The impact of timing of maternal influenza immunisation on infant antibody levels at birth

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Abstract

Pregnant women and infants are at an increased risk of severe disease after influenza infection. Maternal immunisation is a potent tool to protect both of these at-risk groups. Whilst the primary aim of maternal influenza vaccination is to protect the mother, a secondary benefit is the transfer of protective antibodies to the infant. A recent study using the tetanus, diphtheria and acellular pertussis (Tdap) vaccine indicated that children born to mothers immunised in the second trimester of pregnancy had the highest antibody titres compared to children immunised in the third trimester. The aim of the current study was to investigate how timing of maternal influenza immunisation impacts infant antibody levels at birth. Antibody titres were assessed in maternal and cord blood samples by both IgG-binding ELISA and haemagglutination inhibition assay (HAI). Antibody titres to the H1N1 component were significantly higher in infants born to mothers vaccinated in either the second or third trimesters than infants born to unvaccinated mothers. HAI levels in the infant were significantly lower when maternal immunisation was performed less than four weeks before birth. These studies confirm that immunisation during pregnancy increases the antibody titre in infants. Importantly antibody levels in cord blood were significantly higher when mother was vaccinated in either trimester two or three, though titres were significantly lower if the mother was immunised less than 4 weeks before birth. Based on this data, seasonal influenza vaccination should continue to be given in pregnancy as soon as it becomes available.

Introduction

Pregnant women and their infants, especially neonates, are at a higher risk of severe illness caused by influenza virus infection [1, 2]. During the 2009 H1N1 pandemic, pregnant women were 7.2 times more likely to be admitted to hospital due to serious influenza associated disease compared to non-pregnant women [3]. Furthermore, maternal influenza infection has been associated with serious complications, such as stillbirth and pre-term delivery [4]. Influenza infection is also more severe in infancy; for children under 2 years of age, influenza infection is one of the most significant causes of hospitalisation, with infants under 6 months of age experiencing the highest rates of morbidity and mortality [5, 6].

The influenza vaccine remains the best way to protect high risk populations [7]. Multiple randomised controlled trials and observational studies have shown that vaccinating pregnant women with influenza vaccine is safe throughout pregnancy for both the mother and the neonate [8-10]. Maternal influenza immunisation is also effective; vaccine-induced antibody protects the mother against influenza infection [10]. Maternal influenza immunisation has also been probabilistically linked to reduced risk of stillbirth [11] confirmed in a meta-analysis [12]. Additionally, a study from Bangladesh showed that infants born during influenza season to vaccinated mothers were 70% less likely to be born premature and 69% less likely to be small for gestational age [4]. The published studies support immunisation as an effective strategy to protect pregnant women against influenza infection and to improve pregnancy outcomes for women and their infants.

Current influenza vaccines are not licensed for infants younger than 6 months of age, a period of high susceptibility for influenza infection. However, we can take advantage of a facet of the immune system during pregnancy: maternally derived IgG is actively transported via the placenta from mother to foetus, offering passive immunity against infections to infants up to 6 months [13]. This means that immunising the mother has the potential to protect the infant from influenza infection via passive protection with maternal antibody [7, 14]. This principle is well established for the prevention of neonatal tetanus and also more recently for pertussis [15]. Since 2005, immunisation of pregnant women with inactivated influenza vaccine has been recommended by the World Health Organisation to protect both mothers and their infants. Maternal influenza vaccination significantly increases the antibody titre in the infant at birth and up to 2-3 months of age, but reduces the risk of infection for up to 6 months of age [16]. This vaccine-induced increase in antibody is clinically efficacious in protecting infants, with 45-63% reduction in laboratory-confirmed influenza among newborns and infants depending on models [10, 17, 18].

Whilst it is clear that maternal immunisation is beneficial, recent studies have raised questions about the best timing of immunisation for transfer of protection to the infant. Eberhardt et al. suggest that antigen-specific cord blood antibody titres were greater following maternal immunisation with the tetanus, diphtheria and acellular pertussis (Tdap) vaccine in the second rather than the third trimester [19, 20]. The results from published studies on timing of maternal influenza vaccination paint an incomplete picture. One study observed no difference in antibody levels in cord blood following vaccination in different trimesters [16] and another study observed significantly lower antibody titres if vaccination was given less than 15 days before birth [21]. However, other studies have not observed statistically significant differences in post-partum IgG levels in maternal blood [22, 23]. It is therefore important to further investigate the effect of maternal vaccination timing on anti-influenza antibody in their offspring.

The aim of the current study was to evaluate the effect of timing of inactivated influenza vaccination during pregnancy on the antibody level in both the mother and the newborn. This was to improve understanding of influenza vaccination pregnancy but also to see if there are general lessons that can be learnt with regards the timing of maternal vaccination.

Methods

Recruitment

The study was an opportunistic study nested within a larger prospective cohort study of maternal immunisation (MatImms@Imperial) investigating the effects of maternal pertussis vaccination. Samples were collected between November 2013 and October 2016; this period covered four different influenza seasons, September to March in the northern hemisphere, and four different vaccine composition recommendations (Table S1). Samples from 96 women, 61 vaccinated and 35 unvaccinated were included in this sub-study out of 383 recruited to the overall MatImms@Imperial study. Samples were selected based on availability of material for analysis with an aim to get even numbers of participants from each of the three trimesters, aliquots of the samples were used for the ELISA and HAI analyses. 26% (n=17) of participants were vaccinated in trimester one (weeks 0-13), 23% (n=14) in trimester two (weeks 14-26) and 49% (n=30) in trimester three (weeks 27-42) with samples selected from the larger study to reflect a range of trimesters of immunisation. The supplier of the influenza vaccine was not recorded. Healthy pregnant women were recruited antenatally and gestational age was determined by antenatal ultrasound. Healthy, low risk pregnancies with babies born at term- above 36 weeks and a birth weight above 2.5 kg were included. Mothers with chronic infections known to be transferred to the infant (HIV, hepatitis B and syphilis), chronic conditions (e.g. diabetes, autoimmune diseases, high blood pressure) or twin pregnancies were excluded. On recruitment, women were asked if they had experienced any illness during their pregnancy. Only those who reported no significant illness were recruited. We cannot exclude that some women experienced sub-clinical flu during pregnancy. The study was approved by the National Research Ethics Service (NRES), NHS, UK (REC 13/LO/1712). Written informed consent was obtained from all women and samples were stored under the Imperial College Healthcare Tissue Bank (ICHTB).

Sampling procedure and laboratory assays

Blood samples were collected from the mother at or within 2 days of delivery. Umbilical cord blood samples were collected at delivery. Blood was collected into Z Serum Sep Clot Activator tubes (Greiner Bio-One). All samples were processed in the laboratory within 4 hours and sera were stored at -80°C until further analysis. Laboratory staff were blinded to all clinical data.

Influenza antigen-specific ELISA

A standardised ELISA assay [24, 25] was used to measure antibodies (IgG) specific to the Influenza strains present in the vaccine (A/California/7/2009, A/Texas/50/2012 or B/Massachusetts/2/2012). Plates were coated with purified influenza virus antigens (GSK, Sienna) diluted 1µg/ml in PBS. A serial dilution of human IgG from serum (Sigma-Aldrich) was used for the standard curve in order to

quantify the influenza virus specific IgG in the sample, captured with anti-human Kappa (Southern Biotech Cat No 2060-01) and anti-human Lambda (Southern Biotech Cat No 2070-01). The bound IgG was detected by HRP-conjugated goat anti-human IgG antibodies (Sigma-Aldrich, Cat No AP112P) and the colour change in TMB after H_2SO_4 stop was read at 450nm.

