			54	58 (46-73)	194 (153-246)	3	35	78	16	63

\*A/California/04/2009.

<sup>¶</sup> A titer of 1280 was used for all samples with a titer of ≥1280.

<sup>\*\*</sup> Confidence interval.

