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Use of traditional serological methods and oral fluids to assess immunogenicity in children aged 2–16 years after successive annual vaccinations with LAIV

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ABSTRACT

Background: The UK introduced quadrivalent live attenuated influenza vaccine (qLAIV) for children in 2013/2014. The impact of annual vaccination on effectiveness and immunogenicity is being assessed.
Method: A phase III/IV open-label study of the immunogenicity of annual vaccination with qLAIV (Fluenz™) was conducted over three consecutive years (2014/15–2016/17) in 254, 249 and 162 children respectively. Serum responses to vaccine components were measured by Haemagglutination Inhibition (HAI) and anti-A(H1N1)pdm09 Neuraminidase (NAI) assays, stratified according to previous receipt of $AS03_B$-adjuvanted A(H1N1)pdm09 pandemic vaccine in 2009/10. Antibody levels to the A(H1N1)pdm09 and H3N2 vaccine components in oral fluids (OF) were explored using an ELISA.
Findings: More paired pre- and post-vaccination oral fluids (96%) than paired sera (87%) were obtained. Geometric mean titre rises using HAI assays were limited, with maximum rises seen in year one for both influenza B strains when 39% and 43% of subjects seroconverted (95% confidence interval 33–46% and 36–50%, respectively) and year two for influenza H3N2, when 40% (33–46%) individuals seroconverted. Prior pandemic vaccine receipt resulted in higher pre- and post-vaccination A(H1N1)pdm09 HAI titres and lower pre-and post-vaccination NAI (N1 neuraminidase) titres in all three years. OF results were congruent with HAI results; assay specificity compared to HAI was 88.1 and 71.6 percent, and sensitivity was 86.4 and 74.8 percent respectively for A(H1N1)pdm09 and H3N2.
Conclusion: In all three study years, vaccination with qLAIV resulted in poor antibody responses. However, OFs are an alternative specimen type that allows self sampling, can easily be obtained from children, and their analysis leads to similar conclusions as classic serology by HAI. Their suitability for seroprevalence studies should be investigated. We demonstrated a sustained effect from prior receipt of the $AS03_B$-adjuvanted A(H1N1)pdm09 vaccine, even after repeat vaccination with qLAIV indicating that early exposure to influenza antigens has a significant long lasting effect.

1. Introduction

In the United Kingdom, the childhood influenza vaccination programme using live attenuated influenza vaccine (LAIV) is being implemented as a phased national roll-out which began in the 2013/14 season in children aged 2–3 years. Extension to older birth cohorts has taken place in successive years [1], currently (2019/20 season) including children up to 10 years of age [2]. A single dose of the quadrivalent LAIV (qLAIV) containing an A(H1N1)pdm09, A/H3N2 and two B strains is offered to eligible age groups irrespective of prior vaccination history.

A cohort of children was established in 2014/15 to measure serum antibody responses to successive qLAIV vaccinations over a period of three years. The study was also designed to investigate whether previous vaccination of children with monovalent pandemic vaccine in 2009/10 ($AS03_B$ adjuvanted A(H1N1)pdm09 pandemic vaccine; Pandemrix™) affected responses to qLAIV. Receipt

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of Pandemrix™ was previously shown to influence responses to all three components of trivalent inactivated vaccine (TIV) [3] received in the following year. In addition, oral fluids (OF) were collected from the study cohort at the same time as the serum samples to explore the potential of this non-invasive sample to assess antibody responses to influenza A haemagglutinins in vaccine using an in-house Enzyme Linked Immunosorbent Assay (ELISA).

In this paper we report the serum antibody (HAI) responses of children in our study cohort over successive years for influenza A and B, and also by NAI for A(H1N1)pdm09. In addition, we report the sensitivity and specificity of the OF ELISA when compared to HAI for assessing vaccine responses.

2. Materials and methods

2.1. Trial approvals

The study was sponsored by Public Health England (PHE) and approved by the UK Medicines and Healthcare Products Regulatory Agency (EudraCT number 2013-003592-35), the NRES Committee London - West London & GTAC (14/LO/0227) and was given local NHS approval.

2.2. Study design

Study participants were recruited and vaccinated by research nurses based in primary care in Gloucestershire, Hertfordshire and North West London with written informed consent given by parent/legal guardian. Approximately equal numbers of children in previous receipt of Pandemrix™ and those naïve to Pandemrix™ were sought, so that the inclusion criterion for age was 5 to <12 years on the day of recruitment. The participants were followed up on an annual basis, so that they were included in the trial for a maximum of three years (two years for those joining in year two; 2015/16), receiving one dose qLAIV in each year of the trial.

Exclusion criteria were hypersensitivity to any of the vaccine constituents, clinical immuno-deficiencies, recipients of salicylate therapy, or any other vaccine within a month prior to LAIV or any contraindication to vaccination as specified in the "Green Book"- Immunisation against Infectious Disease [4]. For each trial year, blood and oral fluid samples (OF, crevicular fluid; using Oracol swabs from Malvern Medical Developments) were collected on the day of immunization and on day 21 after vaccination. The oral fluids were collected by the research nurse, parent or child by passing the swab across the tooth/gum line for about one to two minutes in an action similar to brushing teeth. The collected fluid is a serum transudate and contains mainly IgG [5].

2.3. Vaccine

Participants were given qLAIV (Fluenz Tetra®, AstraZeneca UK Limited) intra-nasally as a 0.1 mL spray dose into each nostril. The composition of the vaccine in different study years is listed (together with WHO vaccine recommendation) in Table 1.

2.4. Laboratory analysis

Serological analysis by HAI using vaccine-matched influenza A (H1N1)pdm09, A(H3N2) and B strains and quantification of NAI antibody by Enzyme linked lectin assay (ELLA) for A(H1N1) pdm09 were performed as previously described [6]. Antigens for all assays were egg-grown and the antigen for influenza B antigens were Tween80/Diethyl-Ether extracted (details for strain choice by year in Table 1).

For the influenza specific ELISA, commercial recombinant HA1 subunit of the haemagglutininins (HA) were purchased. A/California/06/2009(H1N1) virus HA1 (amino acid 1-344; Accession# ACP41935.1; $HA1_{H1N1}$) with a C-terminal polyhistidine tag and the C-terminal 6x His-tagged influenza hemagglutinin HA1 of A/Hong Kong/4801/2014(H3N2) virus (amino acid 17-345; Accession# EPI653201; $HA1_{H3N2}$) – both expressed in HEK293 cell culture - were purchased from SinoBiological Inc (Beijing, P.R. China) and eEnzyme LLC (Gaithersburg, U.S.A), respectively and used to coat Nunc MaxiSorp® flat-bottomed, polystyrene 96-well microtitre plates by diluting 50 ng recombinant $HA1_{H1N1}$ or 15 ng recombinant $HA1_{H3N2}$ protein/well in sterile PBS; pH7.2 ± 0.05 (-CaCl2, -MgCl2), (GIBCO Invitrogen) and incubating at 4–8 °C for a minimum of 16 h.

