Anamnestic immune response and safety of an inactivated quadrivalent influenza vaccine in primed versus vaccine-naïve children

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Conflict of interest

C Claeys, V Chandrasekaran, D Friel, T Ollinger, J Soni and A Schuind are employees of the GSK group of companies. P Li reports she was employed by the GSK group of companies at the time of the study and is currently employed by Pfizer. BL Innis reports he was employed by the GSK group of companies at the time of the study and is currently employed by PATH. VK Jain reports she was employed by the GSK group of companies at the time of the study and is currently employed by Bill and Melinda Gates Foundation. C Claeys, A Schuind, P Li, and VK Jain hold shares in the GSK group of companies. AJ Pollard reports grants from the GSK group of companies for the submitted work, and previous grants from Okairos, and Pfizer outside the submitted work in the last 36 months. AJ Pollard also reports he is chair of the UK Department of Health and Social Care's (DHSC) Joint Committee on Vaccination and Immunisation (JCVI) and a member of WHOs SAGE. The views expressed in this manuscript do not necessarily reflect the views of DHSC or JCVI. F Martinon-Torres reports grants to his institution from the GSK group of companies for this study and reports grants from the GSK group of companies, Pfizer, Sanofi Pasteur, MSD, Janssen, Ablynx, and Novartis, outside the submitted work; his research activities are funded by Instituto Carlos III (Intensificación de la actividad

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Abstract

Background: It has not yet been demonstrated whether two doses of inactivated quadrivalent influenza vaccine (IIV4) prime a booster response in infants. We evaluated the anamnestic immune response to an IIV4 in children aged 17−48 months.

Methods: Children were randomized to two doses of IIV4 or control in the primary phase III study (NCT01439360). One year later, in an open-label revaccination extension study (NCT01702454), a subset of children who received IIV4 in the primary study (primed group) received one IIV4 dose and children who received control in the primary study (unprimed) received two IIV4 doses 28 days apart. The primary objective was to evaluate hemagglutination inhibition (HI) antibody titers 7 days after first IIV4 vaccination in the per-protocol cohort (N=224 primed; N=209 unprimed). Neutralizing and anti-neuraminidase antibodies were also measured. Safety was analyzed in the total vaccinated cohort (N=241 primed; N=229 unprimed).

Results: An anamnestic response was observed in primed children relative to unprimed controls, measured by age-adjusted geometric mean HI titer ratios against strains homologous (A/H1N1: 9.0; B/Victoria: 3.9) and heterologous (A/H3N2: 2.7; B/Yamagata: 6.7) to those in the primary vaccination series. The anamnestic response in primed children included increases in neutralizing antibodies (mean geometric increase: 5.0–10.6) and anti-neuraminidase antibodies (4.9–8.8). No serious adverse events related to vaccination were reported.

Conclusion: In this study, 2-dose priming with IIV4 induced immune memory that was recalled with 1-dose IIV4 the following year to boost HI, anti-neuraminidase, and neutralizing antibodies, even though the IIV4 strain composition partially changed.

Word count: 248 (max 250)

Introduction

Influenza has a high incidence and burden of disease in children[1-3] and vaccination is recommended by the World Health Organization.[4] Suboptimal vaccine protection may occur if there is a mismatch between the circulating virus strains and the strains contained in the vaccine. This can be a particular problem with regard to the vaccine B strains, because two antigenically distinct lineages of influenza B circulate worldwide, the Yamagata lineage and the Victoria lineage. Mismatch between the circulating lineage and the vaccine lineage reduces the degree of protection offered by the vaccine.[5-8]

Until recently, vaccination strategies used a trivalent influenza vaccine containing two influenza A strains (H1N1 and H3N2 subtypes) and one influenza B strain. Quadrivalent influenza vaccines containing B strains from both lineages offer broader protection and lessen the problem of mismatching due to B lineage. They may be particularly useful in children because, although vaccinated adults show moderate cross-reactive antibody responses against the alternative B lineage,[9] the responses of children show poor cross-reactivity.[10, 11] Indeed, a meta-analysis of vaccine trials in young children found that efficacy was substantially reduced against influenza B strains of the alternative lineage to that contained in the vaccine compared with the same lineage.[8]

The World Health Organization recommends that children less than 9 years of age are given two doses of influenza vaccine during their first season of vaccination to optimize the immune response.[4] Thereafter, children are considered to be primed

and require only one dose of influenza vaccine per season. This strategy relies on the ability of influenza vaccine given in two doses to establish immune memory and subsequently drive an acceptable anamnestic response when boosted with a single dose the following year. However, published evidence on immune memory and anamnestic response elicited by inactivated quadrivalent influenza vaccine (IIV4) in children is lacking. We therefore conducted the present study to evaluate the humoral anamnestic response to a candidate IIV4 in children 17–48 months of age. This IIV4 (*Fluarix Quadrivalen*t) is licensed in the US and Europe for use in adults and children from 6 months of age. The primary objective of the study was to assess the anamnestic immune response to the IIV4 in terms of hemagglutination inhibition (HI) antibody titer in children 17–48 months of age.

Materials and methods

The trial was sponsored by GlaxoSmithKline Biologicals SA, and approved by independent ethics committees and/or institutional review boards, conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonisation Good Clinical Practice guidelines, and all applicable regulatory requirements. Parents provided written informed consent prior to participation of their child. The trial was registered with clinicaltrials.gov: NCT01702454.

Study design and participants

The present revaccination study was an extension of the first seasonal cohort (northern hemisphere influenza season 2011-2012) of a primary phase III study[12] designed to evaluate the efficacy of the candidate IIV4, in which healthy children (6–35 months of age) were randomized 1:1 to receive IIV4 or non-influenza control vaccine (NCT01439360) (Text, Supplemental Digital Content 1).

The revaccination study (2012–2013 season) enrolled a convenience sample of children who had received two doses of study vaccine (IIV4 or control) in the primary study during the previous year. Children were 17–48 months of age at enrollment into the revaccination study (stratified into 17–29 months and 30–48 months). Because more children in the older age group (30−48 months) participated in the first cohort of the primary study, to ensure an adequate balance between age groups, parents of children in the younger group (17−29 months) were contacted first to invite their children to participate in the revaccination study. Parents of older children

were contacted in a second wave. All parents were contacted in the same order as the randomization list of the primary study.

In the open-label revaccination study, children retained their randomly allocated treatment group from the primary study. Children who were randomly allocated to the IIV4 group in the primary study and had received two IIV4 doses were given one dose of IIV4 (the primed group); children who were randomly allocated to the control vaccine group in the primary study and had received two doses of the control vaccine were given two doses of IIV4 28 days apart (the unprimed group). The IIV4 used in the primary study and in the revaccination study was administered intramuscularly in a 0.5 mL dose. Thirty three centers in the Czech Republic, Poland, Spain and the UK participated in the study.

The IIV4 (*Fluarix Quadrivalen*t, GSK, Dresden, Germany) was prepared from influenza viruses propagated in embryonated chicken eggs. Each of the four viruses was purified by zonal centrifugation using a linear sucrose density gradient solution containing detergent to split the virions, further purified by diafiltration, and inactivated by the consecutive effects of sodium deoxycholate and formaldehyde. The IIV4 was formulated to contain 15 µg hemagglutinin antigen per strain of the following recommended influenza strains: A/Christchurch/16/2010 (A/H1N1; an A/California/7/2009-like strain), A/Victoria/361/2011 (A/H3N2), B/Brisbane/60/2008 (B/Victoria), and B/Hubei-Wujiagang/158/2009 (B/Yamagata). Two strains were updated between the primary study and the revaccination study: the two different

strains in the primary study were A/Victoria/210/2009 (A/H3N2; an A/Perth/16/2009-like virus) and B/Brisbane/3/2007 (B/Yamagata; a B/Florida/4/2006-like virus).

Study endpoints

Immunogenicity

Blood samples were taken before and at Day 7 after administration of the first IIV4 dose in the revaccination study (the first dose of IIV4 ever for unprimed children and the third dose for primed children [after an interval of approximately 1 year]). Immunogenicity was evaluated at Day 7 because an anamnestic response is characterized by an early and sharp rise in antibody titers. All samples were tested by HI assay and a random subset was tested by microneutralization (MN) assay and neuraminidase inhibition (NI) assay (Text, Supplemental Digital Content 2).

The following parameters were derived from HI titers: (1) geometric mean titer (GMT); (2) seropositivity rate; (3) seroconversion rate (SCR); (4) seroprotection rate (SPR); (5) mean geometric increase (MGI). The seropositivity rate was defined as the percentage of children with HI titer equal to or above the assay cut-off value. The SCR was defined as the percentage of children with either (a) pre-vaccination titer <1:10 and a post-vaccination titer ≥1:40; or (b) pre-vaccination titer ≥1:10 and a minimum 4-fold increase in post-vaccination titer. Although there is no accepted criterion for seroprotection in children, the SPR was defined as the percentage of children with HI titer ≥1:40 that is usually accepted as indicating protection in 50% of adult vaccinees.[13] The MGI was defined as the fold increase in HI GMTs post-

vaccination compared with pre-vaccination. The GMT and MGI were also calculated for neutralizing and anti-neuraminidase antibody titers.

Safety

Parents recorded solicited injection site reactions (pain, redness and swelling) and solicited systemic reactions (drowsiness, fever, irritability/fussiness, loss of appetite) in a diary card every day up to Day 7 after the first vaccination. They recorded other adverse events (spontaneously reported AEs) up to Day 28 after the first vaccination. Medically attended AEs and serious AEs (SAEs) were reported throughout the study until the final telephone contact at approximately Day 180.