Haemagglutination inhibition (HAI) assay

All samples were analysed by HAI assay using A/California/7/2009 (H1N1) virus strain. Virus was grown in Madin-Darby Canine Kidney (MDCK) cells (gift from W. Barclay), in serum-free DMEM supplemented with 1µg/ml trypsin. The virus was harvested 3 days after inoculation and stored at -80°C. Viral titre was determined by haemagglutination assay (HA). Cord and maternal serum samples were pre-treated with Receptor Destroying Enzyme (RDE: Denka Seiken) for 18 hours at 37°C before inactivating the enzyme at 56°C for one hour. All treated sera were tested for non-specific agglutination before analysing with HAI assay. RDE-treated serum was two-fold serially diluted across the plate with PBS and incubated with pre-diluted 4 haemagglutinating unit virus per well for 15 minutes at room temperature. 100µl of 0.5% turkey erythrocytes diluted in PBS was then added to each well, and the plate was incubated for 30 minutes at room temperature before scoring the response. Seroprotection was defined as an HAI titre ≥40 based on challenge studies [26].

Statistical analysis

Descriptive statistics were carried out to investigate any differences in baseline characteristics between the vaccinated and un-vaccinated groups using the Student's t-test. For comparisons of trimester on antibody titre, ANOVA followed by Bonferroni's multiple comparison test was used to account for multiple comparisons; specific pairwise comparisons are described in figure legends. Fisher's exact test was used compare proportions of seroprotection.

Univariate analyses were conducted to assess associations of mean H1N1 maternal and cord antibody titres with a range of socio-demographic and clinical risk-factor variables. Crude associations between vaccination status, trimester of vaccination, and other variables, with mean antibody titres were obtained using two sided Student's t-test; datasets were tested for normality using the D'Agostino & Pearson test.

Variables that showed evidence of association with mean antibody titres at the p≤0.1 level in the univariate analysis, were analysed further in multivariable analyses using logistic regression models. In multivariable analyses the relationship between vaccination status; trimester of vaccination; and season of birth; and H1N1 cord blood titre were quantified using a logistic regression model. The outcome was defined as the upper 50th centile of H1N1 antibody titre in cord blood. All tests were performed using GraphPad Prism 7.02 (GraphPad, USA). Transfer ratios were calculated as the

maternal titre divided by the cord blood titre. Post hoc power analysis was performed with Gpower [27].

Data availability statement

The complete raw dataset is available in supplementary data file (File S1).

Result

Study population

63.5% (n=61) of the study population had received seasonal influenza vaccine during pregnancy, 35% (n=35) were unvaccinated (Table 1). There were no significant differences in age, BMI, ethnicity or parity between vaccinated and unvaccinated pregnant women. Of the vaccinated mothers, 5% of study population received the vaccine in the 2013-14 influenza season, 62% in the 2014-15 season, 31% in the 2015-16 season and 2% in the 2016-2017 season: it is of note that the same H1N1 antigen was used in all of the seasons included in the study (Table S1). For the newborns, there were no significant differences in terms of gestational week, birth weight or sex between those born to vaccinated mother compared to newborns with unvaccinated mother. When infants were grouped by maternal vaccination trimester, there were no significant differences in terms of gestational age, birth weight or sex ratio (Table 2). All infants included in the study were delivered at term (mean gestational age 39.9 weeks) and no infants were categorised as low birth weight (defined as less than 2.9kg for male children or 2.8kg for female children at 40 weeks).

Vaccination in the second and third trimesters significantly elevates H1N1 specific IgG at birth in mothers and newborns

ELISA was used to quantify the influenza specific IgG titre in cord and maternal serum samples collected at the time of birth. H1N1 specific IgG titres were significantly higher in the cord blood of babies born to vaccinated rather than unvaccinated mothers (*P*=0.016, Figure 1A). The cord samples had significantly higher antibody titres than the maternal samples (*P*=0.031). The transfer ratio for H1N1 in matched vaccinated pairs (3.199 ± 3.203) was significantly greater (*P*=0.0023) than in unvaccinated pairs (1.397 ± 0.9174). There was no significant difference in anti-H1N1 binding antibodies measured by ELISA between vaccinated and unvaccinated mothers.

Analysis of antibody titres on cord blood samples was carried out in relation to the stage of pregnancy at the time of immunisation compared to the unvaccinated group (Table 2); mothers and their infants were grouped based on whether they were vaccinated in the first (n=17), second (n=15) or third trimester (n=29). Cord blood samples from infants born to mothers vaccinated in the second (*P*=0.0336) or third (*P*=0.0084) trimester had significantly higher H1N1 specific IgG titre at birth compared to infants from unvaccinated mothers (Figure 1B). There was no significant difference in the antibody titre in babies born to mothers vaccinated in the first trimester compared to those born to non-vaccinated mothers. In the maternal samples, H1N1 specific IgG responses in mothers vaccinated in the second trimester were significantly higher than in unvaccinated mothers

(*P*=0.0036, Figure 1C). The transfer ratio for H1N1 specific antibody in matched vaccinated pairs was significantly different between T1 (3.61 ± 2.289, *P*=0.024), and T3 (3.15± 3.758, *P*=0.0337) and unvaccinated pairs (1.397 ± 0.9174), but not T2 (2.819± 3.042).

Vaccination in the second and third trimesters significantly elevates HAI at birth in mothers and newborns

To further dissect the level of protection provided by maternal vaccination, we measured the degree of haemagglutination inhibition (HAI) in the samples. The HAI titre that is currently used as a correlate of influenza protection was defined in the 1970's in a series of human influenza challenge studies [26]. Since the H1N1 vaccine strain was unchanged through the course of the study, we focussed the HAI on a representative H1N1 virus (A/California/7/2009). The HAI titre in cord blood of babies born to vaccinated mothers was significantly higher than in infants born to unvaccinated mothers (Figure 1D). The HAI titre was also higher in cord blood from vaccinated mothers than in the maternal blood. The HAI titre in cord blood of babies born to mothers vaccinated in the second (*P*=0.0006) or third (*P*=0.0006) trimesters of pregnancy was significantly greater than those born to unvaccinated mothers (Figure 1E). The maternal HAI titre at the time of birth of mother immunised in T3 was significantly higher than unvaccinated mothers (*P*=0.0294, Figure 1F). The level of antibody measured by HAI or ELISA correlated significantly in both the cord (R^2=0.3277, *P*<0.0001, Fig S1A) and maternal (R^2=0.1697, *P*= 0.0003, Fig S1B) samples.

We evaluated the seroprotection rate (proportion of individuals with HAI titre ≥ 40): in infants born to vaccinated mothers, the rate was 47%, which was significantly higher than in infants born to unvaccinated mothers (Figure 1E, 0%, *P*<0.001). Likewise, significantly more vaccinated mothers (18%) had an HAI titre ≥ 40 compared to unvaccinated mothers (Figure 1F, 0%, *P*=0.0063). Comparing the proportion by trimester, there was a significantly greater proportion (*P*<0.001) of cord blood samples with HAI ≥ 40, if the mothers were immunised in either T1, T2 or T3 than in samples from unvaccinated mothers. Likewise the proportion of mothers with HAI ≥ 40 were significantly greater than unvaccinated mothers if they were immunised in T1 (*P*=0.01), T2 (*P*=0.0047) or T3 (*P*=0.041).

