THE LANCET
Microbe

Supplementary appendix

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

Supplement to: Calvert A, Amirthalingam G, Andrews N, et al. Optimising the timing of whooping cough immunisation in mums (OpTIMUM) through investigating pertussis vaccination in pregnancy: an open-label, equivalence, randomised controlled trial. *Lancet Microbe* 2023; published online April 17. https://doi.org/10.1016/S2666-5247(22)00332-9.

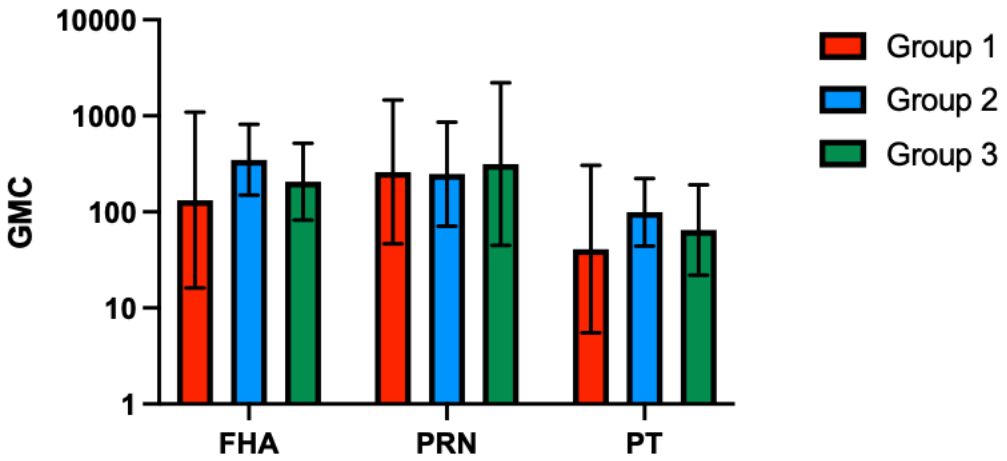
Supplementary material

Contents

Supplementary table 1: GMCs for FHA, PRN and PT in serum from cord blood of term infants

	FHA (n=278)			PRN (n=279)			PT (n=276)		
Study group	**GMC IU/ml (95% CI)**	**Ratio to grp 3 (28-31+6w)**	**P value**	**GMC IU/ml (95% CI)**	**Ratio to grp 3 (28-31+6w)**	**P value**	**GMC IU/ml (95% CI)**	**Ratio to grp 3 (28-31+6w)**	**P value**
1	189.1 (163.2-219.1)	0.59 (0.48-0.72)	**<0.001**	268.9 (205.6-351.8)	0.87 (0.58-1.30)	0.49	51.2 (43.7-60.1)	0.76 (0.61-0.95)	**0.02**
2	232.4 (202.2-267.2)	0.72 (0.58-0.89)	**0.003**	271.4 (200.7-367.0)	0.88 (0.58-1.32)	0.53	61.8 (53.1-72.0)	0.92 (0.73-1.15)	0.47
3	322.3 (272.8-380.6)			309.9 (229.3-418.8)			67.3 (56.7-79.8)		

FHA filamentous haemagglutinin; PRN pertactin; PT pertussis toxin; GMC geometric mean concentration; IU international units



FHA filamentous haemagglutinin; PRN pertactin; PT pertussis toxin; GMC geometric mean concentration

Supplementary figure 1: GMC and 95% CI in preterm infants for FHA, PRN and PT according to study group

Supplementary table 2: GMCs for FHA, PRN and PT in cord blood of preterm infants

	FHA (n=4)			PRN (n=4)			PT (n=4)		
Study group	**GMC IU/ml (95% CI)**	**Ratio to grp 3 (28-31+6w)**	**P value**	**GMC IU/ml (95% CI)**	**Ratio to grp 3 (28-31+6w)**	**P value**	**GMC IU/ml (95% CI)**	**Ratio to grp 3 (28-31+6w)**	**Ratio to grp 3**
1	132.6 (16.1-1088.8)	0.64 (0.19-2.20)	0.48	260.7 (46.5-1462.0)	0.83 (0.19-3.54)	0.80	41.0 (5.5-304.4)	0.63 (0.19-2.13)	0.46
2	349.0 (149.2-816.3)	1.69 (0.49-5.80)	0.40	247.8 (71.1-864.2)	0.79 (0.18-3.37)	0.75	99.4 (44.2-223.8)	1.53 (0.45-5.17)	0.49
3	206.5 (82.1-519.7)			314.4 (44.7-2211.3)			64.9 (22.0-191.7)		

FHA filamentous haemagglutinin; PRN pertactin; PT pertussis toxin; GMC geometric mean concentration; IU international units

Supplementary table 3: Transfer ratios for term and preterm infants for FHA, PRN and PT