Statistics

The study was planned to enrol a sufficient number of children to assess the relative immune response of the vaccine-primed participants versus vaccine-unprimed participants, with at least 80% power in terms of HI GMT ratio (primed/unprimed). Assuming a standard deviation of 0.8 for HI titer in logarithm base 10 for both primed and unprimed groups, and assuming that all four strains in the revaccination vaccine were homologous to those in the primary study vaccine, a total of 184 evaluable subjects for each group gave a global power of at least 80% to detect a difference in terms of GMT ratio (i.e., GMT ratio=1 under Null hypothesis) at Day 7 at the 2.5% significance level, by assuming the observed difference is two-fold (by PASS 2005, one-sided two-sample t-test for a difference of means, one-sided alpha=2.5%).

The objectives were to evaluate after one dose of IIV4 at Day 7: (1) GMTs, SCRs, SPRs, and MGIs in terms of HI titers; (2) priming effect via the GMT ratios of influenza vaccine-primed versus unprimed children and the difference in SCR and SPR between primed and unprimed children (based on HI titers); (3) GMTs and MGIs in terms of neutralizing and anti-neuraminidase antibody titers.

A seronegative participant was defined as having an antibody titer below the assay cut-off value; a seropositive participant was defined as having a titer greater than or equal to the assay cut-off value (Text, Supplemental Digital Content 2). GMT calculations were performed by taking the anti-log of the log titer transformations. Antibody titers below the assay cut-off value were given an arbitrary value of half the cut-off value for the GMT calculation.

The Clopper-Pearson exact 95% confidence interval (CI) for a proportion within a group was calculated.[14] The 95% CI for the mean of log-transformed titer was first obtained assuming that log-transformed values were normally distributed with unknown variance. The 95% CI for the GMTs was then obtained by exponential-transformation of the 95% CI for the mean of log-transformed titer. The group GMT ratio was obtained using an ANCOVA model on the logarithm-transformed titers that included the vaccine group as fixed effect and age as a regressor. The GMT ratio and its 95% CI were derived as exponential-transformation of the corresponding group contrast in the model. The standardized asymptotic 95% CI for the group difference in proportion was based on Method 6 as described by Newcombe.[15]

The primary immunogenicity analysis was based on the per-protocol cohort which included all children who met the eligibility criteria, complied with the procedures and vaccination intervals specified, did not receive a product or have a medical condition leading to elimination from the per-protocol cohort, and who had data available for immunogenicity endpoints against at least one vaccine strain. An exploratory immunogenicity analysis was performed excluding children who had experienced a reverse transciption polymerase chain reaction (RT-PCR)-confirmed influenza infection in the primary study the year before. The safety analysis was based on the total vaccinated cohort which included all children who received at least one vaccine dose.

Results

The parents of 665 children out of 1777 children from the first seasonal cohort of the primary study were contacted regarding participation in the revaccination study, of whom the parents of 473 children agreed and 192 declined. Three children were allocated to a study group but were not vaccinated. Enrollment took place between October and November 2012, and the last visit took place in June 2013.

The total vaccinated cohort included 470 (241 primed and 229 unprimed) children; the per-protocol cohort included 433 (224 primed and 209 unprimed) children (Figure 1). Three primed and eight unprimed children did not complete the study (Figure 1). In the primed group, the mean age was 33.2 months, 47.3% were female, and 97.9% were Caucasian (total vaccinated cohort). Corresponding values in the unprimed group were 32.5 months, 41.9% female and 97.8% Caucasian. Demographics were considered to be representative of the original study cohort enrolled 1 year earlier. A total of 183 and 250 children were included in the 17–29 and 30–48 months age strata, respectively, in the per-protocol cohort.

The exploratory immunogenicity analysis excluding children who experienced an RT-PCR-confirmed influenza infection in the primary study comprised 392 children; 11 children were excluded from the primed group (who had received two doses of IIV4 in the primary study), and 30 children were excluded from the unprimed group (who had received two doses of the control vaccine in the primary study). In the primed group, all 11 children had experienced an infection with influenza A/H3N2; in the unprimed group, one child had an infection with influenza A/H1N1, 27 children with

A/H3N2, one child with B/Yamagata and one child with an unknown subtype or lineage.

Immunogenicity in the per-protocol cohort

HI antibody titers

More primed than unprimed children were seropositive to a vaccine strain before vaccination in the revaccination study (Table, Supplemental Digital Content 3), and pre-vaccination antibody titers were higher in primed than unprimed children except for A/H3N2 (Figure 2).

The primed group mounted an anamnestic response that was detected 7 days after the booster dose of IIV4, with a rise in GMTs for strains that were unchanged from the 2011-2012 season (A/H1N1 and B/Victoria) and strains that had changed compared with the 2011-2012 season (A/H3N2 and B/Yamagata) (Figure 2). It was observed that the lower limit of the 95% CI of the GMT ratio (primed/unprimed) was above 1 for each vaccine strain (Figure 2). The between-group difference in the anamnestic response was also observed in the SCR difference (primed minus unprimed), with the lower limit of the 95% CIs being above zero for all vaccine strains (Figure 2), and in the SPR difference (Table, Supplemental Digital Content 4). The highest post-vaccination GMTs were observed for the A/H1N1 strain (Figure 2), and in children aged 30–48 months (Figures, Supplemental Digital Content 5 and 6).

After one dose of IIV4, 76.5–94.1% of primed children seroconverted per vaccine strain compared with 32.2–38.6% of unprimed children (Table, Supplemental Digital

Content 7). There was little difference in SCR between primed children aged 17–29 months versus 30–48 months, but a higher proportion of unprimed children in the older age group seroconverted versus the younger unprimed children (Table, Supplemental Digital Content 7). A similar pattern was observed for SPR (Table, Supplemental Digital Content 8). Higher MGIs were observed in primed children than in unprimed children (Table, Supplemental Digital Content 9).

Neutralizing and anti-neuraminidase antibody titers

GMTs for neutralizing and anti-neuraminidase antibodies rose after vaccination in both primed and unprimed children (Figure 3a and 3b). GMTs were higher in primed children for the A/H1N1, B/Victoria and B/Yamagata strains. However, there was almost no difference between groups in terms of GMTs for the A/H3N2 strain, although the MGI values were higher in the primed group (Figure 3a and 3b; Tables, Supplemental Digital Content 9).

Exploratory immunogenicity analysis excluding children with RT-PCR-confirmed influenza infection in the primary study

In the analysis excluding children with a RT-PCR-confirmed influenza infection in the primary study, primed children mounted a similar anamnestic response to those in the overall per-protocol cohort. GMTs for HI antibodies were similar to the overall per-protocol cohort in both primed and unprimed children (Figure, Supplemental Digital Content 10). For each vaccine strain, the lower limit of the GMT ratio (primed/unprimed) was above 1 and the lower limits of the SCR and SPR difference

(primed minus unprimed) were above zero (Figure, Supplemental Digital Content 10). Likewise, SPR and SCR were comparable in this exploratory analysis to those in the overall per-protocol cohort (Table, Supplemental Digital Content 11). A similar pattern was observed for neutralizing and anti-neuraminidase antibodies (Figures, Supplemental Digital Content 12 and 13).

Safety

Safety outcomes are shown in Table 1. More children in the primed group experienced injection site adverse events compared with the unprimed group. Fever (temperature ≥37.5°C by any route) during the 7 days post-vaccination period was observed more often in unprimed (11.6%) than primed (5.5%) children. A febrile convulsion was reported for a primed child 100 days after vaccination and was not considered to be causally related to the study vaccine by the investigator. The frequency of spontaneously reported safety endpoints was similar between groups. No SAEs related to vaccination occurred during the study and there were no deaths.

Discussion

This is the first randomized study in children 17−48 months of age to demonstrate an anamnestic immune response to a booster dose of IIV4 in terms of HI, neutralizing and anti-neuraminidase antibodies. The immune response 7 days after the booster dose was higher than the immune response after the first dose in influenza-vaccine naïve children. An anamnestic immune response was observed in both age strata, with little or no difference between children 17–29 and 30–48 months of age.