Immunisation between 24 weeks gestation and 5 weeks before birth led to the highest antibody titre in the cord samples

An alternative consideration to assess the protective potential of a vaccine given in pregnancy is the interval between immunisation and delivery, which can inform us about the kinetics of antibody transfer. In particular we wanted to compare the effect of immunisation very close to the time of

birth (less than 4 weeks) to see whether this affected transfer. When grouped this way, infants from mothers vaccinated either 5-12 (*P*=0.0237) or 13-24 weeks (*P*=0.0107) before delivery had significantly higher H1N1 specific IgG titre at birth compared to those from unvaccinated mothers (Figure 2A). There was no significant difference between infants whose mother received vaccination either less than 4 weeks or more than 24 weeks before birth and infants born to unvaccinated mothers. Mothers vaccinated 13-24 weeks before delivery had the highest IgG response at time of delivery among all vaccination groups, and the titre was significantly higher than unvaccinated mothers (*P*=0.0177, Figure 2B). HAI titres were also compared by interval to delivery. Newborns from mothers vaccinated 5-12 weeks before delivery had the highest HAI titre (Figure 2C) and were significantly greater than titres in newborns vaccinated less than 4 weeks, newborns from mothers vaccinated either 5-12 or 13-24 weeks before birth had significantly higher HAI titres than those from unvaccinated mothers. Maternal HAI titres at birth were not significantly different regardless of the interval between vaccination and birth (Figure 2D). In terms of seroprotection, administration of maternal influenza vaccine 5-12 weeks before delivery conferred the highest percentage of infants with HAI titre ≥ 40 (58.8%). All timings had a significantly greater proportion of infants with HAI titre ≥ 40 than the unvaccinated group.. These data suggest that immunising too close to the time of birth reduces the amount of antibody transferred to the infant.

Season of birth

Due to the seasonality of influenza, we determined if season of birth affected antibody titres. There was no difference in the season of birth between vaccinated and unvaccinated groups (Table 1). The anti-H1 IgG titre was significantly greater at birth in unvaccinated infants born in the influenza season (*P*=0.043, Figure 3A) but not children born to vaccinated mothers (Figure 3B). We then investigated the relationship between month of birth and antibody titre. In children from unvaccinated mothers, there was a weak but significant decline in antibody titre (*P*=0.02, R^2=0.16, Figure 3C) from those born at the start of the influenza season (September) to those born before the beginning of the next season (August). This relationship was not seen in the vaccinated group (*P*>0.05, R^2=0.07, Figure 3D). Since season had an effect on antibody titre we compared month of birth with trimester of vaccination. The majority of mothers were immunised in October, regardless of trimester (Figure 3E). This was subsequently reflected in a significant difference in the median month of birth (Table 2), with children born to mothers immunised in trimester 3 born earlier in the influenza season, than children born to mothers immunised in trimester 1 (Figure 3F).

Impact of timing of maternal vaccination to H3N2 and B components of trivalent influenza vaccine

The influenza vaccine that is routinely used in pregnancy covers three different strains of influenza, normally two influenza A strains and one influenza B strain (Table S1). This gave us the opportunity to investigate whether there were similar effects to co-administered antigens. Analysis was restricted to the 13-14 and 14-15 influenza seasons as the same vaccine composition was used in these two seasons, enabling comparison to the same antigens; this did reduce the number of subjects studied (unvaccinated n=34, vaccinated n=41, T1 n=13, T2 n=9, T3 n=19). Antibody levels to the H3N2 antigen (A/Texas/50/2011) were compared between vaccinated and unvaccinated mothers and their infants; there was no significant difference observed between groups (Figure 4A). No difference was seen when cord (Figure 4B) or maternal (Figure 4C) samples were grouped by trimester, though there was a trend to increased titre in mothers vaccinated at T3 (T3 titre 296.6 ± 382.6, unvaccinated titre 147.2 ± 170.5, adjusted *P*=0.1483). The H3N2 specific antibody transfer rate was not significantly different between groups.

We also measured antibody titres to the influenza B component (B/Massachusetts/2/2012). There was no difference in influenza B specific antibody titre between vaccinated or unvaccinated maternal samples or cord from maternal samples (Figure 4D), though the mean titre in the vaccinated group (482 ± 600) was higher than the mean titre in the unvaccinated group (323 ± 387). There was a trend towards higher antibody titres in the T3 groups in both cord (Figure 4E) and maternal (4F) samples, but this was not significant. In the unvaccinated samples birth timing relative to influenza season had no effect on the H3N2 titre (Figure 4G), but there was a significant difference in the cord sample in children born in influenza season (*P*=0.036, Figure 4H).

Determinants of influenza specific antibody titre in cord and maternal blood samples

Having observed that a number of factors contributed to antibody levels in pregnant women and their infants at birth, we used univariate analysis to determine their relative contributions. Vaccination status significantly impacted cord IgG (Table 3) and HAI titres (Table S2); it also had a significant impact on maternal HAI titre (Table S2). Vaccination trimester and birth season also had a significant impact on cord and maternal antibody titres. Parity, maternal age, maternal BMI, ethnicity and mode of delivery did not significantly impact vaccine-specific antibody levels in infants. Interestingly mothers with BMI≥25 had significantly lower H1N1 titres (*P*=0.04) than mothers with BMI<25, and mothers who gave birth after 40 weeks also had significantly lower IgG titres (*P*=0.0047), though this difference was not seen in the HAI titre.

For multivariable analyses the upper 50th percentile of H1N1 IgG cord blood concentration was used, which ranged from 194.0 – 1205.6 μg/ml (Table 4). It should be noted that due to sample size limitations a broad outcome group (upper 50th percentile H1N1 IgG titre: which represents mothers

with H1N1 antibody titres above the mean of this study population) was used for multivariable analysis, which is important to consider when interpreting these results. 74.1% of cord blood samples with a protective HAI titre >40 fell within this group. Vaccinated mother-infant pairs were 3.7 (1.5-9.3) times more likely to be in the upper half of H1N1 IgG concentration relative to unvaccinated pairs, controlling for season of birth. Mother-infant pairs vaccinated in T1, T2 and T3 were 3.4, 8.1 and 2.5 times more likely to be in the upper H1N1 cord blood concentration group respectively, compared to unvaccinated pairs, after adjustment for season of birth; this was significant only for T2 (*P*= 0.005). Mother-infant pairs that gave birth/were born in the flu season had significantly increased odds of 3.1 (1.1-9.3) of being in the upper H1N1 IgG cord concentration group, relative to those born in the non-flu season, after controlling for vaccination status and trimester of vaccination.

Discussion

The aim of the study was to assess the effect of timing of influenza vaccination during pregnancy on the antibody levels in both infants and mothers. Vaccination significantly increased the antibody titre at the time of birth in both the mother and the infant. It should be noted that unvaccinated mothers still pass influenza specific antibodies to their children, more so in the influenza season probably reflecting natural infection or exposure to virus in the mothers. Infants from mothers that received influenza vaccine at 5-24 weeks before delivery (second trimester to mid third trimester) had significantly higher HAI and specific IgG titres against influenza A (H1N1) than infants from unvaccinated mothers. If their mother was immunised either too far from (>24 weeks) or very close to (<4 weeks) delivery, the antibody titre in the infant was no different to infants from unimmunised mothers. Similar results were reported in a previous study, with significantly increased HAI antibody titre in the cord blood of infants born to mothers vaccinated more than 30 days before delivery [21]. We also observed an association between birth season and antibody titre, but only in children born to unvaccinated mothers.

Previous studies have shown that maternal immune responses to the influenza vaccine are independent of the trimester [22, 23]. Therefore differences in antibody titres seen in newborns after immunisation at the second or third trimester during pregnancy are presumably the result of cumulative antibody transfer across the placenta. Placental transfer of IgG antibody is facilitated by the neonatal Fc receptor (FcRn), with transport efficiency increasing over time [28], and is believed to be maximal in the third trimester. Immunising mid-pregnancy gives enough time for the mother to develop high antibody levels while allowing time for maternal-fetal antibody transfer. Immunisation

at the end stages of pregnancy (less than 4 weeks before delivery) is unlikely to allow sufficient time for the mother to both respond to the vaccine and transfer antibody to the foetus. It is not fully clear why the antibody titre at birth is not significantly greater in mothers vaccinated during T1 compared to unvaccinated mothers. But one consideration is that the comparison is not against an immune naïve population, both vaccinated and unvaccinated mothers will have been exposed to influenza antigens and therefore vaccination is boosting from a baseline, which may wane over time. Since there is minimal antibody transfer in the first trimester[29], by the time of peak antibody transfer later in pregnancy, the titre in the serum of T1 vaccinated mothers may not be different to unvaccinated mothers, especially if the unvaccinated mothers are exposed to influenza during pregnancy.