For both, A(H1N1)pdm09 and H3N2 ELISAs, serum and OF were diluted at a final dilution factor of 1 in 500 and 1 in 50 for sera and OF, respectively. Samples were analysed in duplicate.

For detection of HA1 specific antibody in sera, polyclonal rabbit anti-human-IgG horseradish peroxidase conjugate (Agilent Technologies LDA UK Limited, Stockport, UK) was used, while detection of HA1 specific antibody in OF required a further signal increasing step using goat biotinylated anti-human IgG (eBiosciences, San Diego, U.S.A) followed by addition of Poly-HRP-streptavidin (Thermo Scientific™). The visualisation of antibody detection for both assay formats was performed using TMB (3,3′ 5,5′ - Tetramethylbenzidine) as substrate (Europa Bioproducts Ltd, Ely, UK) and stopped by addition of 0.5 M HCl. The quantification of the colour development was performed using a 96-well plate reader at 450 nm.

Optical density (OD_{450}) data was evaluated by dividing average OD_{450} values for all samples by average OD_{450} of a known calibrator with negative to low specific antibody levels (T/N ratio).

For all OF samples, the total IgG was determined in antibody capture format as previously described [7] with the following modifications: Pre-coated anti-human IgG microplates were purchased from Clin-Tech Ltd (Guildford, UK), from whom we also purchased wash buffer 10 × concentrate and ready-to-use TMB. The samples were diluted 1:10, the standard curve was prepared in the range 2.5 mg/L to 0.0195 mg/L using an IgG calibrator (The Binding Site Ltd., Birmingham, UK). Bound antibodies were detected using peroxidase conjugated rabbit anti-human IgG (Jackson Immuno Research Laboratories Inc, West Grove, U.S.A) after washing plates 5 times with 300ul wash buffer. After addition of TMB substrate, the reaction was stopped using 100ul of 0.5 M HCl.

2.5. Statistical analysis

2.5.1. Assay development

Upon preliminary analysis of the OF data, it became clear that, on average, OF ELISA T/N increased with total IgG content. This is demonstrated using boxplots for both A(H1N1)pdm09 and H3N2 in supplementary Fig. 1, the median OF ELISA T/N on the original scale increases by quartile of IgG content. Using the ladder of powers, we found a transformation as follows to take into account the IgG content,

$$oral\ fluid\ ELISA\ with\ IgG\ correction = \frac{oral\ fluid\ ELISA}{\sqrt{total\ IgG/4}}$$

Transformed OF ELISA T/Ns are referred to as standardised OF ELISA T/Ns. The division by four of total IgG in the equation was to keep the standardised total IgG on the same scale as unstandardized since the geometric mean total IgG was 4.0. Median standardised OF ELISA T/Ns are shown to be roughly stable by IgG content quartile for both A(H1N1)pdm09 and H3N2 in supplementary Fig. 1.

Table 1
Overview of antigens used in vaccines and laboratory testing by year and assay.[a]

Year	Subtype	WHO vaccine recommendation (IIV)	Vaccine composition (LAIV)	Antigen used in HAI (abbreviation in text)	Antigen used in ELISA
Year 1: 2014–2015	H1N1	A/California/7/2009-like	A/California/7/2009, MEDI 228029	A/California/7/2009 ($A/H1N1_{Cal}$)	A/California/7/2009
	H3N2	A/Texas/50/2012-like	A/Texas/50/2012, MEDI 237514	A/Texas/50/2012 ($A/H3N2_{Tex}$) A/Switzerland/9715293/2013 ($A/H3N2_{Switz}$)	A/Hong Kong/4801/2014
	B_{Yam}	B/Massachusetts/2/2012-like	B/Massachusetts/2/2012, MEDI 237751	B/Massachusetts/2/2012 (B_{Bris})	–
	B_{Vic}	B/Brisbane/60/2008-like	B/Brisbane/60/2008, MEDI 228030	B/Brisbane/60/2008 (B_{Mass})	–
Year 2: 2015–2016	H1N1	A/California/7/2009-like	A/Bolivia/559/2013, MEDI 255962	A/California/7/2009 ($A/H1N1_{Cal}$)	A/California/7/2009
	H3N2	A/Switzerland/9715293/2013-like	A/Switzerland/9715293/2013, MEDI 252385	A/Switzerland/9715293/2013 ($A/H3N2_{Switz}$)	A/Hong Kong/4801/2014
	B_{Yam}	B/Phuket/3073/2013-like	B/Phuket/3073/2013, MEDI 254977	B/Phuket/3073/2013 (B_{Phu})	–
	B_{Vic}	B/Brisbane/60/2008-like	B/Brisbane/60/2008, MEDI 228030	B/Brisbane/60/2008 (B_{Bris})	–
Year 3: 2016–2017	H1N1	A/California/7/2009-like	A/Bolivia/559/2013, MEDI 255962	A/California/7/2009 ($A/H1N1_{Cal}$) A/Bolivia/559/2013 ($A/H1N1_{Bol}$)	A/California/7/2009
	H3N2	A/Hong Kong/4801/2014-like	A/New Caledonia/71/2014, MEDI 263122	A/Hong Kong/4801/2014 ($A/H3N2_{HongK}$)	A/Hong Kong/4801/2014
	B_{Yam}	B/Phuket/3073/2013-like	B/Phuket/3073/2013, MEDI 254977	B/Phuket/3073/2013 (B_{Phu})	–
	B_{Vic}	B/Brisbane/60/2008-like	B/Brisbane/60/2008, MEDI 228030	B/Brisbane/60/2008 (B_{Bris})	–

[a] NAI assay only performed for N1 using NIBRG127 in all three study years (=reverse genetic virus with HA from A/Prague/56 (H7N7), NA gene from A/California/7/2009 (H1N1) on A/PR/8/34 (H1N1).

2.5.2. Assay comparison

Results from serum HAI, serum ELISA and OF ELISA taken on the same day from the same individual were used to compare assays. The relationship between HAI titres, serum and OF ELISA T/N (both standardised and unstandardised) was assessed using scatterplots and calculation of Spearman's rank correlation coefficient. All pre- or post- vaccination samples were assessed together in one analysis.

2.5.3. Cut-off determination and analysis

Cut-offs for OF ELISA T/Ns equivalent to a HAI titre of 40 were determined, since we considered HAI titres $\geq$ 40 seroprotective. A receiver operating curve (ROC) analysis was carried out, which indicated optimal equivalent cut-offs in terms of sensitivity and specificity for standardised OF ELISAs of 1.6 and 0.7 for A(H1N1) pdm09 and H3N2, respectively. The same optimal cut-offs were found for unstandardized OF ELISA T/Ns. Taking HAI seroprotective as the gold standard, the sensitivity, specificity and number classified for OF ELISA seroprotective using these cut-offs were calculated.