Immunogenicity of IIV4 has now been widely evaluated in children.[12,16-22] Compared with inactivated trivalent influenza vaccine (IIV3), studies show that IIV4 produces a similar immune response to the common vaccine strains and a superior response to the B lineage not contained in the IIV3. In these previous studies, following a full vaccination course of IIV4, SCRs varied between 74–92% for A/H1N1, 70–88% for A/H3N2, 65–85% for B/Victoria, and 66–94% for B/Yamagata in children from 6 months to 17 years of age.[16-21] Seroconversion rates in the present study were consistent with these previous studies, with values of 77%, 81%, 77%, and 94% observed for A/H1N1, A/H3N2, B/Victoria, and B/Yamagata, respectively, in the primed group. Efficacy of the IIV4 in prevention of mild and moderate-to-severe influenza in children has also been shown.[12,22]

Most primed children were seropositive to a vaccine strain before first vaccination, and pre-vaccination titers were higher in primed than unprimed children, except for influenza A/H3N2. The high pre-vaccination seropositivity and antibody titers in the primed group reflect the persistence of the immune response to the vaccine given

during the previous season in the primary study. It is unclear why no difference between primed and unprimed children in pre-vaccination titers against A/H3N2 was observed in both analyses including and excluding children with a previous influenza illness. It may be related to the update of the A/H3N2 vaccine strain between the 2011−2012 influenza season and the 2012–2013 season; the A/Victoria/210/2009 virus, an A/Perth/16/2009-like virus, was updated to the A/Victoria/361/2011 virus. The B/Yamagata virus was also updated between these seasons; the B/Brisbane/3/2007 virus, a B/Florida/4/2006-like virus, was updated to the B/Hubei-Wujiagang/158/2009 virus. The A/Victoria/361/2011 virus had a 16-fold reduced titer by virus neutralization assay compared with the A/Perth/16/2009 virus.[23] The B/Wisconsin/1/2010 virus, which is the reference strain for the B/Hubei-Wujiagang/158/2009 virus, had an 8-fold reduced HI titer compared with the B/Florida/4/2006 virus.[23] Despite the update, there was an anamnestic response to the A/H3N2 and B/Yamagata strains, indicating that the prior year's strains induced a priming response. This is a relevant observation, because the vaccine strains in the seasonal vaccine change frequently and therefore booster influenza vaccination must be able to drive an effective anamnestic response to newly introduced vaccine strains or even newly emerging drifted strains not present in the vaccine. Two previous studies of IIV3 in children 6–23 months of age showed an anamnestic immune response after priming with heterologous vaccine strains, although the response was lower compared with priming with homologous strains.[10,24]

The demonstration of immunogenicity in terms of both HI and anti-neuraminidase antibodies is important, as it confirms the functional breadth of the immune response

to surface proteins of the vaccine. Anti-neuraminidase antibody has been shown to be an independent predictor of immunity to naturally occurring influenza.[25] Overall, the immune response followed the same pattern with the three different assays (HI, MN, and NI). The booster response appeared to be particularly high for A/H1N1 with the MN assay (reaching titers of 1500). Although the HI and NI assays measure the functional immune response towards the surface proteins of the vaccine, the MN assay may detect a broader range of neutralizing antibodies.[26]

Excluding children with RT-PCR-confirmed influenza infection in the primary study had no significant impact on the analysis of the immune response in either primed or unprimed children. As expected, more children in the unprimed group, who did not receive influenza vaccination in the primary study, experienced an influenza illness than the primed group (30 versus 11 children, respectively). Influenza A/H3N2 was by far the most commonly detected virus in children with influenza illness with all 11 children in the primed group and 27 out of 30 children in the unprimed group experiencing influenza associated with this virus. Prior exposure to infection and natural antibody production in the unprimed group would be expected to mask differences between the primed and unprimed groups. Excluding children with a previous illness may therefore be expected to increase the difference between the primed and unprimed groups. This was indeed observed for A/H3N2, for which the GMT ratio increased from 2.7 in the analysis of all children to 4.2 in the analysis excluding children with a previous illness.

The IIV4 used in the study was given at a dose of 15 µg per antigen (0.5 mL volume), rather than the lower dose of 7.5 µg per antigen (0.25 mL volume) traditionally used in young children. The lower dose was introduced in young children during the 1970s as a response to the high reactogenicity experienced by this age group to the whole virus vaccines available at the time.[27] However, young children mount a variable immune response to the 7.5 µg dose,[10] and current split virus vaccines are much better tolerated than whole virus vaccines.[28] Use of a 15 µg dose has been shown to improve the immune response in young children compared with the 7.5 µg dose.[20,21] The 15 µg dose was shown to be well tolerated in the present study, with a safety profile in line with other studies; the higher antigen content of the IIV4 resulting from the additional B lineage antigen and the 15 µg dose does not appear to adversely affect tolerability in children, including the very young.[12,16-22]

Our study had some limitations. Firstly, the traditional measure of immunogenicity according to European and US licensure criteria is the immune response determined on blood samples collected 1 month after vaccination. Here, we chose to evaluate immunogenicity 1 week after vaccination to measure the anamnestic response which is characterized by an early and sharp rise in antibody titers. To limit the number of blood samples drawn in these young children, no immunogenicity analysis was planned at 1 month. Secondly, although participants were randomized at baseline of the primary study to IIV4 or control vaccine, a convenience sample was enrolled in the revaccination study, with no randomization of the primed and unprimed groups. A relatively small number of children from the first of the five seasonal cohorts of the primary study participated in the revaccination study. Thirdly, children who

participated in the revaccination study may or may not have been part of the immunogenicity subset of the primary study, as this comprised a convenience sample consisting of approximately 250 children out of a total of approximately 1800 enrolled.

In conclusion, the present study has shown that the IIV4 induces anamnestic immune responses in IIV4-primed children to the two major surface proteins of the influenza virus that are important for protection against infection, for strains that are antigenically like the vaccine strains administered in the previous year and for drift variants. The findings indicate the capacity of an annual booster of IIV4 to enhance immunity after primary vaccination of infants and toddlers, with an acceptable safety profile. These data support extending to IIV4 the current use of a 2-dose IIV series followed by one dose in subsequent years in very young vaccine-naïve children who are at increased risk of poor outcomes associated with influenza.

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Contributorship

All authors participated in the design or implementation or analysis, and interpretation of the study; and the development of this manuscript. All authors had full access to the data and gave final approval before submission.

Trademark

Fluarix Quadrivalent is a trademark of the GSK group of companies.

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1 **Table 1. Safety outcomes reported throughout study (total vaccinated cohort)**

	No. (%) children reporting outcome	
	Primed	**Unprimed**
	N=241[1]	**N=229[1]**
Solicited injection site adverse events during 7-day post-vaccination period (dose 1)		
Pain		
All	96 (40.2)	61 (26.8)
Grade 3[2]	2 (0.8)	1 (0.4)
Redness		
All	82 (34.3)	48 (21.1)
Grade 3[2]	2 (0.8)	0
Swelling		
All	49 (20.5)	25 (11.0)
Grade 3[2]	2 (0.8)	0
Solicited systemic adverse events during 7-day post-vaccination period (dose 1)		
Drowsiness		
All	54 (22.7)	44 (19.6)

Grade 3[2]	5 (2.1)	1 (0.4)
Irritability/fussiness		
All	77 (32.4)	59 (26.3)
Grade 3[2]	5 (2.1)	5 (2.2)
Loss of appetite		
All	51 (21.4)	46 (20.5)
Grade 3[2]	8 (3.4)	5 (2.2)
Fever		
All (≥37.5°C)	13 (5.5)	26 (11.6)
Grade 3[2]	2 (0.8)	1 (0.4)
Febrile convulsion	1 (0.4)	0
Spontaneously reported (unsolicited) adverse events during 28-day post-vaccination period (dose 1)		
All	66 (27.4)	66 (28.8)
Grade 3[2]	6 (2.5)	7 (3.1)
Related to vaccine[3]	5 (2.1)	3 (1.3)
Serious adverse event[4] during entire study period		
All	7 (2.9)	8 (3.5)
Related to vaccine	0	0

Medically attended event[5] during entire study period	149 (61.8)	130 (56.8)
Deaths	0	0

[1]The parents of 239 and 238 children in the primed group were compliant in returning the symptom sheets for the solicited injection site and systemic adverse events, respectively. In the unprimed group, the corresponding numbers were 228 and 224, respectively. The number and percentage of children with solicited adverse event is calculated based on these values.

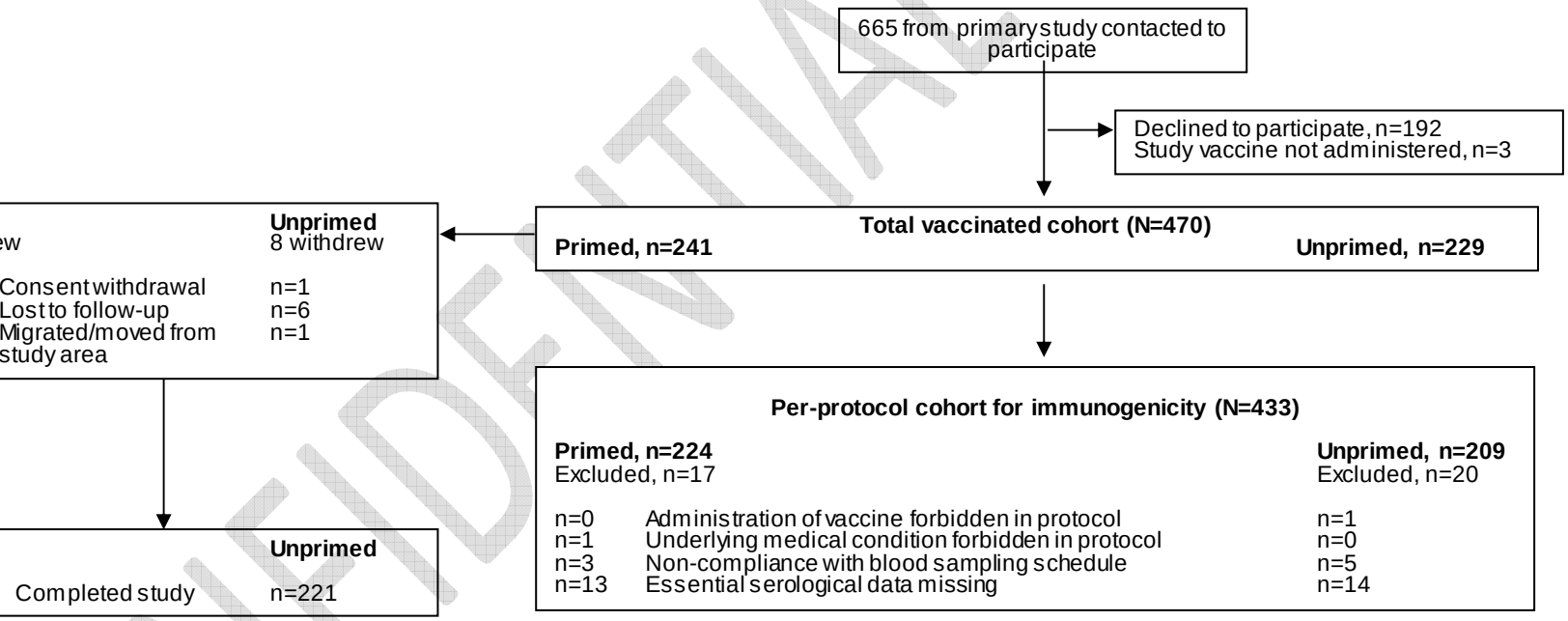
[2]Grade 3 events were defined as follows: pain: child cried when the limb was moved or the limb was spontaneously painful; redness and swelling: >50 mm surface diameter; drowsiness and irritability/fussiness: prevented normal activity; loss of appetite: did not eat at all; fever: >39°C; spontaneously reported: prevented normal activity.