It was of note that antibody levels in cord blood samples were higher than in maternal blood samples. A similar pattern has been observed after immunisation of pregnant women with inactivated monovalent H1N1 [30], pertussis and pneumococcus [31-33] vaccines. This could be a result of either the active transfer of antibody across the placenta or the increased circulating volume in pregnant women at the time of birth which decreases IgG antibody concentration in proportion as pregnancy proceeds [34]. We also observed lower antibody titres in mothers with children born at greater gestational ages, suggesting that increasing serum dilution in the mother over the course of pregnancy might be more important in this difference between the infant and the maternal titres. Another intriguing association was between maternal BMI and antibody titre by ELISA; why this is the case is not clear; there was a discrepancy between the association between titre, HAI and BMI, which is likely caused by the small sample size.

There are two important considerations relating to maternal influenza vaccination when considering optimal timing. Firstly the vaccine for influenza, unlike other pathogens, changes annually to reflect changes in the circulating strains of virus, which restricts the availability of the vaccine to certain times of the year. The current practice is to immunise mothers as soon as the influenza vaccine becomes available (late September, early October) giving them the greatest protection throughout the influenza season, and in our study the majority of individuals were vaccinated in October, at the start of the flu season in the UK. Secondly, the goal of maternal influenza immunisation is to protect both the mother from infection throughout pregnancy and the infant during the first months of life; this is different to experimental Group B streptococcus (GBS) and respiratory syncytial virus (RSV) vaccines where the priority is to protect the infant; though the ongoing Novavax trial is also looking at the effect on maternal RSV infection (NCT02624947), which may provide additional cocooning benefit to the child. Since the aim of maternal influenza immunisation is to protect both mother and child, there is a potential trade-off in the optimal timing between vaccinating early to provide protection to the mother for the course of the pregnancy and vaccinating later to maximise transfer

and longevity of antibody to the child. However the seasonality of infection and the increased antibody level in children born to mothers immunised later in pregnancy mean that vaccinating early in the influenza season achieves the twin goals of protecting both the mother and the child. This is because vaccinated mothers who give birth during the influenza season will mostly be in the later stages of their pregnancy at the time of immunisation and therefore their babies will have more anti-influenza antibody at the time of birth, whereas mothers who are early in pregnancy at the time of vaccination are less likely to give birth during the influenza season, therefore protecting the mother for the course of the pregnancy is the main target of vaccination. This interaction of season and response may only apply in areas with short and reliable influenza seasons. One other consideration is that maternal immunisation later in pregnancy may have a cocooning effect: prevention of maternal infection will reduce transmission from the mother to her infant.

This was a smaller opportunistic study nested within a larger study, the original study was not designed to answer the question about timing of influenza vaccination in pregnancy and the analysis was applied retrospectively to samples collected. As such there are a number of limitations. The donor numbers are small and not evenly spread across the three trimesters so we did not have sufficient power to determine whether there were significant differences between trimesters. While the data suggests an equivalence between T2 and T3 in the anti-H1 titre, a post-hoc power calculation using the data obtained in the Gpower package [27] gives a low power (0.05), which is a 95% chance of a type II error, i.e. a failure to reject the null hypothesis that T2 and T3 are the same. Performing the same analysis to compare T1 and T3 gave a power of 60%. Therefore analysis focused on comparison of the trimesters against the unvaccinated groups, for which the study was adequately powered (power=98% using Gpower). The data presented here can be used to inform power calculations for future studies: to detect a difference between T1 and T2 would have required 42 individuals per arm and T1 and T3 would have needed 56 individuals per arm and for a difference between T2 and T3 14,355 individuals per arm. This problem of small group sizes is exacerbated in the H3N2 and B datasets which are taken from fewer seasons to look at equivalent antigens. We do not know the previous influenza vaccination or infection status of the donors and the seasonality data suggests that infection is a confounding effect. We focussed on IgG ELISA and HAI assays, but other assays to measure antibody function, for example neutralisation assays would also be informative. There was a significant effect of birth season in the unvaccinated mothers, which is most likely driven by natural infection. Ultimately, the study shows that maternal vaccination increases infant anti-influenza titre and given the seasonality of influenza infection, our study supports the administration of influenza vaccine as soon as it becomes available.

Maternal immunisation is one of the safest and most useful tools to address the limited immunity of infants during first months of life, aiming to close the gap in infant protection until it is possible to immunise the infant [14]. The administration of influenza and Tdap vaccines during pregnancy have shown to be clinically efficacious in protecting infants from infections [7]. Developing maternal vaccination for other major pathogens that cause infections in early life, including RSV [35] and GBS [36] is a research priority. The timing of vaccine administration is critical in optimising the windows of protection. Studies in maternal immunisation against pertussis suggest that trimester two is the optimum time for maternal immunisation to maximise protection in the infant [19, 21]. In the current study we observed that immunisation in trimester two or three, both lead to significantly higher titres than in children born to unvaccinated mothers. This suggests that immunisation timing relative to pregnancy may be less important for influenza than pertussis and given the seasonal nature of influenza infection immunising as soon as the vaccine becomes available is the best approach.

Author contributions

ZZ and MH performed the experiments; TR and BD collected samples; M O'D analysed data; KD wrote paper; JT, BH and BK designed study, analysed data and wrote paper.

Conflict of interest

The authors have no have no potential financial or non-financial conflicts of interest.

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Tables

Table 1. Characteristics of the Study Populations

Characteristic	**Vaccinated (n=61)**	**Unvaccinated (n=35)**	***P* Value**
Women			
Age, mean (SD)	32.3 (5.1)	32.3 (4.5)	0.995
BMI, mean (SD)	25.0 (4.4)	24.6 (4.2)	0.7
Parity, mean (SD)	0.56 (0.8)	0.9 (1)	0.061
Ethnicity n (% Caucasian)	38 (62.3)	20 (58.8)	0.74
Newborns			
Gestational age, weeks (SD)	40.0 (1.3)	39.9 (1.1)	0.76
Mean birth weight, g (SD)	3.5 (0.43)	3.5 (0.4)	0.98
Female sex, n (%)	27 (44%)	16 (46%)	0.89
Mode of delivery: Vaginal delivery, n (%)	34 (56%)	23 (65%)	0.13
Season of birth			
Month of birth (Median)	June	July	0.23
Born in flu season (Sept-Mar), n (%)	33 (54%)	18 (51%)	0.8

P Value for Age, BMI, Parity, Gestational Age, Birth weight, month of birth by t-test;

P Value for Ethnicity, female sex, vaginal delivery and birth in flu season by Chi-Square test.