2.5.4. Trial analyses

HAI and NAI titres of $\geq$40 were considered seroprotective, A (H1N1)pdm09 OF ELISA T/Ns of $\geq$1.6 and H3N2 OF ELISA T/Ns of $\geq$0.7 were indicative of seroprotective serum HAI levels. A four-fold rise in antibody titres from pre- to post-vaccination was considered a seroconversion. Proportions seroprotected and geometric mean titres (GMT) pre- and post-vaccination, geometric mean fold rise (GMFR), and the proportion of subjects seroconverted by HAI, NAI and OF ELISA were calculated for each study year with 95% confidence intervals. GMT pre- and post-vaccination as well as GMFR pre- to post-vaccination by HAI and NAI were calculated for each year by prior Pandemrix™ status with 95% confidence intervals; titres were compared by Pandemrix™ status using the Kruskall-Wallis test.

3. Results

3.1. Participants

Fig. 1 describes recruitment and samples available by collection year, vaccination visit and sample type. A total of 256 participants were recruited into the study in its first year (October 2014 to February 2015), two of whom subsequently withdrew prior to vaccination. The remaining 254 participants provided at least one pre-vaccination sample (serum and/or OF) and 249 at least one post-vaccination sample for analysis. One hundred and ninety-six subjects agreed to remain in the trial and were joined by 53 newly recruited individuals for the second year of the study (2015/16). In the third study year (2016/17), 162 previously recruited participants provided at least one pre-vaccination sample and 153 at least one post-vaccination sample for analysis. In total, 127 children were vaccinated in all three study years.

3.2. Sample numbers by type

Comparison of samples by specimen type and study year (Fig. 1, Table 2) showed that more individuals accepted the collection of OFs compared with serum collection by venepuncture. 96% of subjects provided paired pre-and post OF compared with 87% of subjects who provided paired pre- and post-vaccine sera.

3.3. Serological responses measured by HAI and NAI

In all study years, the pre-vaccination influenza antibody levels by HAI were high; between 51% and 98% participants had titres $\geq$ 40 to all the vaccine strains (Table 3). Vaccination with qLAIV did generally not result in significant rises in GMT except in the first study year for both influenza B strains and second study year for influenza H3N2, when GMFRs of 2.99 to 3.71 were observed between pre-and post- vaccine titres. Similarly, seroconversion rates were generally low, with the exception of study year one when 39.3% and 42.9% individuals converted with B_{Bris} and B_{Mass}, respectively and study year two when 39.5% individuals seroconverted with H3N2.

Differences were observed in all three years according to previous receipt of pandemic vaccine for influenza A, mainly with influenza A(H1N1)pdm09 where consistently higher pre- and post-vaccination HAI titres were observed in individuals who had received Pandemrix™ 5–7 years earlier (reaching significance post-vaccine in year 2 and pre-and post-vaccine in year 3, Fig. 4, Supplement Table 3). Children who had received Pandemrix™ also had lower pre-and post NAI titres, with the difference being significant in years 1 and 2 at both time points. In year 2, children with-

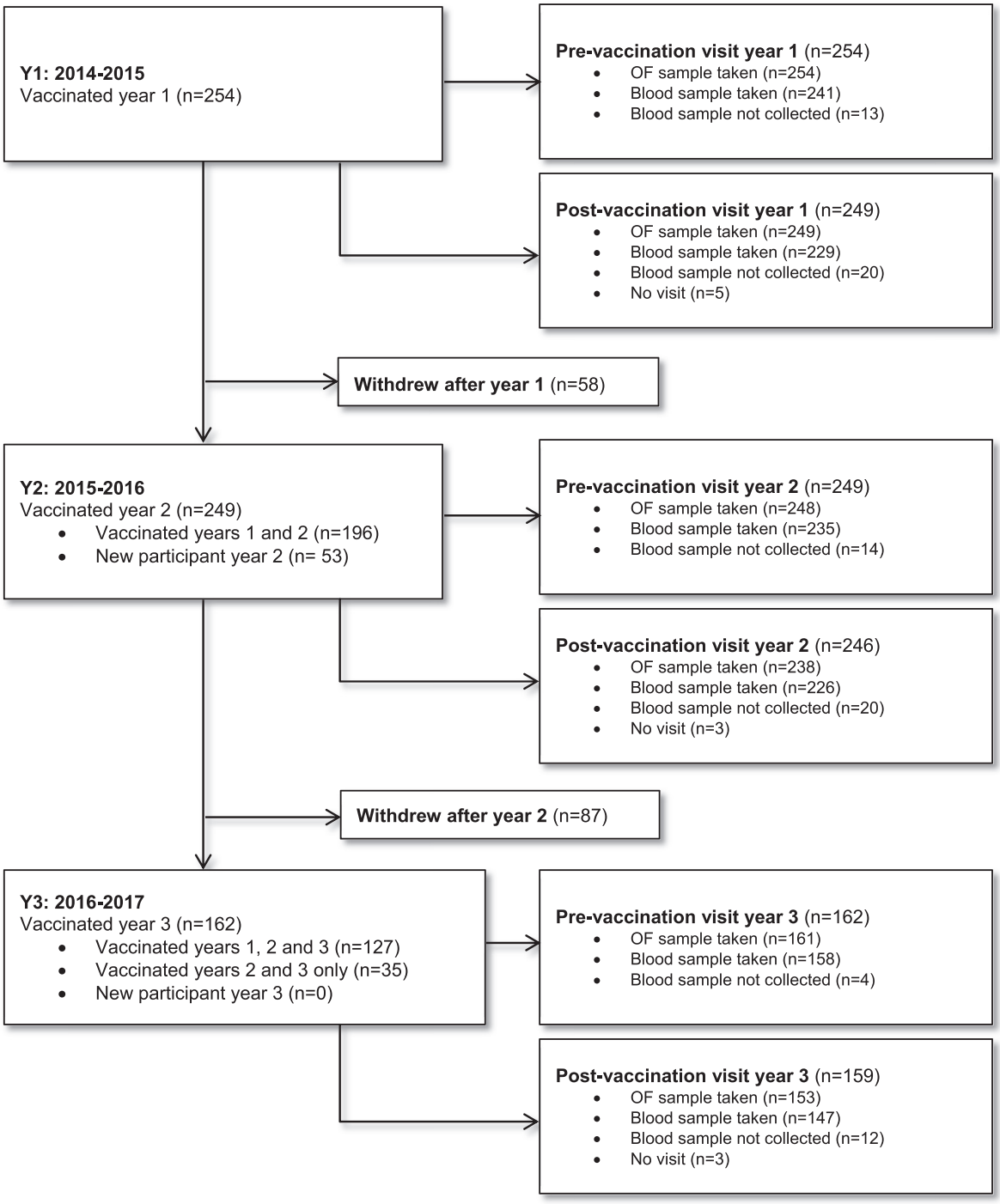

Fig. 1. Flow of participants. Children were recruited to two groups, which were defined by their vaccination history – vaccinated with Pandemrix™ (n = 97), or naïve to pandemic influenza vaccine (n = 157). (All participants received the same treatment; (EudraCT number 2013-003592-35).)

out previous pandemic vaccine receipt reached significantly higher HAI post vaccination to H3N2.