	FHA						PRN						PT					
	Term (n= 266)			Preterm (n=11)			Term (n=267)			Preterm (n=11)			Term (n=264)			Preterm (n=11)		
Study group	GMTR (95% CI)	**Ratio to grp 3 (28-31+6w)**	P value	GMTR (95% CI)	**Ratio to grp 3 (28-31+6w)**	P value	GMTR (95% CI)	**Ratio to grp 3 (28-31+6w)**	P value	GMTR (95% CI)	**Ratio to grp 3 (28-31+6w)**	P value	GMTR (95% CI)	**Ratio to grp 3 (28-31+6w)**	P value	GMTR (95% CI)	**Ratio to grp 3 (28-31+6w)**	P value
1	1.88 (1.77-2.00)	1.05 (0.95-1.15)	0.34	1.19 (0.70-2.03)	0.88 (0.60-1.30)	0.53	1.84 (1.74-1.95)	1.06 (0.97-1.16)	0.23	1.06 (0.98-1.16)	0.81 (0.50-1.31)	0.39	1.84 (1.73-1.95)	1.07 (0.98-1.18)	0.14	1.14 (1.04-1.26)	0.82 (0.51-1.34)	0.43
2	1.97 (1.83-2.11)	1.09 (0.99-1.20)	0.07	0.96 (0.76-1.23)	0.71 (0.50-1.02)	0.07	1.91 (1.78-2.06)	1.10 (1.00-1.20)	0.05	1.13 (0.67-1.92)	0.86 (0.55-1.34)	0.51	1.87 (1.74-2.00)	1.09 (0.99-1.20)	0.08	1.02 (0.58-1.82)	0.74 (0.47-1.16)	0.18
3	1.80 (1.68-1.93)			1.35 (0.77-2.36)			1.74 (1.63-1.87)			1.31 (0.69-2.50)			1.71 (1.58-1.85)			1.39 (0.75-2.56)		

FHA filamentous haemagglutinin; PRN pertactin; PT pertussis toxin; GMTR geometric mean transfer ratio. GMTR calculated using cord samples

Supplementary table 4: GMCs at baseline for FHA, PRN and PT according to number of vaccinations recently received

Antigen	No recent vaccination		1 recent previous vaccination		>1 recent vaccination		P value		
	N	GMC (95% CI)	N	GMC (95% CI)	N	GMC (95% CI)	1 vs 0	2 vs 0	2 vs 1
FHA	235	19.5 (17.5-21.8)	98	59.3 (49.8-70.7)	12	54.9 (37.0-81.3)	<0.001	<0.001	0.95
PRN	235	9.6 (7.9-11.6)	98	82.7 (63.2-108.4)	12	74.0 (27.0-203.0)	<0.001	<0.001	0.97
PT	235	8.3 (7.3-9.5)	98	15.7 (13.2-18.7)	12	18.6 (8.5-40.5)	<0.001	0.02	0.85

FHA filamentous haemagglutinin; PRN pertactin; PT pertussis toxin; GMC geometric mean concentration

Supplementary table 5: GMCs two weeks following vaccination for FHA, PRN and PT according to number of vaccinations recently received

Antigen	No recent vaccination		1 recent vaccination		>1 recent vaccination		P value		
	N	GMC (95% CI)	N	GMC (95% CI)	N	GMC (95% CI)	1 vs 0	2 vs 0	2 vs 1
FHA	223	196.6 (176.7-218.7)	95	160.4 (140.7-182.9)	12	142.4 (107.6-188.4)	0.07	0.32	0.86
PRN	223	192.8 (160.5-231.5)	95	266.6 (219.5-323.9)	12	227.0 (113.4-454.5)	0.10	0.90	0.91
PT	223	52.0 (46.7-57.9)	95	50.5 (42.3-60.2)	12	62.4 (38.6-100.9)	0.96	0.74	0.68

FHA filamentous haemagglutinin; PRN pertactin; PT pertussis toxin; GMC geometric mean concentration

Supplementary table 6: GMCs (IU/ml) following primary infant vaccination series for FHA, PRN and PT

	FHA (n=69)			PRN (n=69)			PT (n=69)		
Study group	GMC IU/ml (95% CI)	Ratio to grp 3 (95% CI)	P value	GMC IU/ml (95% CI)	Ratio to grp 3 (95% CI)	P value	GMC IU/ml (95% CI)	Ratio to grp 3 (95% CI)	P value
1 (n=19)	72.1 (57.1-90.9)	1.25 (0.90-1.73)	0.18	43.6 (29.3-64.9)	0.87 (0.53-1.43)	0.58	43.4 (32.0-58.8)	1.12 (0.74-1.70)	0.60
2 (n=26)	52.2 (41.3-66.0)	0.91 (0.67-1.22)	0.52	46.0 (31.4-67.4)	0.92 (0.58-1.46)	0.71	35.6 (26.6-47.8)	0.92 (0.62-1.35)	0.67
3 (n=24)	57.6 (45.9-72.4)			50.2 (37.3-67.5)			38.8 (28.8-52.2)		