Table 2. Characteristics of vaccinated pregnant women in the study by the stage of pregnancy at immunisation

Characteristics	**Maternal flu immunisation in First trimester (n=17)**	**Maternal flu immunisation in Second trimester (n=14)**	**Maternal flu immunisation in Third trimester (n=30)**	***P* value**
Mothers				
Age, year, mean (SD)	31.7 (4.7)	30.9 (6.0)	33.3 (4.8)	0.28
Parity, mean (SD)	0.53 (0.8)	0.5 (0.9)	0.6 (0.9)	0.99
Influenza season at immunisation (%)				
2013-2014	1 (6%)	1 (7%)	1 (3%)	
2014-2015	12 (70%)	8 (57%)	18 (60%)	
2015-2016	4 (24%)	5 (36%)	10 (34%)	
2016-2017			1 (3%)	
Newborns				
Gestational age, weeks (SD)	40.2 (0.9)	40.2 (1.2)	39.7 (1.5)	0.3
Season of birth:				
Birth month (median)	June	April	December	**<0.0001**
Born in flu season, n (%)	2 (12%)	4 (29%)	25 (83%)	**<0.0001**
Month of vaccination (median)	October	October	October	0.9
Mean birth weight, g (SD)	3.5 (0.4)	3.5 (0.5)	3.5 (0.5)	0.9
Female sex	9 (53%)	6 (57%)	12 36%)	0.7
Mode of delivery: Vaginal delivery, n (%)	11 (65%)	9 (64%)	14 (47%)	0.38

P Value by ANOVA and bonferroni post test.

Table 3. Univariate analysis of factors determining the H1N1 antibody titre rate in cord and maternal blood samples

			H1N1 Cord Titre			H1N1 Maternal Titre	
		N	*Mean (95%CI)*	*P value*	*N*	*Mean (95%CI)*	*P value*
Vaccination Status	Vaccinated	61	349.3 (271-426)	**0.0073**	61	221.7 (151.8-291.5)	0.062
	Un-vaccinated	35	191.7 (117.78-265.7)		35	133.3 (92.3-174.2)	
Season of Birth	April-Aug	45	223.1 (145.0-301.3)	**0.026**	41	133.7 (70.0-197.4)	**0.029**
	Sept-Mar	51	351.2 (268.9-433.5)		47	234.5 (169.7-299.2)	
Trimester of vaccination	Un-vaccinated	35	188.3 (113.9-262.7)	-	35	135.5 (93.5-177.5)	-
	Trim 1	17	227.7 (120.3-335)	0.547	17	86.8 (34.5-139.1)	0.168
	Trim 2	15	403.8 (234.8-572.8)	**0.0069**	15	357.8 (156.9-558.7)	**0.0015**
	Trim 3	29	392.2 (264.3-520.1)	**0.0041**	29	225.1 (128.9-321.2)	0.067
Parity	Primiparous	53	266.7 (188.2-345.2)	0.398	53	152.5 (93.5-211.6)	0.098
	Multiparous	43	316.2 (229.3-403.1)		43	229.5 (156.7-302.3)	
Mother's age	20-34	63	296.8 (223.0-370.6)	0.704	63	117.3 (122.7-231.9)	0.552
	35-42	33	273.7 (178.3-369.1)		33	206.3 (119.0-293.6)	
Ethnicity ****	Caucasian	58	299.5 (221.6-377.4)	0.739	58	194.6 (129-259.3)	0.77
	Other	37	279.5 (190.8-368.3)		37	180.5 (113.8-247.1)	
Mode of Delivery **	Vaginal	45	322.2 (243.9-400.5)	0.323	45	236 (161.2-310.8)	0.063
	C-Section	49	264.2 (176.2-352.1)		49	148.2 (90-206.5)	
Gestation	35-39 weeks	35	323.3 (230.4-416.3)	0.35	35	270.6 (176.5-364.7)	**0.0047**
	≥40 weeks	61	267.8 (193.3-342.4)		61	137.7 (93.0-182.3)	
Maternal BMI ***	<25	52	294.7 (219.8-369.5)	0.83	52	227.6 (161.3-294)	**0.04**
	≥25	41	268.1 (175-350)		41	131.9 (70.3-193.4)	

P* values are results of paired sample t-test relative to the un-vaccinated group. ** Delivery mode not recorded for 2 donors. * BMI not recorded for 3 donors. **** Ethnicity not recorded for 1 donor.

Table 4. Crude and adjusted odds ratios for being in the upper 50th percentile of H1N1 IgG cord blood concentration.

[+] The vaccination status adjusted OR was controlled for the effect of birth season.

		Odds Ratio (95%CI)	*P* Value	Adjusted Odds Ratio (95%CI)	*P* Value
Vaccination status[+]	Unvaccinated	1	-	1	-
	Vaccinated	3.6 (1.5-8.9)	**0.005**	3.7 (1.5-9.3)	**0.006**
Trimester of vaccination*	Unvaccinated	1	-	1	-
	Trimester 1	2.2 (0.6-7.8)	0.228	3.4 (0.9-13.9)	0.084
	Trimester 2	5.9 (1.6-23.1)	**0.009**	8.1 (1.9-34.3)	**0.005**
	Trimester 3	3.6 (1.2-10.8)	**0.020**	2.5 (0.8-8.0)	0.120
Birth Season [x]	April-August	1	-	1	-
	September-March	2.29 (0.9-5.4)	0.057	3.1 (1.1-9.3)	**0.046**

*The trimester adjusted OR was controlled for the effect of birth season.

[x] The birth season adjusted OR was controlled for the effect of vaccination status and trimester of vaccination.

Figure 1. Effect of maternal immunisation on maternal and cord ELISA titres. Blood was collected from mothers and cord at the time of delivery. Donors were grouped by vaccination status of the mother. Antibody titre to H1N1 influenza by ELISA in all individuals (A), or in cord (B) and maternal samples (C) by stage of pregnancy at immunisation. HAI titres to H1N1 influenza (D), the dotted line represents the level of seroprotection (HAI titre ≥1:40), numbers above are proportion of individuals that sero-converted. HAI titres by stage of pregnancy at immunisation in cord (E) and maternal samples (F). One way ANOVA and Bonferroni's test : A, D comparison between all groups: B, C, E, F comparisons against unvaccinated group. Line represents mean,* $P<0.05$, ** $P\leq0.01$, *** $P\leq0.001$.

Figure 2. The effect of time interval between maternal vaccination and delivery on anti-influenza titres. Individuals were grouped by the time interval between vaccination and delivery. H1N1 specific IgG titres were measured by ELISA in cord (A) and maternal serum (B). H1N1 specific HAI titre was measured by HAI assay in cord (C) and maternal (D) serum samples. The dotted line represents the level of seroprotection (HAI titre ≥1:40). One way ANOVA and Bonferroni's test, comparisons between all groups and the unvaccinated and 0-4 groups. Line represents mean,* $P<0.05$, ** $P\leq0.01$, *** $P\leq0.001$.

Figure 3: Effect of season of birth and immunisation on antibody titre in mother and cord. Maternal and cord samples were grouped by the season of birth comparing those in flu season (born Sept-March) to those outside it (Apr-Aug). Antibody titres were compared by birth season in unvaccinated (A) and unvaccinated donors (B). Titres in unvaccinated (C) and vaccinated (D) groups were compared against month of birth, ordered from the start of the influenza season (Sept). Month of vaccination (E) and month of birth (F) for children born to mothers vaccinated in different trimesters. One way ANOVA and Bonferroni's test : A, B pairwise comparison between cord and maternal groups, line represents mean,* $P<0.05$, ** $P\leq0.01$, *** $P\leq0.001$.

Figure 4. Effect of maternal immunisation on maternal and cord ELISA titres to H3N2 and B antigens. Blood was collected from mothers and cord at the time of delivery. Donors were grouped by vaccination status of the mother. Antibody titre to H1N1 influenza by ELISA in all individuals (A), or in cord (B) and maternal samples (C) by stage of pregnancy at immunisation. Antibody titre to B influenza by ELISA in all individuals (D), or in cord (E) and maternal samples (F) by stage of pregnancy at immunisation. Maternal (Δ) and cord (●) samples were grouped by the season of birth comparing titres to H3N2 (G) or influenza B (H) in flu season (born Sept-March, closed symbols) to those outside it (Apr-Aug, open symbols). One way ANOVA and Bonferroni's test : A, D comparison between all groups; B, C, E, F comparisons against unvaccinated group; G, H pairwise comparison between cord and maternal groups. Line represents mean,* $P<0.05$.