3.4. Analysis of oral fluids. Total immunoglobulin (IgG) content in oral fluids

Analysis of all 1297 available OFs indicated that total IgG content increases with age. Samples taken by the participating child contained more IgG than those taken by the study nurses in years 1 and 2, with no differences observed in year 3 (data not shown), indicating that self-sampling was at least as good as health care worker sampling; the geometric mean IgG content for all samples was 4 mg/L.

3.4.1. Correlation between serum ELISA and HAI

We used data from samples for which paired HAI and serum ELISA analysis was available. Comparison of the 255 available data pairs (HAI titres and T/N ELISA ratios) indicated strong correlation

Table 2
Summary of participant characteristics and available results by assay and specimen type by year.

Year	1	2	3	total
Number of participants vaccinated	254	249	162	665
Record of post-vaccination visit	249	246	159	654
Male (%)	124 (49%)	121 (49%)	80 (49%)	325 (49%)
Age at vaccination median (min - max)	8 (5–11)	9 (6–11)	9 (7–12)	9 (5–12)
Interval between samples median (min - max)	22 (18–57)	26 (21–43)	27 (21–49)	25 (18 – 57)
Received prior Pandemrix (%)	104 (41%)	110 (44%)	75 (46%)	289 (43%)
Paired pre- and post-vaccination samples:				
HAI result available	217	215	144	576 (87%)
OF ELISA result available	249	237	152	638 (96%)

between the two assay formats evidenced by Spearman R coefficients of 0.86 and 0.78 for A(H1N1)pdm09 and H3N2, respectively (Supplement Table 1a, original scale and Figs. 2 and 3).

3.4.2. Correlation between serum and OF ELISA

Paired data was available from 255 individuals. Analysis indicated strong correlation with Spearman's R coefficients of 0.83 and 0.79 for A(H1N1)pdm09 and H3N2, respectively (Supplement Table 1a, original scale and Figs. 2 and 3).

3.4.3. Correlation between serum HAI and OF ELISA

Overall correlation between serum HAI and OF ELISA was 0.72 and 0.68 for A(H1N1)pdm09 and H3N2, respectively; following standardisation of OF ELISA titres, correlation coefficients increased to 0.78 and 0.73 for A(H1N1)pdm09 and H3N2, respectively. (Supplement Table 1a and Figs. 2 and 3.)

3.4.4. OF ELISA sensitivity and specificity

Sensitivity, specificity and percentage with congruent results are shown in Supplement Table 1b. Using standardised OF ELISA data for all three years combined, specificity was 88.1 and 71.6 percent for A(H1N1)pdm09 and H3N2, respectively and sensitivity was 86.4 and 74.8 percent, respectively for A(H1N1)pdm09 and H3N2.

3.4.5. Evaluation of vaccination responses using OF ELISA data compared to serum HAI

Evaluation of the vaccine responses using standardized OF data compared to analysis by HAI (only available for influenza A), found overall comparable trends (Table 3). As with HAI, we found a generalised lack of responses to influenza A in years 1 and 3, and a weak response in year 2, where a GMFR of 1.53 for the antibody to H3N2 was observed in OF though the seroconversion rate was at 12% lower than the seroconversion rate by HAI (39.5%).

4. Discussion

This proof of principle study confirms the feasibility of collection and analysis of OFs as an alternative specimen type for assessment of antibody in children following vaccination with LAIV. Self-sampling of OFs is achievable even by children. Our results demonstrate in principle that analysis of oral fluids leads to similar conclusions as classic serology by HAI regarding subtype specific immunogenicity and show that qLAIV vaccination results in poor humoral antibody responses. Furthermore, we observed that prior receipt of the $AS03_B$-adjuvanted A(H1N1)pdm09 vaccine has a sustained effect on antibody quality and quantity even after repeat vaccination with qLAIV, leading to higher pre-vaccination A (H1N1)pdm09 HAI titres and lower pre-and post-vaccination NAI (N1 neuraminidase) titres in those previously vaccinated with pandemic vaccine. This is consistent with our previous results [6] and demonstrates the impact and duration of the effect of immune priming by early receipt of adjuvanted vaccines.

Our study design to investigate the long-term effects of previous vaccination with Pandemrix™ led to an increased age in our participants compared to the target group for use of qLAIV in the general population – which in 2014/15 – at the start of the study was two to four years in non-pilot and 4–11 or 11–13 years in pilot areas. Based on observations from previous studies, subject age does influence baseline antibody titres as well as sero-responses in a subtype specific manner reflecting the previous exposure of the individual and is also inversely correlated to vaccine virus shedding [8,9].

LAIV is believed to work through a combination of stimulation of cell mediated (especially T-cell), local (such as IgA) [10] and humoral responses in an age dependent fashion [11] and thus, clinical studies on LAIV have described these different aspects of the immune response [12]. More recently, the association and quantitation of viral shedding with immune measures pre- and post-vaccination have been explored in attempts to explain vaccine performance [9,13,14]. Although the link between these different parameters and vaccine effectiveness are not completely understood, it is important to keep the older age of our cohort in mind when trying to put our results into context with the national vaccine program.

Recent observations of low vaccine effectiveness (VE) of LAIV, especially from the US, and other countries with emergent childhood influenza vaccination programs highlight need for further ongoing monitoring of vaccine performance [15–18]. Relatively few studies have assessed immunogenicity of LAIV in post licensure trials or explored immunogenicity data in parallel to field estimates of vaccine effectiveness. Examination of risk factors for vaccine failure in older children in the 2013–14 season, showed minimal HAI antibody response after receipt of LAIV and that LAIV recipients had significant increased risk of A (H1N1)pdm09 infection compared to IIV recipients, but could not identify titre levels required for protection [19]. Supplementary Table 2 shows the strain specific qLAIV vaccine effectiveness estimates from the UK during the three study years and the seroconversion rates we measured to the same strains. While previously published data for year one [6] showed immunogenicity trends for H3N2 and B strains that were comparable to UK vaccine effectiveness, the relationship was less clear in subsequent years with, for example only 2.1% seroconverting with H3N2 in year three, for which the observed effectiveness was 57% (7.7–80.0). Therefore, caution needs to be exercised in interpretation of the relationship between immunogenicity and effectiveness based on observations in a single year, especially given the wide confidence intervals for field measurements of VE. The correlates of protection for LAIV thus remain unclear and suggest that serological measurements are not highly predictive of performance of vaccines in the field.

Table 3
Pre-vaccination and post-vaccination proportions with titres ≥ 40 and geometric mean titres (GMTs) with 95% confidence intervals (CIs) as measured by HAI, NAI and standardised OF ELISA together with geometric mean fold changes pre- to post-vaccination and proportions seroconverting (≥4 fold rise in titre).