[3]Primed children: cough and rhinorrhea (reported in the same child), vomiting, headache, sleep terror, and rash; unprimed children: cough and wheezing (reported in the same child), nasopharyngitis, and upper respiratory tract infection.

[4]Serious adverse events were defined as any untoward medical occurrence that results in death, is life-threatening, requires hospitalization or prolongs hospitalization, or results in disability or incapacity.

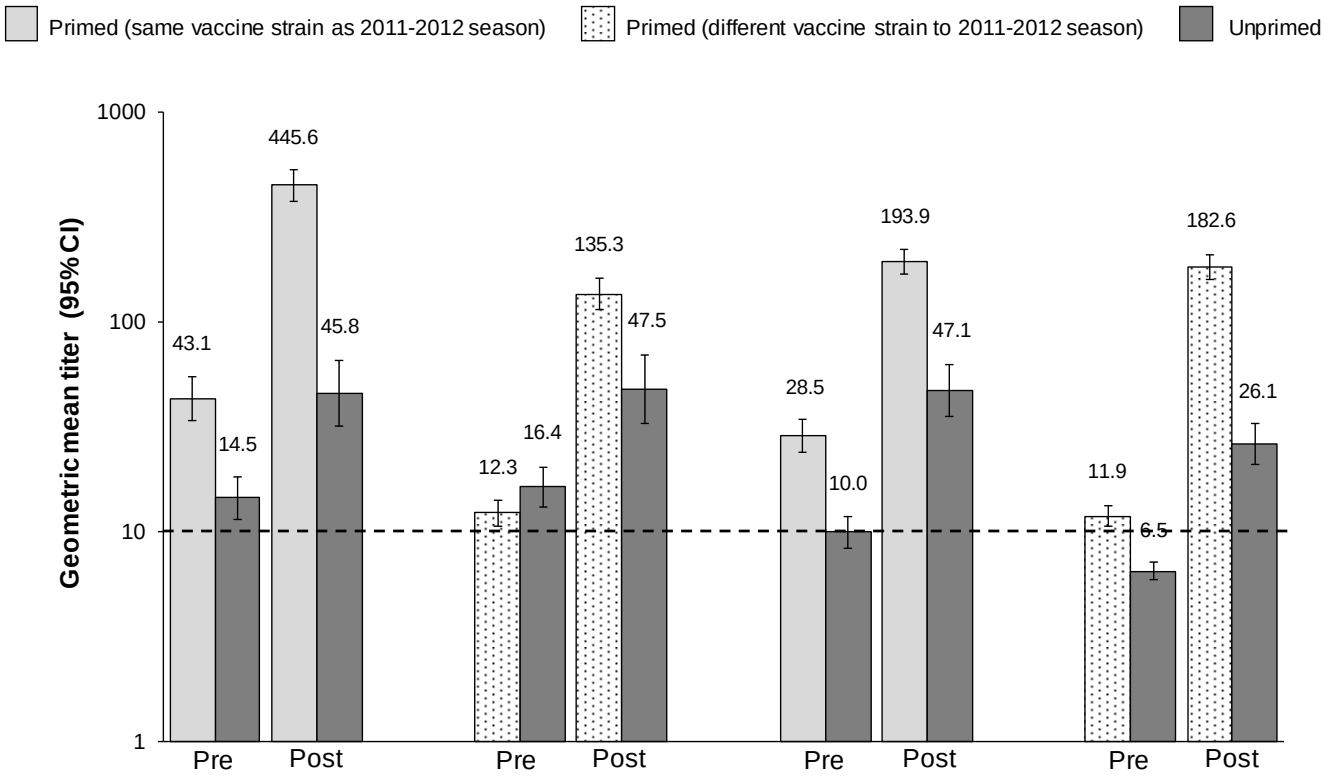
[5]Hospitalization, emergency room visit, physician/nurse practitioner/healthcare worker visit.

1. Participant disposition



2. GMT pre-vaccination and 7 days after first vaccination, GMT ratio and SCR difference for HI antibodies in

nation study (per-protocol cohort)



	A/H1N1	A/H3N2	B/Victoria	B/Yamagata
MT ratio (95% CI)[1]	8.97 (6.21-12.96)	2.70 (1.81-4.02)	3.94 (2.89-5.37)	6.71 (5.21-8.63)
ference (95% CI)[2]	44.74 (35.87-52.84)	45.31 (36.58-53.30)	37.86 (28.83-46.26)	56.00 (48.32-63.04)

atio adjusted for age (primed/unprimed); [2]Difference in SCR (primed minus unprimed)

ine represents the assay cut-off (10 1/dil). GMT values are shown above the bars.

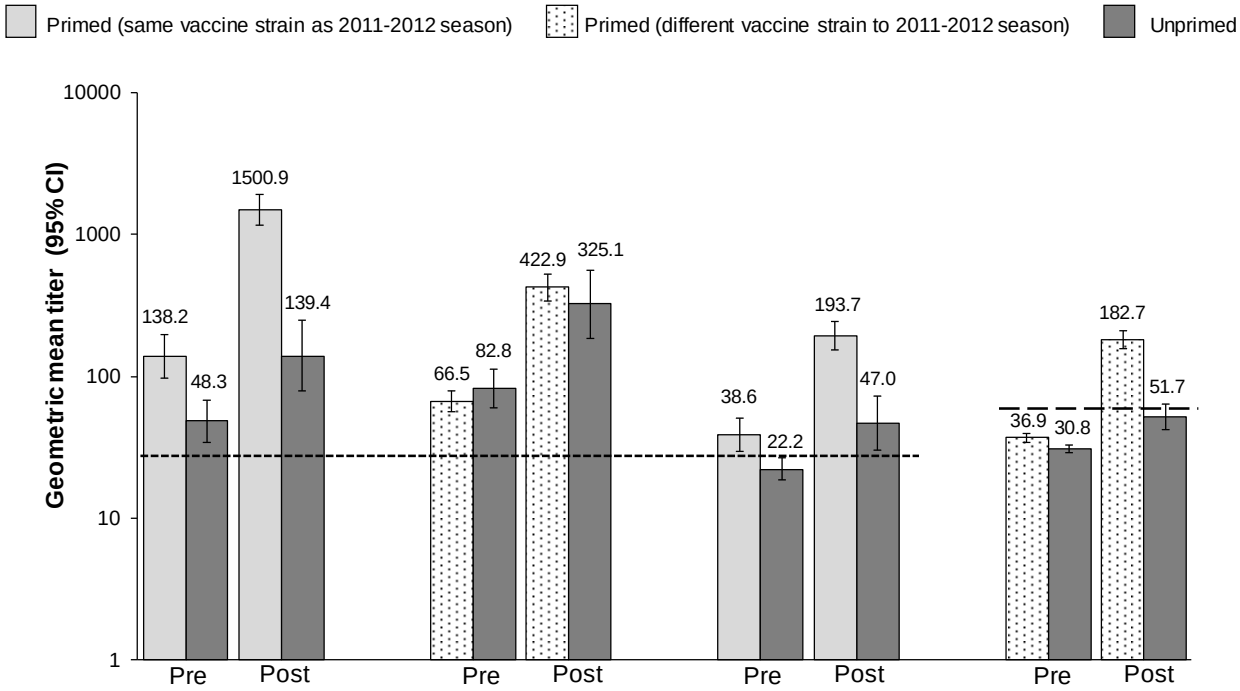
idence interval; GMT: geometric mean titer; HI: hemagglutination inhibition; Pre: pre-vaccination; Post: 7 days follow

tion; SCR: seroconversion rate

3. GMT pre-vaccination and 7 days after first vaccination and MGI for neutralizing and anti-neuraminidase

ies in the revaccination study (per-protocol cohort)