Figure S1. Correlation between HAI and ELISA. Antibody titres measured by different approaches were correlated in cord blood samples (A) and maternal blood samples at the time of birth (B).

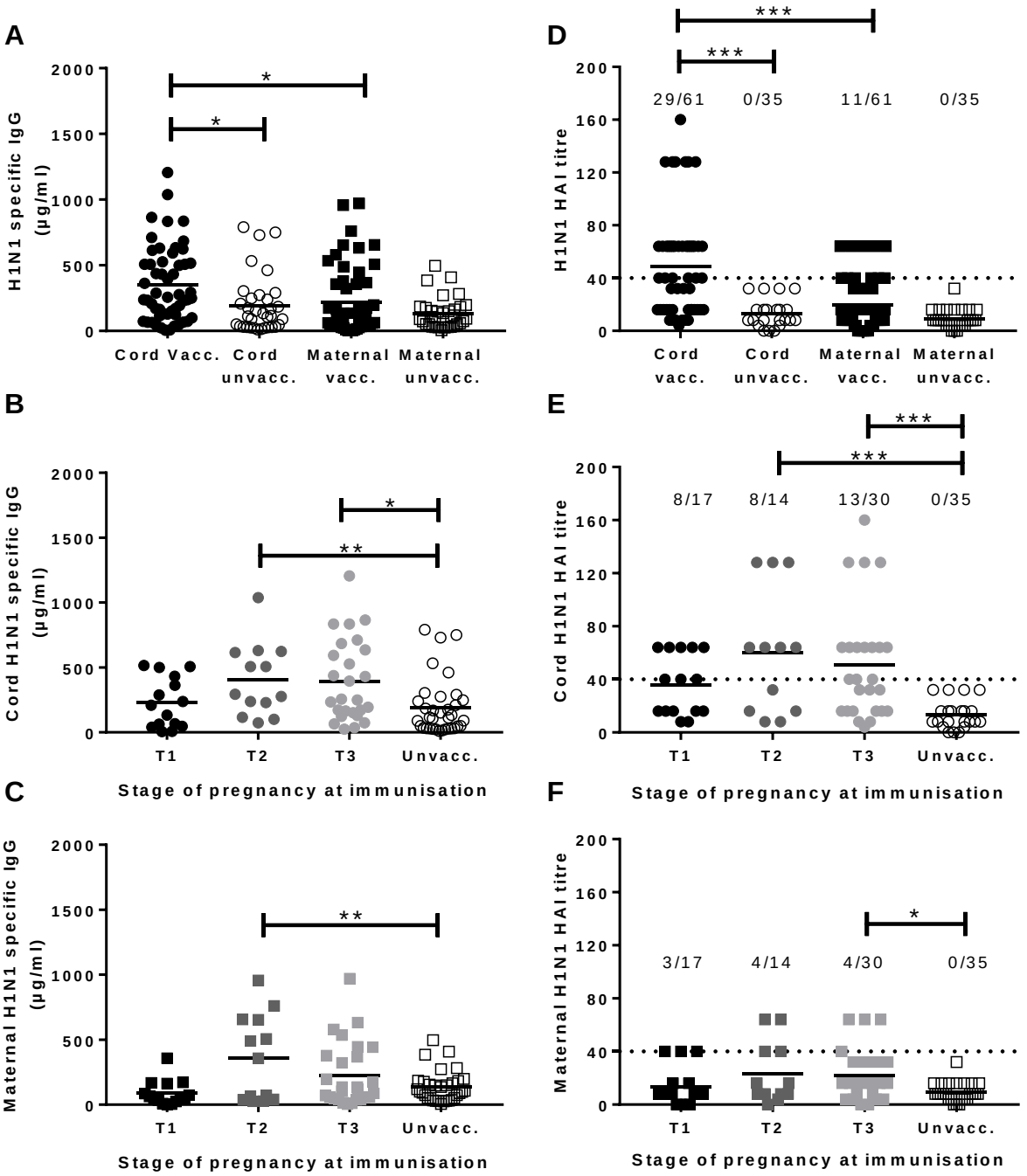

Figure 1. Effect of maternal immunisation on maternal and cord ELISA titres. Blood was collected from mothers and cord at the time of delivery. Donors were grouped by vaccination status of the mother. Antibody titre to H1N1 influenza by ELISA in all individuals (A), or in cord (B) and maternal samples (C) by stage of pregnancy at immunisation. HAI titres to H1N1 influenza (D), the dotted line represents the level of seroprotection (HAI titre ≥1:40), numbers above are proportion of individuals that sero-converted. HAI titres by stage of pregnancy at immunisation in cord (E) and maternal samples (F). One way ANOVA and Bonferroni's test : A, D comparison between all groups: B, C, E, F comparisons against unvaccinated group. Line represents mean,* $p<0.05$, **$p\leq0.01$, *** $p\leq0.001$.

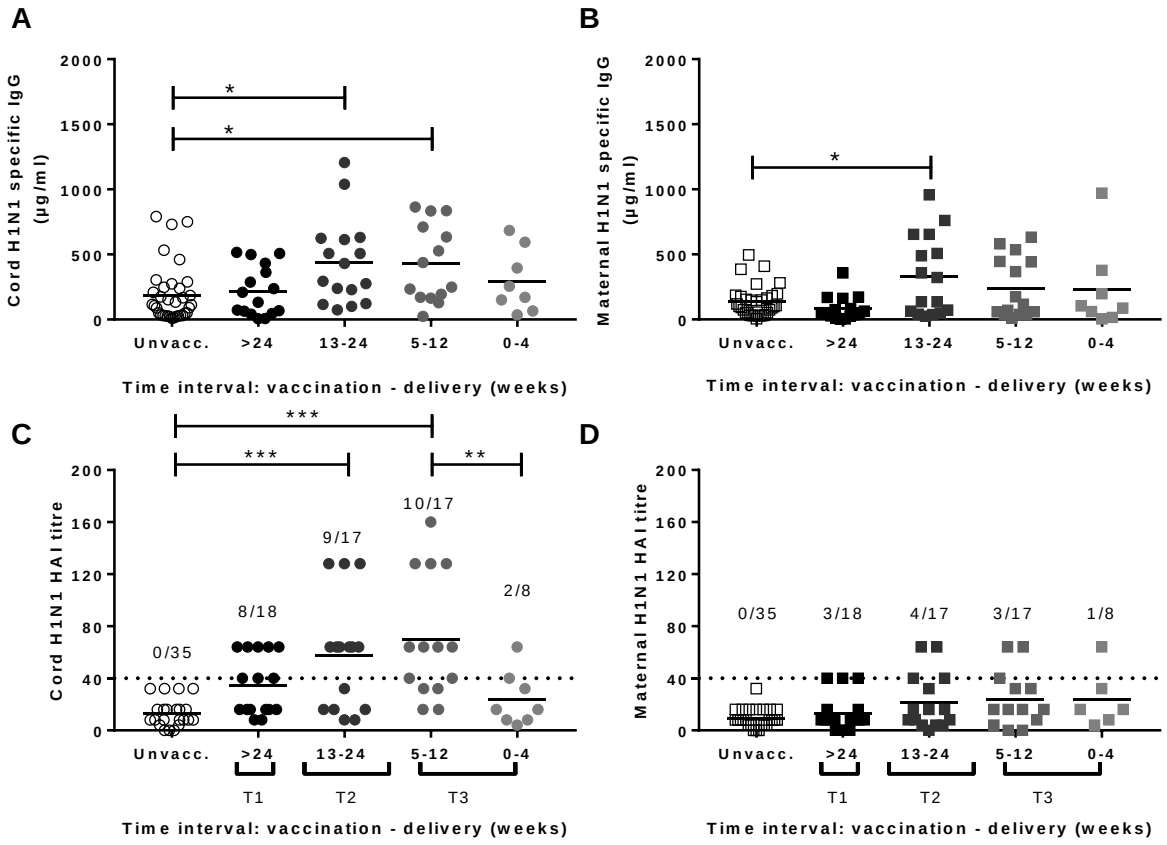

Figure 2. The effect of time interval between maternal vaccination and delivery on anti-influenza titres. Individuals were grouped by the time interval between vaccination and delivery. H1N1 specific IgG titres were measured by ELISA in cord (A) and maternal serum (B). H1N1 specific HAI titre was measured by HAI assay in cord (C) and maternal (D) serum samples. The dotted line represents the level of seroprotection (HAI titre ≥1:40). One way ANOVA and Bonferroni's test, comparisons between all groups and the unvaccinated and 0-4 groups. Line represents mean,* $p<0.05$, **$p\leq0.01$, *** $p\leq0.001$.