	Pre-vaccination		Post-vaccination			
Test: strain	Sero-positive count/total Proportion [%] (95% CI)[a]	Pre-vaccination GMT (95% CI)	Sero-positive count/total Proportion [%] (95% CI)[a]	Post-vaccination GMT (95% CI)	GMFR (95% CI)	Seroconversion rate[b,c] (95% CI)
Year 1: 2014–2015						
HAI: A/H1N1$_{Cal}$	190/240 79.2% (73.5–84.1)	84.3 (69.9–101.6)	186/227 81.9% (76.3–86.7)	90.8 (75.7–109.0)	1.10 (1.04–1.18)	4/217 1.8% (0.5–4.7)
NAI: A/H1N1$_{Cal}$	184/240 76.7% (70.8–81.9)	239.4 (177.3–323.2)	172/227 75.8% (69.7–81.2)	270.1 (195.7–372.7)	1.20 (1.08–1.33)	17/217 7.8% (4.6–12.2)
OF: H1N1	163/252 64.7%(58.4–70.6)	2.4 (2.1–2.8)	152/246 61.8% (55.4–67.9)	2.4 (2.1–2.7)	0.99 (0.90–1.08)	8/245 3.3% (1.4–6.3)
HAI: A/H3N2$_{Tex}$	215/240 89.6% (85–93.1)	168.5 (139.7–203.3)	214/227 94.3% (90.4–96.9)	224.2 (189.9–264.7)	1.26 (1.12–1.43)	18/217 8.3% (5.0–12.8)
HAI: A/H3N2$_{Switz}$	147/240 61.3% (54.8–67.4)	44.6 (36.3–54.7)	157/227 69.2% (62.7–75.1)	59.7 (48.5–73.5)	1.28 (1.13–1.44)	20/217 9.2% (5.7–13.9)
OF: H3N2	116/252 46.0% (39.8–52.4)	0.8 (0.7–0.9)	122/246 49.6% (43.2–56.0)	0.9 (0.7–1.0)	1.08 (0.98–1.20)	21/245 8.6% (5.4–12.8)
HAI: B$_{Bris}$	119/233 51.1% (44.5–57.7)	31.0 (24.9–38.5)	163/220 74.1% (67.8–79.7)	93.8 (75.5–116.5)	**3.01 (2.56–3.55)**	81/206 39.3% (32.6–46.3)
HAI: B$_{Mass}$	135/233 57.9% (51.3–64.4)	40.8 (32.8–50.8)	194/219 88.6% (83.6–92.5)	148.5 (123.6–178.4)	**3.71 (3.05–4.51)**	88/205 **42.9% (36.1–50)**
Year 2: 2015–2016						
HAI: A/H1N1$_{Cal}$	192/235 81.7% (76.2–86.4)	107.6 (87.9–131.8)	202/226 89.4% (84.6–93.1)	150.2 (124.7–181)	1.29 (1.17–1.42)	16/215 7.4% (4.3–11.8)
NAI: A/H1N1$_{Cal}$	174/229 76.0% (69.9–81.4)	129.1 (99.1–168.2)	180/224 80.4% (74.5–85.3)	186.9 (144.1–242.3)	1.38 (1.23–1.54)	20/208 9.6% (6.0–14.5)
OF: H1N1	184/248 74.2% (68.3–79.5)	2.8 (2.5–3.2)	194/238 81.5% (76.0–86.2)	3.2 (2.9–3.6)	1.14 (1.06–1.22)	7/237 3.0% (1.2–6.0)
HAI: A/H3N2$_{Switz}$	175/235 74.5% (68.4–79.9)	56.1 (48.1–65.4)	218/226 96.5% (93.1–98.5)	165.7 (146–188.2)	**2.99 (2.56–3.48)**	85/215 39.5% (33–46.4)
OF: H3N2	183/248 73.8% (67.9–79.2)	1.1 (1.0–1.2)	218/238 91.6% (87.3–94.8)	1.7 (1.5–1.8)	1.53 (1.42–1.65)	29/237 12.0% (8.4–17.1)
HAI: B$_{Bris}$	182/235 77.4% (71.6–82.6)	89 (73.5–107.7)	214/226 94.7% (90.9–97.2)	204.8 (178.4–235.1)	2.16 (1.88–2.48)	55/215 25.6% (19.9–32)
HAI: B$_{Phu}$	128/234 54.7% (48.1–61.2)	32.6 (27.7–38.3)	171/226 75.7% (69.5–81.1)	57.5 (49.6–66.8)	1.79 (1.6–2.02)	43/214 20.1% (14.9–26.1)
Year 3: 2016–2017						
HAI: A/H1N1$_{Cal}$	118/154 76.6% (69.1–83.1)	64.7 (50.8–82.5)	117/143 81.8% (74.5–87.8)	75.8 (59.9–96.1)	1.11 (1.05–1.18)	3/141 2.1% (0.4–6.1)
HAI: A/H1N1$_{Bol}$[d]	111/152 73.0% (65.2–79.9)	52.2 (41.9–65.1)	112/140 80.0% (72.4–86.3)	66 (53.9–80.7)	1.21 (1.12–1.31)	7/138 5.1% (2.1–10.2)
NAI: A/H1N1$_{Cal}$	129/157 82.2% (75.3–87.8)	172.9 (128.2–233)	128/145 88.3% (81.9–93.0)	228.4 (172.4–302.8)	1.25 (1.15–1.36)	6/143 4.2% (1.6–8.9)
OF: H1N1	126/160 78.8% (71.6–84.8)	3.3 (2.8–3.9)	126/153 82.4% (75.4–88.0)	3.3 (2.8–3.8)	0.98 (0.92–1.06)	1/151 0.7% (0.0–3.6)
HAI: A/H3N2$_{HongK}$	154/157 98.1% (94.5–99.6)	191.7 (164.9–223)	143/146 97.9% (94.1–99.6)	198.6 (172.5–228.6)	1.06 (1.00–1.13)	3/144 2.1% (0.4–6.0)
OF: H3N2	119/160 74.4% (66.9–80.9)	1.4 (1.2–1.6)	117/153 76.5% (68.9–82.9)	1.3 (1.2–1.5)	0.99 (0.92–1.06)	3/151 2.0% (0.4–5.7)
HAI: B$_{Bris}$	134/157 85.4% (78.8–90.5)	80.7 (67.5–96.5)	142/145 97.9% (94.1–99.6)	149.6 (132.9–168.4)	1.83 (1.57–2.14)	23/143 16.1% (10.5–23.1)
HAI: B$_{Phu}$	117/157 74.5% (67–81.1)	53.5 (45.1–63.5)	133/146 91.1% (85.3–95.2)	89.7 (76.6–104.9)	1.71 (1.52–1.93)	21/144 14.6% (9.3–21.4)

Geometric, rather than arithmetic means were taken between the two available assay results, leading to minor discrepancies between these results and those published in Hoschler et al.
OF = standardised oral fluid ELISA.
[a] Seropositive is defined as HAI titre ≥ 40, H1 OF ELISA titre ≥ 1.6 or H3 OF ELISA titre ≥ 0.7.
[b] For those with paired samples only, numbers as in seroconversion rate column.
[c] Seroconversion for OF ELISA is defined as a 3 fold increase, whereas seroconversion for HAI titres is defined as a 4 fold increase.
[d] Data only available for year 3 due to delay in strain availability.