FHA filamentous haemagglutinin; PRN pertactin; PT pertussis toxin; GMC geometric mean concentration

Supplementary table 7: Comparison of fever and local reactions in participants who had recently received DTP vaccination or not

		Fever					**Redness**					**Swelling**					**Tenderness**				
		0	**1**	**2**	**3**	**P value**	**0**	**1**	**2**	**3**	**P value**	**0**	**1**	**2**	**3**	**P value**	**0**	**1**	**2**	**3**	**P value**
Recent DTP vacc	Y	105	1	1	0	0.79	91	10	2	4	0.81	90	5	4	8	0.64	5	44	53	5	**0.04**
	N	224	2	1	0		196	15	5	11		201	8	7	11		25	113	80	9	

Supplementary table 8: Serious Adverse Events

	Details of SAE	Number
Maternal (n=23)	Non-reassuring fetal status	1
	Mastitis	2
	Pre-eclampsia	2
	Sepsis	2
	Preterm rupture of membranes	1
	Central serous retinopathy	1
	Unwell	1
	Suspected pulmonary embolism	1
	Retained product of conception	1
	Obstetric haemorrhage	1
	Preterm delivery	1
	Postpartum haemorrhage	5
	Cord prolapse	1
	Hypertension	2
	Failed trial without catheter	1
Infant (n=18)	Respiratory distress	3
	Neonatal fever	1
	Weight loss	2
	Jaundice	4
	Preterm birth	3
	Suspected sepsis	1
	Risk factors for sepsis	1
	Congenital anomalies	1
	Vomiting	1
	Suspected NEC	1

Supplementary material 9: Statistical Analysis Plan

1. Introduction

This is the statistical analysis plan for the OpTIMUM trial: Optimising the Timing of whooping cough Immunisation in MUMs. NCT03908164. It will follow Protocol Version 2.1, Date 16/07/2019.
Any changes to the plan prior to analysis will be documented and justified at the end of this plan.

2. Design in brief

When the pertussis vaccination in pregnancy programmes were first implemented, vaccination was recommended in the third trimester (UK 28-32 weeks). This decision was based on the hypothesis that vaccination would be most effective if it was timed to allow the peak in maternal antibody levels after vaccination to coincide with the time of most efficient placental transport. Since 2016 the recommendations in the UK have changed to 16-32 weeks to allow increased coverage. There is however, a significant lack of agreement on optimal timing of pertussis vaccination in pregnancy due to conflicting data. This is the rationale for this randomised controlled trial.
This is a randomised trial which will be performed in locations across England (London, Southampton, Oxford, Bristol, Manchester). We will recruit 354 pregnant women. These women will be recruited in pregnancy prior to receiving a pertussis containing vaccine and will be randomised into one of three groups receiving the vaccine at three different time periods: ≤23+6 GW, 24-27+6 GW and 28-31+6 GW. Women will be involved in the study from the time of enrolment at around 20 weeks to the time of delivery (a period of around 20-22 weeks in total), and their infants will be involved in the study from birth until the age of 5 months (around 5 months in total). The design is an equivalence trial.

Each group will have visits as below.

Visit Number	**Screening visit**	**Vaccination visit**	**Follow up visit**	**Delivery visit**	**Infant visit**
Timing	At or before 23+6 weeks	According to study allocation	V+14 (+/- 2)	Delivery	28-42 days following third pertussis vaccination
Activity	Screening & enrolment	Maternal blood sampling and vaccination. Diary card provided. Questionnaire.	Maternal blood sampling. Diary card collected.	Maternal blood and cord blood sampling	Infant blood sampling

3. Objectives

Primary, secondary and exploratory objectives are given below.

3.1 Primary objective

PO1: To determine if pertussis vaccination at different time points in pregnancy results in equivalent concentrations of pertussis specific antibodies in the term infant at birth.