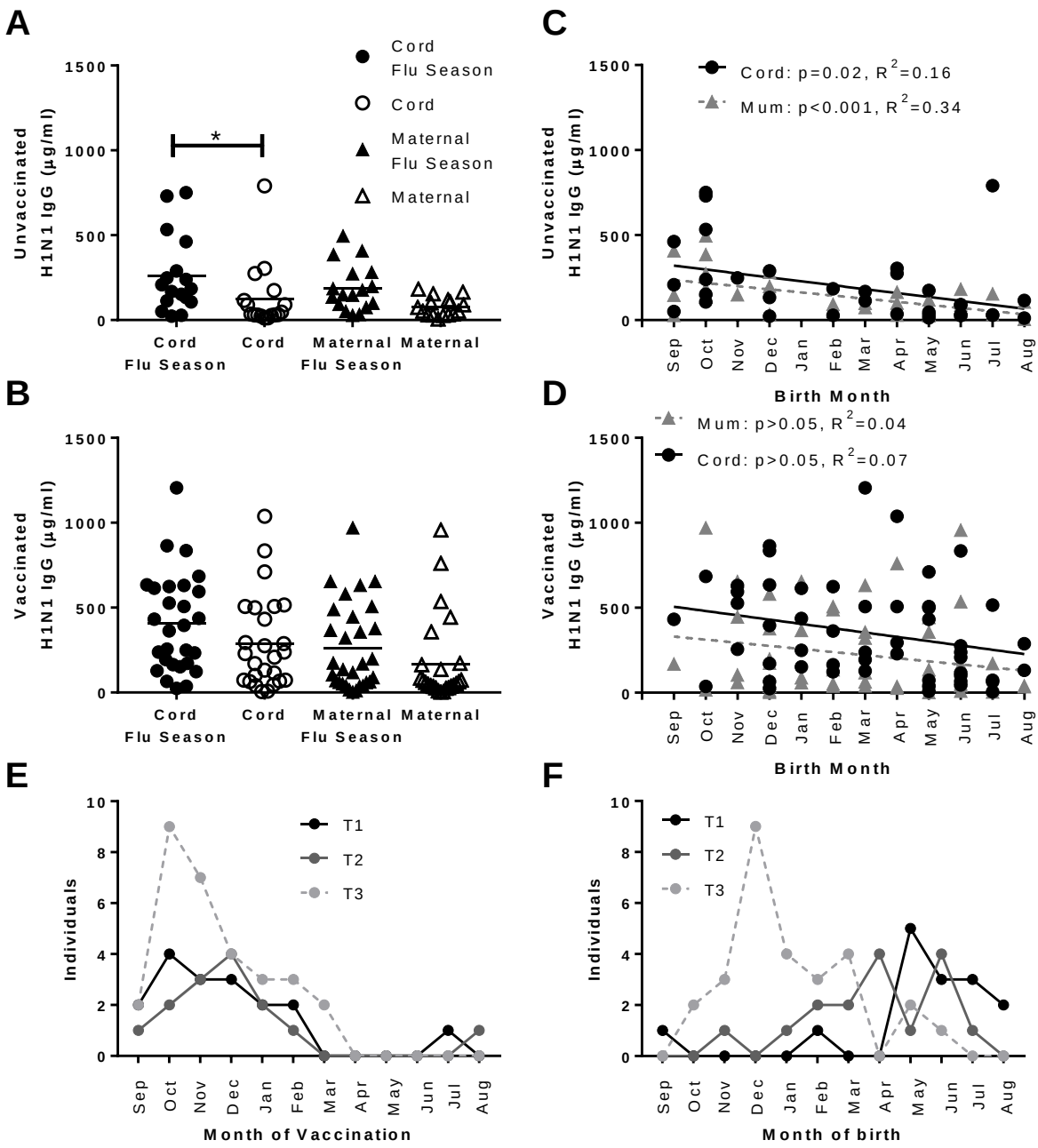

Figure 3: Effect of season of birth and immunisation on antibody response in mother and cord. Maternal (Δ) and cord (●) samples were grouped by the season of birth comparing those in flu season (born Sept-March, closed symbols) to those outside it (Apr-Aug, open symbols). Antibody titres were compared by birth season in unvaccinated (A) and unvaccinated donors (B). Antibody titres in unvaccinated (C) and vaccinated (D) groups were compared against month of birth, ordered from the start of the influenza season (Sept). Month of vaccination (E) and month of birth (F) for children born to mothers vaccinated in different trimesters. One way ANOVA and Bonferroni's test : A, B pairwise comparison between cord and maternal groups, line represents mean,* $p<0.05$, **$p\leq0.01$, *** $p\leq0.001$.