Similar to observation in study year one (2014/15), serological responses to qLAIV measured with classic assays such as HAI and NAI were generally poor across all three seasons and resulted in a very limited number of children seroconverting to both influenza A subtypes. With respect to influenza A(H1N1)pm09, less than 10% of individuals seroconvert either by HAI or NAI, with the two assays following comparable trends in all three study years. The seroconversion rate increases slightly as a result of a strain replacement (to improve thermal stability of the cold adapted, attenuated A(H1N1)pm09 strain) from A/California/7/2009 to A/Bolivia/559/2013 in year 2.

For H3N2, responses vary over the study period - these are low in year 1 and 3, but a significant antibody increase and a seroconversion rate of 39.5% is observed for study year 2 (2015/16), after a

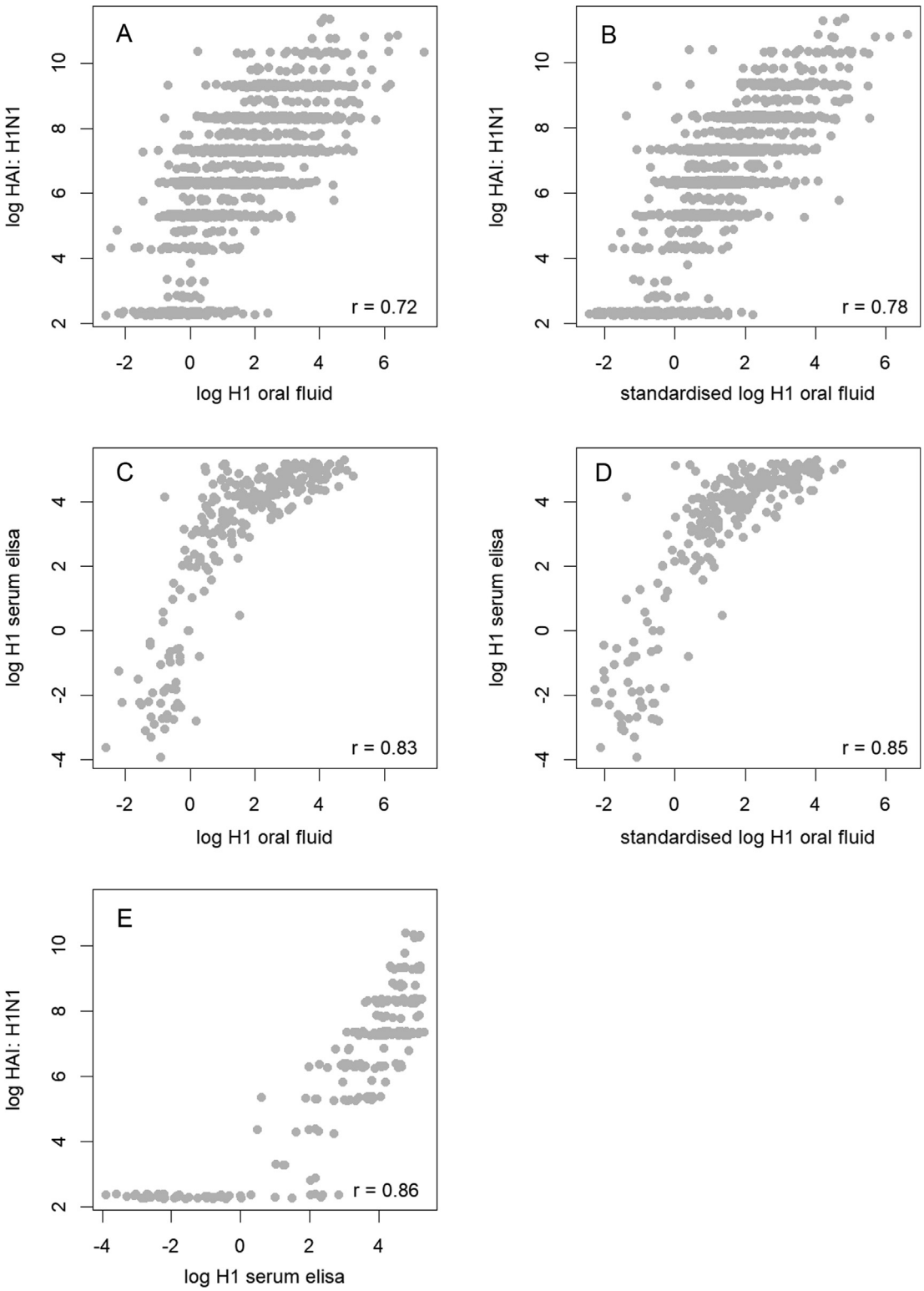

Fig. 2. Scatterplots of log (base 2) H1 HAI titre, H1 OF and H1 serum ELISA. Panel A: OF and HAI titre, panel B: standardised OF and HAI titre, panel C: OF and serum ELISA, panel D: standardised OF and serum ELISA, panel E: serum ELISA and HAI titre. Spearmans rank correlation coefficients (r) are shown in the bottom right corner of each plot.

recommended vaccine strain update (Supplement Table 2). In the 2014/15 winter, ca. 52% of the isolated viruses from the UK were antigenically related to the vaccine strain, A/Texas/50/2013, while later in the season, strains antigenically similar to A/Switzerland/9715293/2013 emerged – this mismatch and domination of H3N2 circulation contributed to poor vaccine effectiveness for 2014/15 in the UK. An additional H3N2 strain update in the following year to A/New Caledonia/71/2014-like (an A/Hong Kong/4801/2014-like virus), did not lead to a further increase in seroconversion rates or GMFR. We consider this is related to