tralizing antibodies

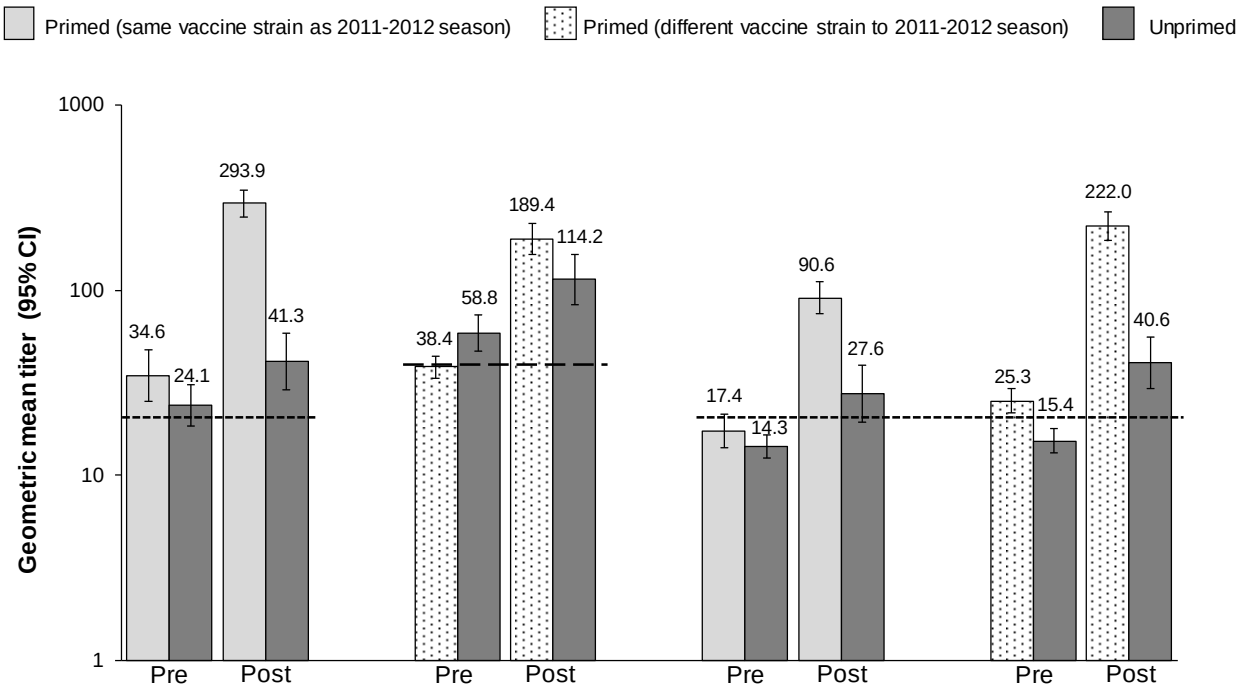


	A/H1N1	A/H3N2	B/Victoria	B/Yamagata
% (95% CI), primed	10.6 (8.2-13.7)	6.4 (5.4-7.6)	5.0 (4.3-5.8)	5.0 (4.3-5.7)
95% CI), unprimed	3.1 (2.3-4.2)	4.5 (3.2-6.2)	2.1 (1.6-2.8)	1.7 (1.4-2.0)

ine represents the assay cut-off for the A/H1N1, A/H3N2 and B/Victoria strains (28 1/dil) and the B/Yamagata strain

MT values are shown above the bars.

i-neuraminidase antibodies



	A/H1N1	A/H3N2	B/Victoria	B/Yamagata
% (95% CI), primed	8.3 (6.5-10.7)	4.9 (4.2-5.8)	5.2 (4.4-6.0)	8.8 (7.5-10.2)
95% CI), unprimed	1.8 (1.5-2.1)	2.0 (1.7-2.3)	1.9 (1.5-2.4)	2.7 (2.1-3.4)

ine represents the assay cut-off for the A/H1N1, B/Victoria and B/Yamagata strains (20 1/dil) and the A/H3N2 strain

MT values are shown above the bars.

idence interval; GMT: geometric mean titer; MGI: mean geometric increase; Pre: pre-vaccination; Post: 7 days follow

tion

Supplemental Digital Content

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Supplemental Digital Content 1

Control vaccine schedule used in the primary study

Vaccine	Schedule
Vaccine-primed children ≥12 months	1 dose of hepatitis A vaccine at Day 0
Unprimed children ≥12 months	1 dose of hepatitis A vaccine at Day 0 and 1 dose of varicella virus vaccine at Day 28
Children <12 months (all considered unprimed)	2 doses of pneumococcal vaccine at Days 0 and 28

Vaccine-primed or unprimed refers to priming for the influenza vaccine

Hepatitis A vaccine: *Havrix* (GSK); Pneumococcal vaccine: *Prevenar13* (Pfizer); Varicella virus vaccine: *Varilrix* (GSK)

Havrix and *Varilrix* are trademarks of the GSK group of companies. *Prevenar13* is a trademark of Pfizer.

Supplemental Digital Content 2

Hemagglutination inhibition assay

Hemagglutination inhibition (HI) antibody titers were determined using a method derived from the WHO Manual on Animal Influenza Diagnosis and Surveillance. Measurements were conducted on thawed frozen serum samples with a standardized and comprehensively validated micro method using two hemagglutination units (2 HAU) of the appropriate antigens and a 0.45% chicken erythrocyte suspension. Non-specific serum inhibitors were removed by heat treatment and receptor-destroying enzymes. Starting with an initial dilution of 1:10, a dilution series (by a factor of 2) was prepared up to an end dilution of 1:10240. The titration end-point was taken as the highest dilution step that showed complete inhibition of hemagglutination. All assays were performed in duplicate. The assay cut-off value was 10 1/dil.

Microneutralization assay

Microneutralization (MN) assay was used to determine the neutralizing antibody titer as previously described (Hehme et al 2004). Thawed frozen serum samples were heat inactivated for 30 minutes at 56°C. A standardized amount of virus was mixed with serial dilutions of serum and incubated to allow binding of the antibodies to the virus. A cell suspension containing a defined amount of Madin-Darby Canine Kidney cells was then added to the mixture of virus and antiserum, and incubated at 37°C for 7 days. After the incubation period, virus replication was visualized by

hemagglutination of chicken red blood cells. The 50% neutralization titer of a serum sample was calculated as the geometric mean titer between the highest serum dilution able to totally neutralize the virus and the next serum dilution where viruses remained detectable. Each serum sample was tested once.

The assay cut-off value was 28 1/dil for the A/H1N1, A/H3N2 and B/Victoria strains. In the course of the annual strain revalidation process for the assay, a run effect was observed for two of the seven runs performed, in which approximately half of the supposedly negative samples tested positive for the B/Yamagata (Hubei-Wujiagang/158/2009) strain. The cause of the run effect could not be determined. The Limit of Blank (LOB) was 49 1/dil. In order to be conservative, the cut-off value for this strain was raised to 57 1/dil.

Neuraminidase inhibition assay

The neuraminidase inhibition (NI) antibody titer was determined using an enzyme linked lectin assay (Ella) as previously described (Hehme et al 2004). In this assay, the bottom of enzyme-linked immunosorbent assay (ELISA) plates was coated with a fetuin substrate. The assay is based on the neuraminidase enzymatic activity which releases N-acetyl neuraminic acid from fetuin substrate. After cleavage of the terminal neuraminic acid, ß-D-galactose-N-acetyl-galactosamin is unmasked. Peroxidase-labelled peanut agglutinin binds specifically to the galactose residues and the enzymatic desialylation can be detected and quantified by a colorimetric reaction using 3.3´5,5´-Tetramethylbenzidin (TMB) as a substrate. The neuraminidase inhibition titer of a serum sample was measured by mixing a standard

amount of neuraminidase with serial dilutions of serum, and was set as the reciprocal of the serum dilution that reduced the colorimetric signal resulting from desialylation by 50%. The assay was performed with wild-type whole virus.

The assay cut-off value was 20 1/dil for the A/H1N1, B/Victoria and B/Yamagata strains. As part of the validation process, limits below the classical cut-off value were explored to better support assay specificity. For the A/H3N2 (A/Victoria/361/2011) strain, assay specificity fell short of the target (50–60% instead of ≥80%) with the standard cut-off of 20 1/dil. The LOB equalled 28 1/dil. The cut-off value was increased to 40 1/dil, the first measurable titer above 28 1/dil.

The whole virus antigen used for the enzyme-linked lectin NI assay may have overestimated neuraminidase antibody responses, as anti-HA antibodies may inhibit neuraminidase-mediated activation of the lectin by steric hindrance. Whether such potentially beneficial inhibition would occur *in vivo* is unknown.