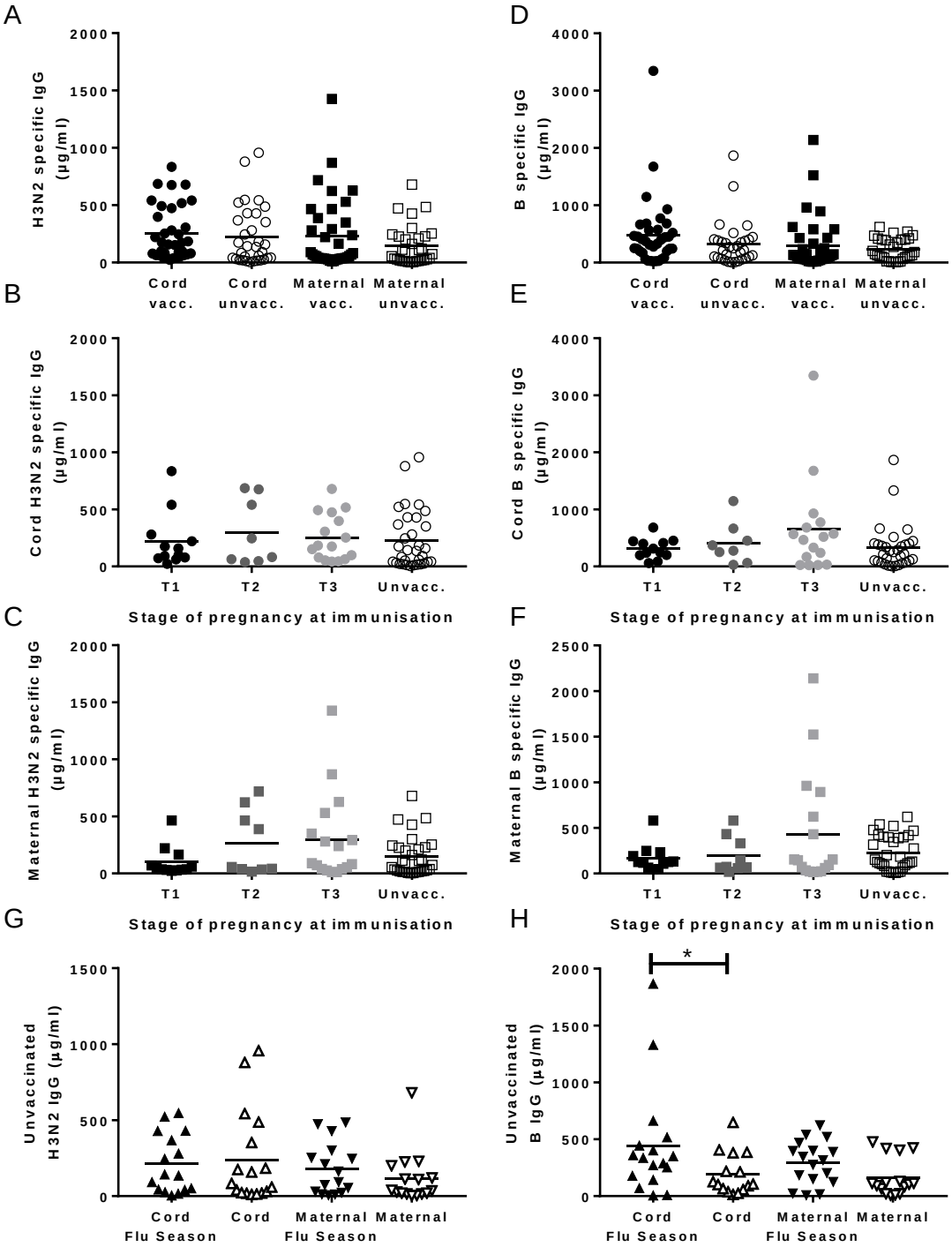

Figure 4. Effect of maternal immunisation on maternal and cord ELISA titres to H3N2 and B antigens. Blood was collected from mothers and cord at the time of delivery. Donors were grouped by vaccination status of the mother. Antibody titre to H1N1 influenza by ELISA in all individuals (A), or in cord (B) and maternal samples (C) by stage of pregnancy at immunisation. Antibody titre to B influenza by ELISA in all individuals (D), or in cord (E) and maternal samples (F) by stage of pregnancy at immunisation. Maternal (Δ) and cord (●) samples were grouped by the season of birth comparing titres to H3N2 (G) or influenza B (H) in flu season (born Sept-March, closed symbols) to those outside it (Apr-Aug, open symbols). One way ANOVA and Bonferroni's test : A, D comparison between all groups; B, C, E, F comparisons against unvaccinated group; G, H pairwise comparison between cord and maternal groups. Line represents mean,* $p<0.05$.

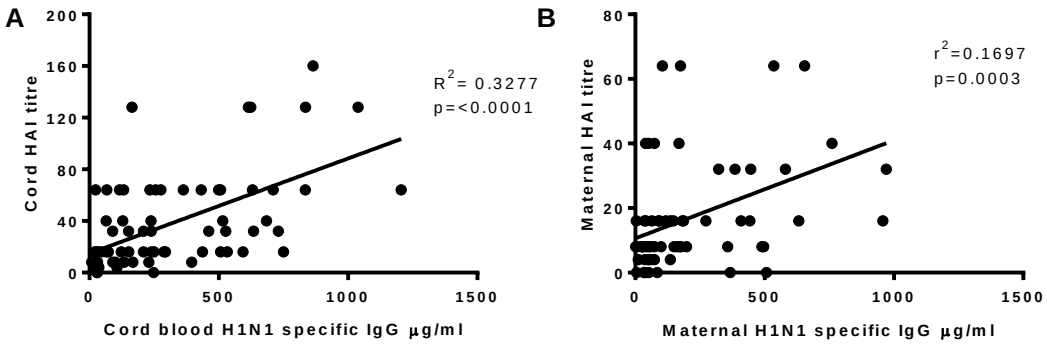

Figure S1. Correlation between HAI and ELISA. Antibody responses measured by different approaches were correlated in cord blood samples (A) and maternal blood samples at the time of birth (B).

Table S1. Seasonal influenza virus vaccine composition (2013-2016)

Flu season	Influenza virus vaccines composition
2013 – 2014	- A/California/7/2009 (H1N1) pdm09-like virus - A/Texas/50/2012 (H3N2)-like virus - B/Massachusetts/2/2012-like virus
2014 – 2015	- A/California/7/2009 (H1N1) pdm09-like virus - A/Texas/50/2012 (H3N2)-like virus - B/Massachusetts/2/2012-like virus
2015 – 2016	- A/California/7/2009 (H1N1) pdm09-like virus - A/Switzerland/9715293/2013 (H3N2)-like virus - B/Phuket/3073/2013-like virus
2016 – 2017	- A/California/7/2009 (H1N1) pdm09-like virus - A/Hong Kong/4801/2014 (H3N2)-like virus - B/Brisbane/60/2008 (Vic)-like virus

Table S2. Univariate analysis of factors determining the HAI titre in cord and maternal blood samples

			HAI Cord Titre			HAI Maternal Titre	
		N	*Mean (95%CI)*	*p value*	*N*	*Mean (95%CI)*	*p value*
Vaccination Status	Vaccinated	61	48.7 (37.8-59.6)	**<0.0001**	61	19.4 (13.9-24.8)	**0.016**
	Un-vaccinated	35	13.1 (8.4-17.8)		35	9.4 (6.5-12.4)	
Season of Birth	April-Aug	45	31.8 (21.6-41.9)	0.17	45	12.2 (7.4-17.1)	0.074
	Sept-Mar	51	43.6 (30.1-57.1)		51	19.2 (13.4-25.1)	
Trimester of vaccination	Un-vaccinated	35	13.1 (8.41-17.8)	-	35	9.4 (6.5-12.4)	-
	Trim 1	17	35.7 (22.9-48.6)	**0.0003**	17	13 (5.4-20.6)	**0.3**
	Trim 2	15	60 (30.3-89.7)	**<0.0001**	15	23 (8.4-37.6)	**0.01**
	Trim 3	29	50.9 (33.7-68.2)	**0.0002**	29	21.9 (13.2-30.6)	**0.0056**
Parity	Primiparous	53	39.5 (28-50.9)	0.774	53	13.3 (8.8-17.9)	0.146
	Multiparous	43	37.0 (23.6-50.4)		43	19 (12.5-25.5)	
Ethnicity ****	Caucasian	58	38 (28.1-48)	0.864	58	16.3 (12-20.5)	0.997
	Other	37	39.6 (22.8-56.4)		37	16.3 (8.3-24.2)	
Mother's age	20-34	63	37.9 (27.3-48.4)	0.9	63	14.6 (10.1-19)	0.27
	35-42	33	38.9 (23.2-54.5)		33	19.1 (11.2-26.9)	
Mode of Delivery **	Vaginal	45	38.2 (27-49.5)	0.9	45	16.4 (11.3-21.6)	0.9
	C-Section	49	39.2 (24.8-53.6)		49	15.9 (9.4-22.3)	
Gestation	35-39 weeks	35	33.7 (21.6-45.9)	0.4	35	19.1 (11.2-27.0)	0.27
	≥40 weeks	61	41.2 (29.1-53.4)		61	14.6 (10.2-19.0)	
Maternal BMI ***	<25	52	38.6 (25.5-51.8)	0.93	52	17.1 (11.3-22.8)	0.56
	≥25	41	17.1 (11.3-22.8)36.6 (24.2-48.5)		41	14.8 (9.6-19.9)13.2 (9.1-17.2)	

*p values are results of unweighted t-test, for trimester of vaccination this is to the unvaccinated group.

** Delivery mode not recorded for 2 donors

*** BMI not recorded for 3 donors

**** Ethnicity not recorded for 1 donor