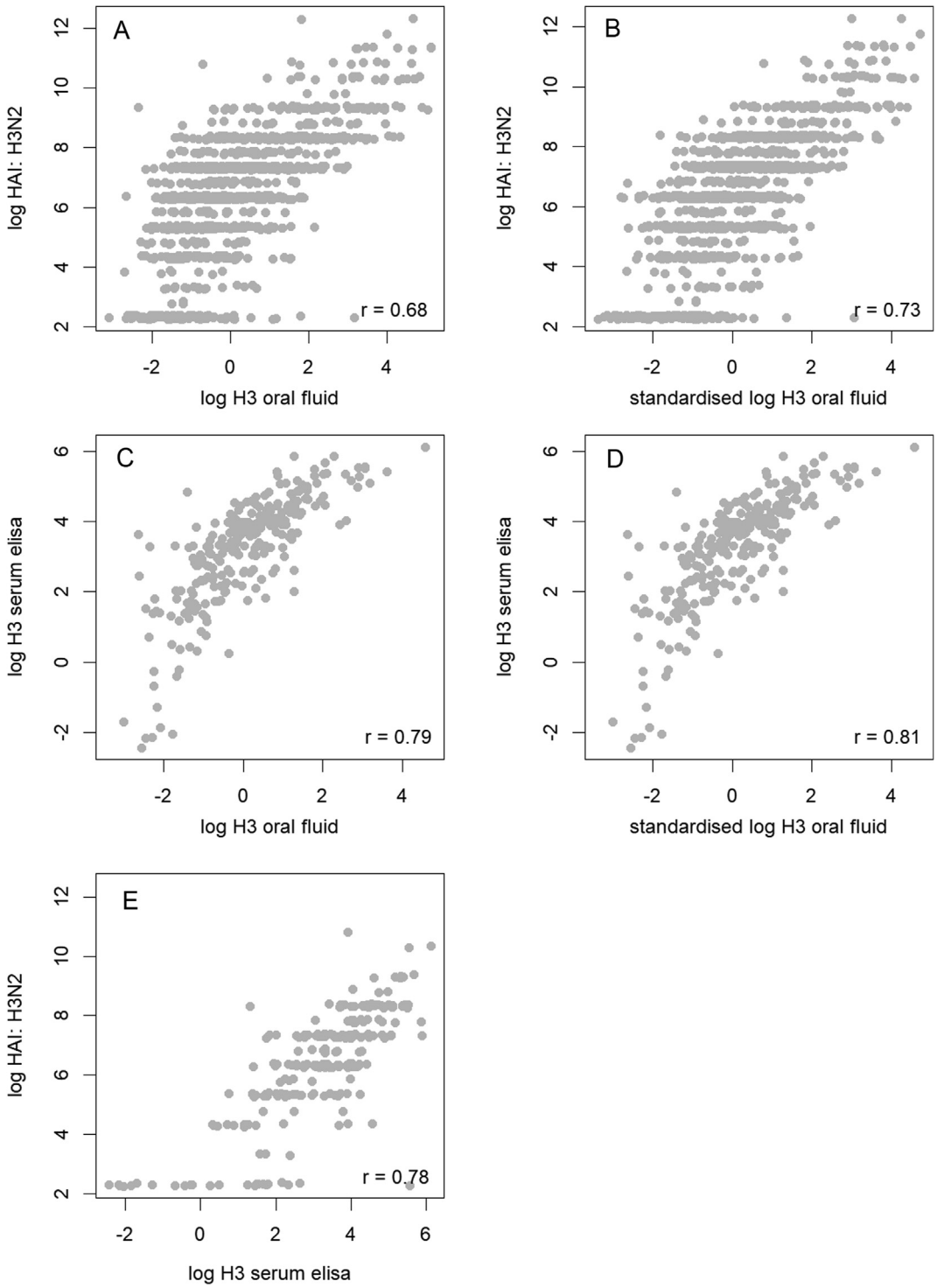

Fig. 3. Scatterplots of log (base 2) H3 HAI titre, H3 OF and H3 serum ELISA. Panel A: OF and HAI titre, panel B: standardised OF and HAI titre, panel C: OF and serum ELISA, panel D: standardised OF and serum ELISA, panel E: serum ELISA and HAI titre. Spearmans rank correlation coefficients (r) are shown in the bottom right corner of each plot.

already elevated pre-vaccine antibody levels and the limited antigenic differences between the 2015/16 and 2016/17 H3N2 components.

As observed in our previous analysis of year 1 samples, receipt of Pandemrix™ had a long lasting immunological impact leading to significantly lower NAI levels pre- and post-qLAIV vaccination and higher HAI titres in Pandemrix™ vaccinated than ~ naïve children in all three study years (Fig. 4, Supplement Table 3). We cannot fully explain this effect nor its possible consequence in terms of long term immunity from influenza. We interpret the difference between the two study groups across the years as indication that the mechanism for this effect is not the impairment of response to Neuraminidase. However, the persistent absolute differences antibody levels to A(H1N1)pm09 as well as the significant differences with H3N2 in study year 3 warrant further examination and investigation of this phenomenon.

In this study we assessed the utility of an alternative sample matrix such as OFs to monitor immunogenicity of LAIV. This partic-

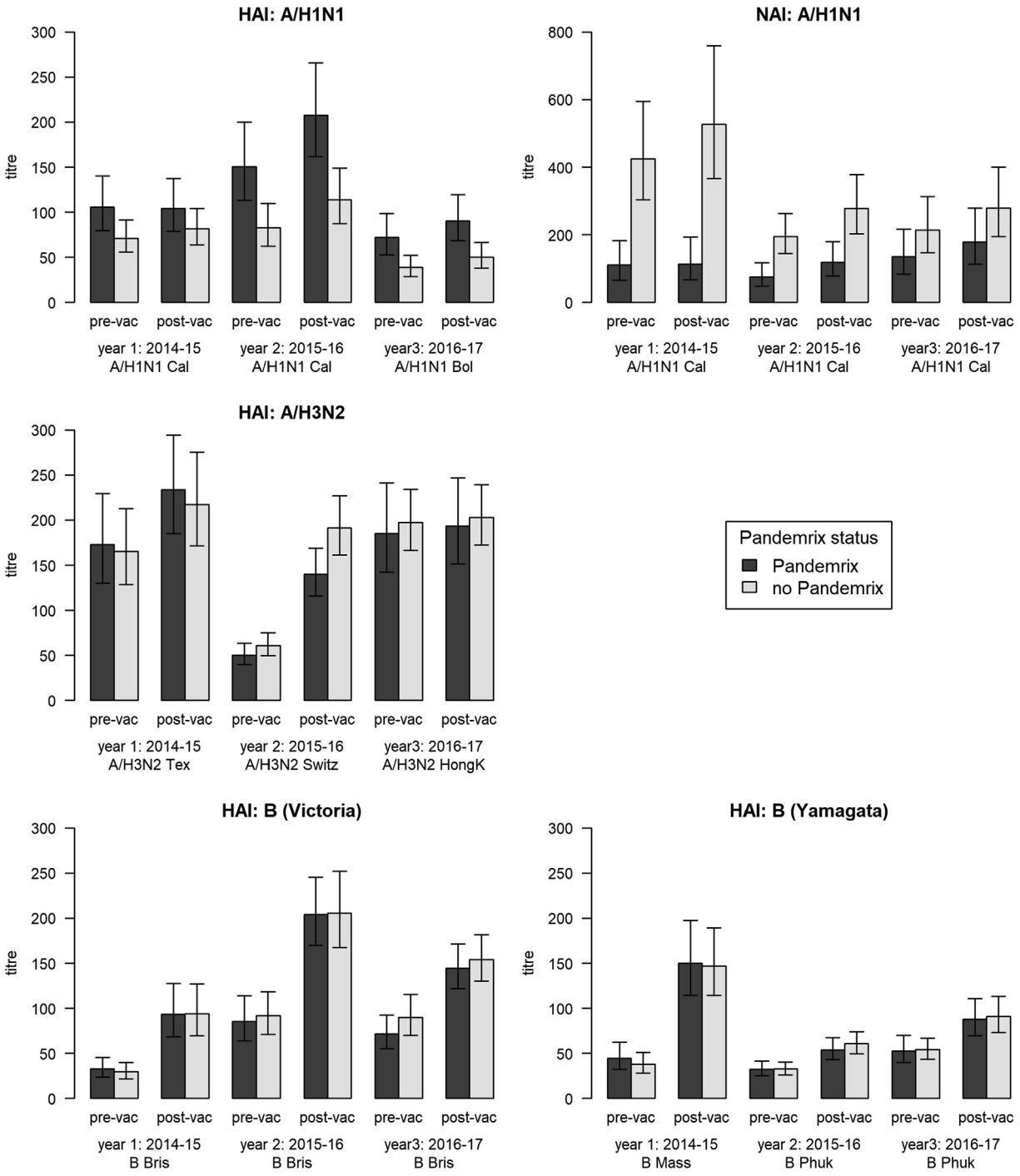

Fig. 4. Geometric mean titres pre and post vaccination (with 95% confidence intervals) for children with and without history of prior Pandemrix™ vaccination.