Reference

Hehme N, Engelmann H, Kuenzel W, Neumeier E, Saenger R. Immunogenicity of a monovalent, aluminium-adjuvanted influenza whole virus vaccine for pandemic use. *Virus Res* 2004;103:163-171

Supplemental Digital Content 3

Difference in seroprotection rates for HI antibodies between primed and unprimed children, 7 days after first vaccination in the revaccination study (per-protocol cohort)

	Difference in SPR, % (95% CI)[1]
A/H1N1	62.43 (55.27-68.89)
A/H3N2	47.40 (39.08-55.06)
B/Victoria	56.68 (49.44-63.43)
B/Yamagata	56.72 (49.41-63.49)

[1]Difference in SPR (primed minus unprimed), %

CI: confidence interval; SPR: seroprotection rate

Supplemental Digital Content 4

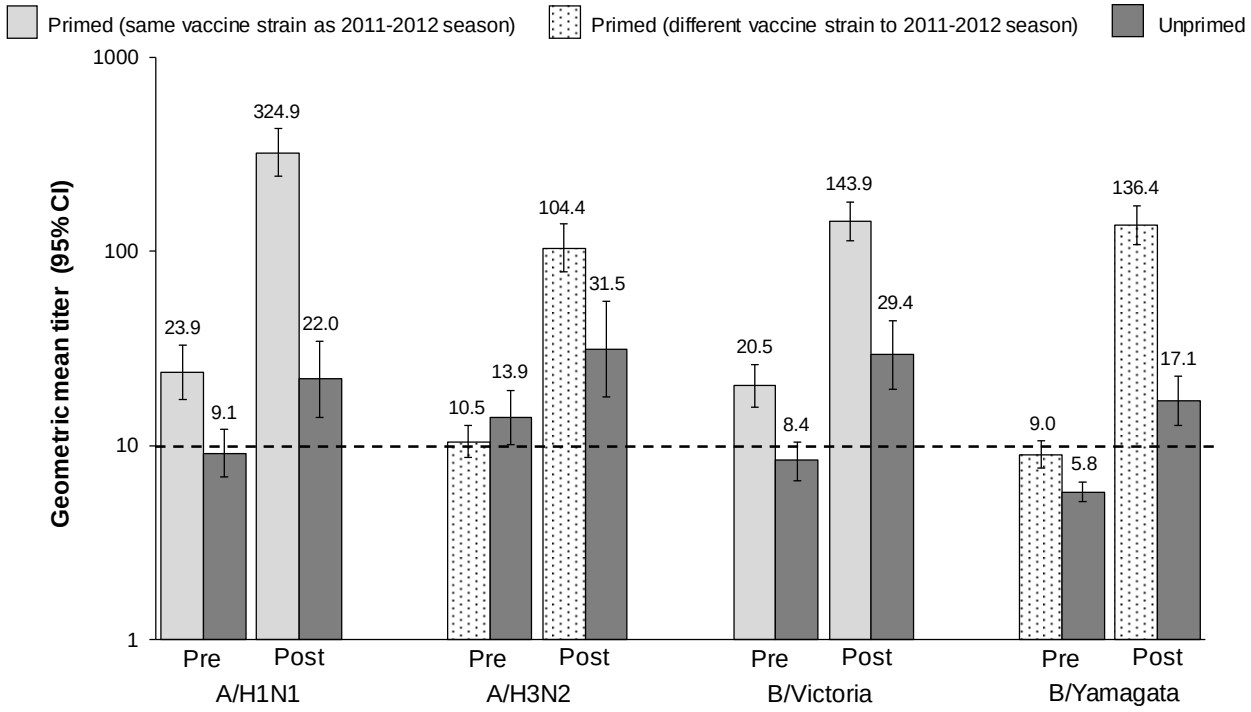
Seropositivity rate for HI antibodies pre-vaccination and 7 days after first vaccination in the revaccination study, by age strata (per-protocol cohort)

	Primed			Unprimed		
	N	**n**	**Seropositivity rate, % (95% CI)**	**N**	**n**	**Seropositivity rate, % (95% CI)**
17-29 months						
A/H1N1						
Pre-vaccination	91	73	80.2 (70.6-87.8)	89	17	19.1 (11.5-28.8)
Post-vaccination	91	88	96.7 (90.7-99.3)	92	55	59.8 (49.0-69.9)
A/H3N2						
Pre-vaccination	91	48	52.7 (42.0-63.3)	89	31	34.8 (25.0-45.7)
Post-vaccination	91	87	95.6 (89.1-98.8)	92	38	41.3 (31.1-52.1)
B/Victoria						
Pre-vaccination	91	71	78.0 (68.1-86.0)	89	20	22.5 (14.3-32.6)
Post-vaccination	91	91	100 (96.0-100)	92	68	73.9 (63.7-82.5)
B/Yamagata						
Pre-vaccination	91	41	45.1 (34.6-55.8)	89	9	10.1 (4.7-18.3)
Post-vaccination	91	89	97.8 (92.3-99.7)	92	59	64.1 (53.5-73.9)
30-48 months						
A/H1N1						
Pre-vaccination	130	116	89.2 (82.6-94.0)	113	47	41.6 (32.4-51.2)
Post-vaccination	133	132	99.2 (95.9-100)	117	82	70.1 (60.9-78.2)
A/H3N2						
Pre-vaccination	130	83	63.8 (55.0-72.1)	113	48	42.5 (33.2-52.1)
Post-vaccination	133	131	98.5 (94.7-99.8)	117	61	52.1 (42.7-61.5)
B/Victoria						
Pre-vaccination	130	116	89.2 (82.6-94.0)	113	38	33.6 (25.0-43.1)
Post-vaccination	133	133	100 (97.3-100)	117	106	90.6 (83.8-95.2)
B/Yamagata						
Pre-vaccination	130	93	71.5 (63.0-79.1)	113	27	23.9 (16.4-32.8)
Post-vaccination	133	133	100 (97.3-100)	117	85	72.6 (63.6-80.5)

CI: confidence interval; HI: hemagglutination inhibition

Supplemental Digital Content 5

GMT for HI antibodies pre-vaccination and 7 days after first vaccination in the revaccination study in children 17−29 months of age (per-protocol cohort)

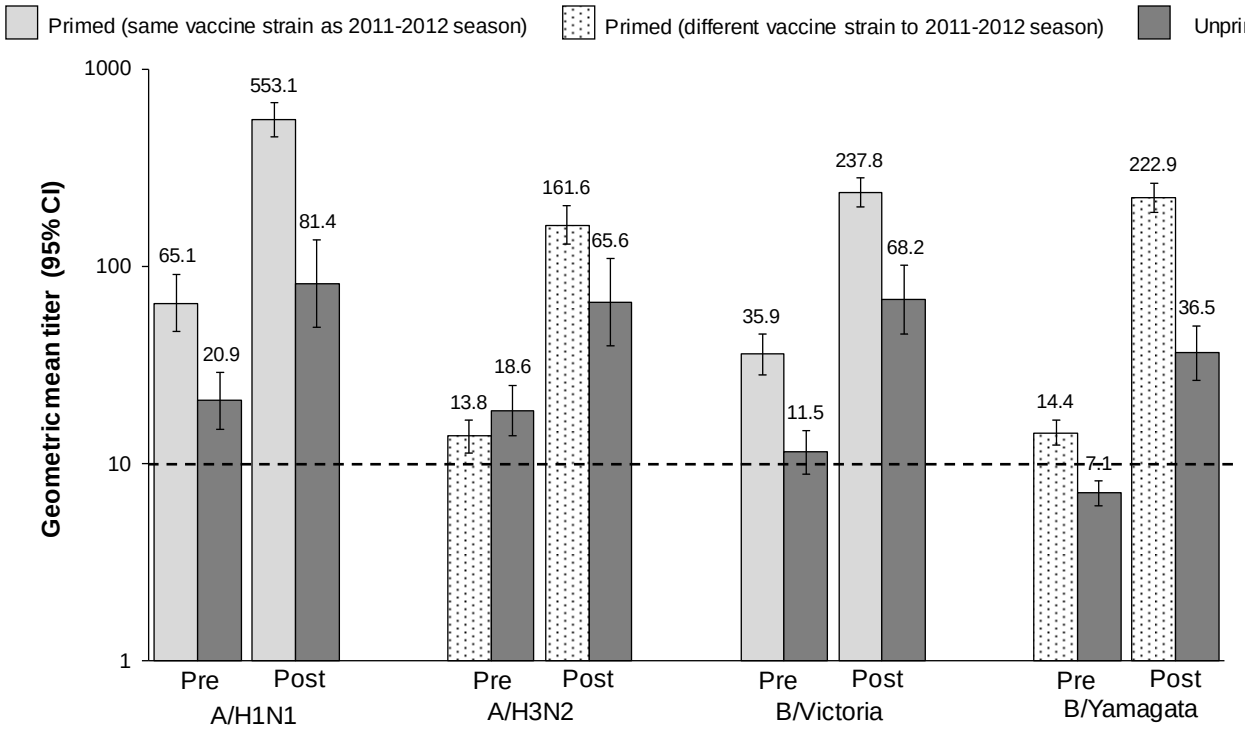


Dotted line represents the assay cut-off (10 1/dil). GMT values are shown above the bars.

CI: confidence interval; GMT: geometric mean titer; HI: hemagglutination inhibition; Pre: pre-vaccination; Post: 7 days following vaccination

Supplemental Digital Content 6

GMT for HI antibodies pre-vaccination and 7 days after first vaccination in the revaccination study in children 30−48 months of age (per-protocol cohort)



Dotted line represents the assay cut-off (10 1/dil). GMT values are shown above the bars.

CI: confidence interval; GMT: geometric mean titer; HI: hemagglutination inhibition; Pre: pre-vaccination; Post: 7 days following vaccination

Supplemental Digital Content 7

Seroconversion rate for HI antibodies 7 days after first vaccination in the revaccination study, overall and by age strata (per-protocol cohort)

	Primed			Unprimed		
	N	**n**	**SCR, % (95% CI)**	**N**	**n**	**SCR, % (95% CI)**
All ages						
A/H1N1	221	170	76.9 (70.8-82.3)	202	65	32.2 (25.8-39.1)
A/H3N2	221	180	81.4 (75.7-86.3)	202	73	36.1 (29.5-43.2)
B/Victoria	221	169	76.5 (70.3-81.9)	202	78	38.6 (31.9-45.7)
B/Yamagata	221	208	94.1 (90.2-96.8)	202	77	38.1 (31.4-45.2)
17-29 months						
A/H1N1	91	77	84.6 (75.5-91.3)	89	19	21.3 (13.4-31.3)
A/H3N2	91	72	79.1 (69.3-86.9)	89	24	27.0 (18.1-37.4)
B/Victoria	91	72	79.1 (69.3-86.9)	89	27	30.3 (21.0-41.0)
B/Yamagata	91	84	92.3 (84.8-96.9)	89	25	28.1 (19.1-38.6)
30-48 months						
A/H1N1	130	93	71.5 (63.0-79.1)	113	46	40.7 (31.6-50.4)
A/H3N2	130	108	83.1 (75.5-89.1)	113	49	43.4 (34.1-53.0)
B/Victoria	130	97	74.6 (66.2-81.8)	113	51	45.1 (35.8-54.8)
B/Yamagata	130	124	95.4 (90.2-98.3)	113	52	46.0 (36.6-55.6)

CI: confidence interval; HI: hemagglutination inhibition; SCR: seroconversion rate

Supplemental Digital Content 8

Seroprotection rate for HI antibodies 7 days after first vaccination in the revaccination study, overall and by age strata (per-protocol cohort)

	Primed			Unprimed		
	N	**n**	**SPR, % (95% CI)**	**N**	**n**	**SPR, % (95% CI)**
All ages						
A/H1N1	224	217	96.9 (93.7-98.7)	209	72	34.4 (28.0-41.3)
A/H3N2	224	193	86.2 (80.9-90.4)	209	81	38.8 (32.1-45.7)
B/Victoria	224	217	96.9 (93.7-98.7)	209	84	40.2 (33.5-47.2)
B/Yamagata	224	216	96.4 (93.1-98.4)	209	83	39.7 (33.0-46.7)
17-29 months						
A/H1N1	91	86	94.5 (87.6-98.2)	92	20	21.7 (13.8-31.6)
A/H3N2	91	75	82.4 (73.0-89.6)	92	27	29.3 (20.3-39.8)
B/Victoria	91	85	93.4 (86.2-97.5)	92	30	32.6 (23.2-43.2)
B/Yamagata	91	85	93.4 (86.2-97.5)	92	26	28.3 (19.4-38.6)
30-48 months						
A/H1N1	133	131	98.5 (94.7-99.8)	117	52	44.4 (35.3-53.9)
A/H3N2	133	118	88.7 (82.1-93.5)	117	54	46.2 (36.9-55.6)
B/Victoria	133	132	99.2 (95.9-100)	117	54	46.2 (36.9-55.6)
B/Yamagata	133	131	98.5 (94.7-99.8)	117	57	48.7 (39.4-58.1)

CI: confidence interval; HI: hemagglutination inhibition; SPR: seroprotection rate

Supplemental Digital Content 9

Mean geometric increase for HI, neutralizing and anti-neuraminidase antibodies 7 days after first vaccination in the revaccination study, overall and by age strata (per-protocol cohort)

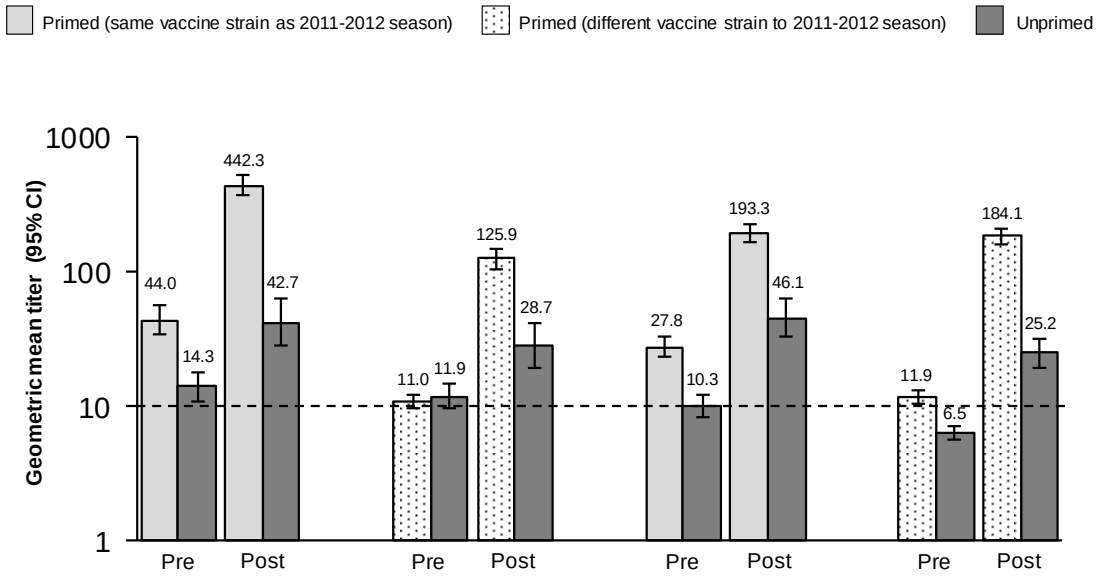
	Primed		Unprimed	
	N	**MGI (95% CI)**	**N**	**MGI (95% CI)**
HI antibodies				
All ages				
A/H1N1	221	10.3 (8.5-12.4)	202	3.2 (2.6-3.9)
A/H3N2	221	10.9 (9.4-12.6)	202	2.9 (2.4-3.6)
B/Victoria	221	6.7 (5.9-7.6)	202	4.6 (3.8-5.5)
B/Yamagata	221	15.2 (13.3-17.3)	202	4.0 (3.3-4.9)
17-29 months				
A/H1N1	91	13.6 (10.3-18.0)	89	2.4 (1.8-3.2)
A/H3N2	91	9.9 (7.7-12.7)	89	2.2 (1.6-3.0)
B/Victoria	91	7.0 (5.7-8.6)	89	3.3 (2.5-4.4)
B/Yamagata	91	15.1 (11.9-19.0)	89	2.9 (2.2-3.7)
30-48 months				
A/H1N1	130	8.4 (6.5-10.9)	113	4.0 (3.1-5.3)
A/H3N2	130	11.6 (9.7-13.8)	113	3.7 (2.8-4.9)
B/Victoria	130	6.5 (5.5-7.7)	113	6.0 (4.8-7.5)
B/Yamagata	130	15.2 (13.1-17.7)	113	5.2 (4.0-6.9)
Neutralizing antibodies				
All ages				
A/H1N1	97	10.6 (8.2-13.7)	89	3.1 (2.3-4.2)
A/H3N2	97	6.4 (5.4-7.6)	94	4.5 (3.2-6.2)
B/Victoria	107	5.0 (4.3-5.8)	108	2.1 (1.6-2.8)
B/Yamagata	107	5.0 (4.3-5.7)	105	1.7 (1.4-2.0)
17-29 months				
A/H1N1	47	13.1 (9.2-18.8)	44	2.0 (1.4-3.0)
A/H3N2	48	6.1 (4.8-7.7)	46	3.4 (2.1-5.4)
B/Victoria	53	5.4 (4.4-6.5)	53	1.8 (1.2-2.6)

B/Yamagata	53	4.4 (3.8-5.2)	51	1.5 (1.2-2.0)
30-48 months				
A/H1N1	50	8.7 (6.0-12.5)	45	4.7 (3.0-7.3)
A/H3N2	49	6.7 (5.1-8.8)	48	5.9 (3.8-9.2)
B/Victoria	54	4.7 (3.8-5.8)	55	2.5 (1.6-3.8)
B/Yamagata	54	5.5 (4.5-6.9)	54	1.8 (1.4-2.4)
Anti-neuraminidase antibodies				
All ages				
A/H1N1	105	8.3 (6.5-10.7)	106	1.8 (1.5-2.1)
A/H3N2	107	4.9 (4.2-5.8)	106	2.0 (1.7-2.3)
B/Victoria	105	5.2 (4.4-6.0)	106	1.9 (1.5-2.4)
B/Yamagata	105	8.8 (7.5-10.2)	106	2.7 (2.1-3.4)
17-29 months				
A/H1N1	53	11.4 (8.2-15.8)	52	1.5 (1.2-1.9)
A/H3N2	53	4.6 (3.6-5.8)	52	1.7 (1.4-2.0)
B/Victoria	53	5.2 (4.3-6.3)	52	1.5 (1.1-2.1)
B/Yamagata	53	8.8 (7.2-10.8)	52	2.2 (1.5-3.2)
30-48 months				
A/H1N1	52	6.0 (4.1-8.8)	54	2.1 (1.7-2.7)
A/H3N2	54	5.3 (4.2-6.8)	54	2.3 (1.9-2.8)
B/Victoria	52	5.1 (4.1-6.5)	54	2.3 (1.6-3.4)
B/Yamagata	52	8.7 (6.9-11.0)	54	3.2 (2.3-4.5)

CI: confidence interval; HI: hemagglutination inhibition; MGI: mean geometric increase

Supplemental Digital Content 10

GMT pre-vaccination and 7 days after first vaccination, GMT ratio, SCR difference and SPR difference for HI antibodies in the revaccination study (per-protocol cohort excluding children with an RT-PCR-confirmed influenza infection in the primary study)



	A/H1N1	A/H3N2	B/Victoria	B/Yamagata
GMT ratio (95% CI)[1]	9.4 (6.4-13.9)	4.2 (2.8-6.2)	4.0 (2.9-5.5)	6.9 (5.3-9.0)
SCR difference (95% CI)[2]	45.0 (35.6-53.5)	54.1 (45.1-62.0)	39.0 (29.5-47.8)	56.2 (48.0-63.8)
SPR difference (95% CI)[3]	63.8 (56.1-70.6)	55.3 (46.6-63.0)	57.6 (49.8-64.8)	57.1 (49.3-64.4)

[1]GMT ratio adjusted for age (primed/unprimed); [2]Difference in SCR (primed minus unprimed); [3]Difference in SPR (primed minus unprimed)

Dotted line represents the assay cut-off (10 1/dil). GMT values are shown above the bars.