ular specimen type is widely used in England for infectious disease surveillance [5]. The testing of OFs was a proof of principle analysis which we integrated into an already existing phase III/IV immunogenicity and safety study. This led to several compromises in the design of the Oral fluid assay, so that questions that might be of potential interest in the context of vaccination with LAIV could not be assessed in this study.

Firstly, mucosal antibody, which is of interest in the assessment of responses to LAIV cannot be assessed with the OFs we collected (gingival crevicular fluid) as it is a serum transudate and contains mainly IgG. Secondly, most immunogenicity data on influenza vaccines is generated by HAI, and we had performed this analysis as a primary objective from the sera collected during the vaccine trial. We limited the ELISA antigen to the globular head domain (HA1) of the haemagglutinin where most of the relevant strain specific epitopes are located which play a role in the HAI. Limitation of the antigen to HA1 enabled us to compare the T/N with HAI, and increases the positive predictive value of the ELISA (data not shown) but also reduces sensitivity as we had observed previously [20]. This strategy omits assessment of additional antibody targets which potentially play a role in protection from influenza such as stem specific or anti-Neuramindase antibody, which may be of interest when assessing antibody to LAIV and will be the subject of future studies. Our proof in principle that OF can be used to indi-

rectly assess antibodies present in serum, provides the basis to look for further antibody specificities.

Our data illustrates for the first time, that using OFs it is possible to deduce the presence of seroprotective HAI antibody levels (ie. titre $\geq$ 40) in individuals to influenza A(H1N1)pdm09 and H3N2 in serum with an overall accuracy of 86.7% and 74% respectively. To achieve this, we corrected the data for IgG levels and performed ROC curve analyses to determine optimal cut-offs equivalent to those used for HAI. Generally, the ELISA antibody titres correlate with the corresponding HAI titre in the same individual, however the combination of specimen type substitution and change of assay format results in a generally lower sensitivity and specificity of the ELISA compared to the gold standard analysis.

At this proof of principle stage, we also limited the use of antigens to one per subtype. Consequently, the antigens for the H3N2 comparisons between HAI and ELISA are not matched in all years. Antigens are also mismatched by expression system – we used an egg grown vaccine strain for the HAI assay (which included known egg adaptation mutations such as 160 K) versus the recombinant antigen expressed in HEK293 cells based on a cell grown isolate of A/Hong Kong/4801/2014. Both factors are likely to have had a negative influence on the correlation of data between assays particularly for H3N2, leading to the potential underestimation of responses and assay correlations. Nevertheless, it is apparent from the general trends that the antigen mismatch is not the only factor eliciting the consistently poorer correlation in H3 compared to H1. The two subtypes do differ in their antigenic diversity and resulting antibody repertoire in the human host – there has been less drift of the H1N1(pdm09) subtype compared to H3N2.

We do not envisage the main use of the OF ELISA in the analysis of antibody to circulating seasonal viruses or of seasonal vaccine trials as the assay shows the typical limitations that other investigators have observed when using ELISA in the assessment of immunity to influenza [21–24]. Rather than replacing the HAI, we think that the specimen type and assay could provide an additional tool for rapid assessment of different populations, or in the event of a pandemic, when applied on a naïve population and coupled with available HAI or neutralisation data so that discrepancies could be considered before making attempts to predict immunity. Taking the expense and resistance to collection of blood by venepuncture into account, collection of a non-invasive sample could be of benefit. OFs may be promising candidates - they do not require a phlebotomist to collect specimens, permit self-sampling (keeping costs low), may facilitate testing when patients would refuse sampling by venepuncture and potentially also permit testing in non-clinical settings (near patient tests) [25]. Our study demonstrates that children are capable of self-sample, yielding oral fluids which showed comparable quality to those taken by a medical professional (data not shown).

Finally, the data shows an increasing trend of antibody levels over time, with decrease in influenza A responses, indicative of longer duration effects of repeat vaccination and alteration of herd immunity profiles of widely vaccinated populations. This raises the question of the optimal interval between and strategy for re-vaccination of the paediatric population.

5. Conclusion

In all three study years, vaccination with qLAIV resulted in poor antibody responses to influenza A. We found further evidence for a sustained effect of prior receipt of the AS03$_B$-adjuvanted A(H1N1) pdm09 vaccine even after repeat vaccination with LAIV. The opposing effect on A(H1N1)pdm09 HAI versus A(H1N1)pdm09 NAI and H3N2 HAI titres supports the idea that receipt of this vaccine may lead to an intensification of "antigenic imprinting" [26,27] towards the A(H1N1)pdm09 strain, but the mechanism and long term consequences of these differences *in vivo* are still unclear.

We developed an assay system that allows approximation of serum anti influenza A antibody without the need for venepuncture from assessment of oral fluids, which has the potential to facilitate sample collections from wide range of subjects, including children and thus may be of value in monitoring LAIV immunogenicity, but may also support feasibility of antibody-prevalence studies.

CRediT authorship contribution statement

Katja Hoschler: Methodology, Validation, Investigation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration. **Sunil Maharjan:** Methodology, Investigation, Writing - original draft. **Heather Whitaker:** Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Jo Southern:** Conceptualization, Methodology, Resources, Data curation, Writing - original draft, Supervision, Project administration, Funding acquisition. **Blessing Okai:** Methodology, Investigation, Writing - original draft. **Janice Baldevarona:** Validation, Investigation, Writing - original draft, Supervision. **Paul J. Turner:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing - original draft, Supervision, Project administration, Funding acquisition. **Nick J. Andrews:** Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Supervision, Visualization. **Elizabeth Miller:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Maria Zambon:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

JS & MZ drafted the protocol, JS obtained all governance approvals and oversaw the field work and data entry. PJT provided clinical support. JB, together with KH prepared and organised the laboratory analysis of trial samples, SM and BO developed the OF assays, KH directed the laboratory testing, was involved in data interpretation and produced the first draft of the paper. HW, FW and NA conducted the statistical analysis. EM, MZ designed the study and with NA directed the analyses and interpretation of the results. All authors contributed to, reviewed and approved the final draft of the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2020.02.024.

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