CI: confidence interval; GMT: geometric mean titer; HI: hemagglutination inhibition; Pre: pre-vaccination; Post: 7 days following vaccination; RT-PCR: reverse transcription polymerase chain reaction; SCR: seroconversion rate; SPR: seroprotection rate

Supplemental Digital Content 11

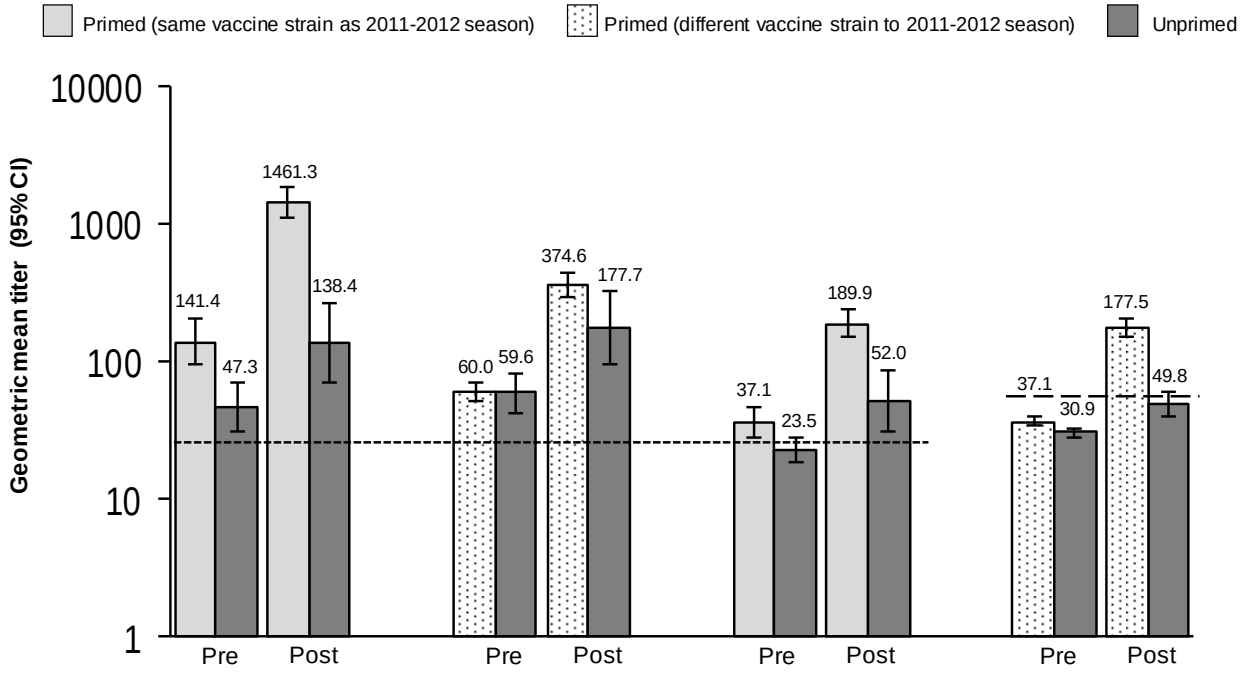
Seroprotection rate and seroconversion rate for HI antibodies 7 days after first vaccination in the revaccination study (per-protocol cohort excluding children with an RT-PCR-confirmed influenza infection in the primary study)

	Primed			Unprimed		
	N	**n**	**Rate, % (95% CI)**	**N**	**n**	**Rate, % (95% CI)**
SPR						
A/H1N1	213	206	96.7 (93.3-98.7)	179	59	33.0 (26.1-40.4)
A/H3N2	213	182	85.4 (80.0-89.9)	179	54	30.2 (23.5-37.5)
B/Victoria	213	206	96.7 (93.3-98.7)	179	70	39.1 (31.9-46.7)
B/Yamagata	213	205	96.2 (92.7-98.4)	179	70	39.1 (31.9-46.7)
SCR						
A/H1N1	210	160	76.2 (69.8-81.8)	173	54	31.2 (24.4-38.7)
A/H3N2	210	173	82.4 (76.5-87.3)	173	49	28.3 (21.7-35.7)
B/Victoria	210	162	77.1 (70.9-82.6)	173	66	38.2 (30.9-45.8)
B/Yamagata	210	197	93.8 (89.6-96.7)	173	65	37.6 (30.3-45.2)

CI: confidence interval; HI: hemagglutination inhibition; RT-PCR: reverse transcription polymerase chain reaction; SCR: seroconversion rate; SPR: seroprotection rate

Supplemental Digital Content 12

GMT pre-vaccination and 7 days after first vaccination and MGI for neutralizing antibodies in the revaccination study (per-protocol cohort excluding children with an RT-PCR-confirmed influenza infection in the primary study)



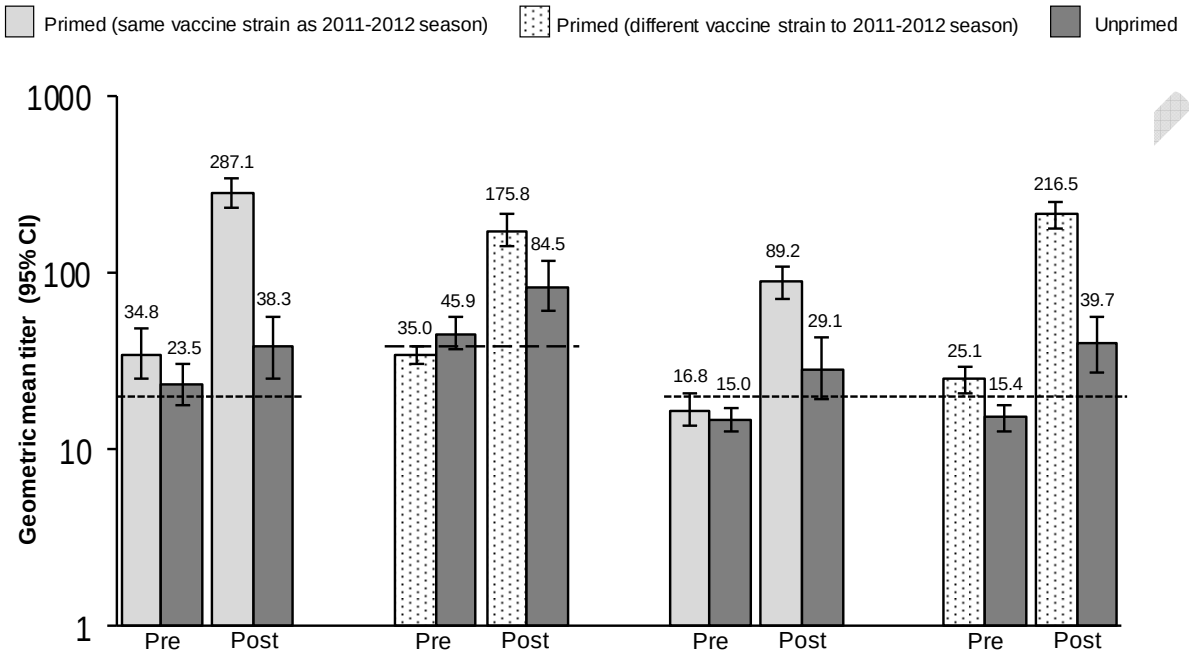
	A/H1N1	A/H3N2	B/Victoria	B/Yamagata
MGI, % (95% CI), primed	10.0 (7.7-13.1)	6.2 (5.2-7.5)	5.1 (4.4-5.9)	4.8 (4.2-5.4)
MGI, % (95% CI), unprimed	3.1 (2.2-4.3)	3.4 (2.4-4.9)	2.2 (1.6-3.0)	1.6 (1.3-1.9)

Dotted line represents the assay cut-off for the A/H1N1, A/H3N2 and B/Victoria strains (28 1/dil) and B/Yamagata strain (57 1/dil). GMT values are shown above the bars.

CI: confidence interval; GMT: geometric mean titer; MGI: mean geometric increase; Pre: pre-vaccination; Post: 7 days following vaccination; RT-PCR: reverse transcription polymerase chain reaction

Supplemental Digital Content 13

GMT pre-vaccination and 7 days after first vaccination and MGI for anti-neuraminidase antibodies in the revaccination study (per-protocol cohort excluding children with an RT-PCR-confirmed influenza infection in the primary study)



	A/H1N1	A/H3N2	B/Victoria	B/Yamagata
MGI, % (95% CI), primed	8.1 (6.2-10.5)	5.2 (4.5-6.1)	8.7 (7.4-10.2)	5.0 (4.2-6.0)
MGI, % (95% CI), unprimed	1.7 (1.4-2.1)	1.9 (1.5-2.5)	2.6 (2.0-3.5)	1.8 (1.6-2.2)

Dotted line represents the assay cut-off for the A/H1N1, B/Victoria and B/Yamagata strains (20 1/dil) and A/H3N2 strain (40 1/dil). GMT values are shown above the bars.

CI: confidence interval; GMT: geometric mean titer; MGI: mean geometric increase; Pre: pre-vaccination; Post: 7 days following vaccination; RT-PCR: reverse transcription polymerase chain reaction