3.2 Secondary Objectives

SO1: To determine if pertussis vaccination at different time points in pregnancy results in equivalent concentrations of pertussis specific antibodies in the preterm infant at birth

SO2: To investigate the rate of fever and local reactions in women receiving the vaccine in pregnancy comparing those who are receiving the vaccine for the first time and those who have previously received the vaccine in pregnancy

SO3: To describe the kinetics of the antibody response to pertussis vaccination during pregnancy

SO4: To describe the placental transfer of antibody following administration of vaccine at three discrete time points

SO5: To explore the impact of repeated vaccination on the antibody response in women who have received a pertussis vaccination in a previous pregnancy

SO6: To evaluate the impact of timing of pertussis vaccination in pregnancy on antibody concentration in the infants following their primary immunisation schedule

3.3 Exploratory objectives

EO1: To assess the functionality of anti- PT antibody in blood samples from women post vaccination, in the cord blood of infants and in the infant post vaccination

EO2: To investigate functional immunity of colostrum within 48 hours of delivery and of breastmilk at 14 days and 5 months following delivery

4. Outcomes and Endpoints

4.1 Primary endpoint/outcome

PE1: Antibody concentrations against Pertussis Toxin (PT), Filamentous Haemagglutinin (FHA) and Pertactin (PRN) in cord blood of term infants at delivery

4.2 Secondary endpoints/outcomes

SE1: Antibody concentrations against Pertussis Toxin (PT), Filamentous Haemagglutinin (FHA) and Pertactin (PRN) in cord blood of preterm infants at delivery

SE2: Rates of fever and local reaction following vaccination

SE3: Antibody concentrations against Pertussis Toxin (PT), Filamentous Haemagglutinin (FHA) and Pertactin (PRN) in maternal blood 14 days after vaccination

SE4: Antibody concentrations against Pertussis Toxin (PT), Filamentous Haemagglutinin (FHA) and Pertactin (PRN) in infants one month after completion of their primary immunisations

4.3 Exploratory endpoints/outcomes

To be added later – functional assay results

5. Sample size calculation

Based on previous studies of cord blood the log10 Standard Deviation is about 0.5 for PT, 0.4 for FHA and 0.55 for PRN. To assess equivalence to within a 1.8 fold margin, and assuming the higher standard deviation of 0.55, a sample size of 100 per group is needed (two sided 95% CI on the fold difference, 80% power) which, allowing for a drop-out rate of around 10% and a rate of prematurity of around 8% would require 354 women to be recruited.

6. Analysis sets, missing data, censored data, significance level

Missing data will be assumed missing at random, there will not be imputation. Analysis will be per-protocol. Major protocol deviations will be described, in particular women not getting vaccinated within the gestational window into which they were randomised.
For safety the analysis set will be all women who received a dose of vaccine. Assay results below assay limits will be assigned a value of half the limit. Significance will be at 5% and 2-sided 95% CI used.

7. Analysis of end points

Summary of baseline data and flow of patients
At the end of the study, a flowchart will be used to summarise the number of women approached, consented, assigned to the different study arms, receiving vaccinations at the planned times, completing the study protocol and analysed for the primary outcome, as recommended by the CONSORT statement (http://www.consort-statement.org/). Baseline data comparing the three trial groups will be summarised in a table format and will compare median age, number of previous pregnancies, recruiting study site, history of previous pertussis containing vaccination and underlying medical conditions.

Primary endpoint analysis

PO1: To investigate if pertussis vaccination at different time points in pregnancy results in equivalent concentrations of pertussis specific antibodies in the term infant at birth
Geometric mean concentrations for each end point in PE1 will be calculated for each group with 95% confidence intervals. To assess equivalence the geometric mean fold ratio with 95% CI will be calculated for group 3/group1 and group 3/group 2 (i.e. late vs. early) and equivalence achieved if the upper end is below 1.8 and the lower end above 1/1.8 (0.55) (the equivalence margin). In addition, groups 1 and 2 will be compared for equivalence and all groups will be compared with one-another for differences using ANOVA on log-concentrations, or Kruskal-Wallis test in the event of non-normal log-concentration distributions. Finally, normal errors regression will be used to compare groups 1 and 2 to group 3 with adjustment for covariates including maternal age, gestation and prior vaccination history.

Secondary endpoint analysis

SO1: To determine if pertussis vaccination at different time points in pregnancy results in equivalent concentrations of pertussis specific antibodies in the preterm infant at birth
The exact number of infants born preterm is not known, but local data suggests a rate of prematurity of about 8%. If this is the case there will be around 30 preterm infants in this study. Geometric mean concentrations will be calculated for each group with 95% confidence intervals. The

groups will be compared according to timing of maternal vaccination as is done with the term infants.

SO2: To investigate the rate of fever and local reactions in women receiving the vaccine in pregnancy comparing those who are receiving the vaccine for the first time and those who have previously received the vaccine in pregnancy
Within each group the rates of fever (>38°C) and local reactions (redness, swelling and tenderness) will be calculated (with 95% exact CIs) and compared using Fisher's exact test.

SO3: To describe the kinetics of the antibody response to pertussis vaccination in pregnancy
This is within the mothers. Within each group geometric mean concentrations at each time point will be calculated with 95% CIs as well as the geometric mean fold change between each successive time points. These will be compared between groups using ANOVA or Kruskal-Wallis test in the event of non-normal log-concentrations distributions. Normal errors regression will be used to compare groups 1 and 2 to group 3 with adjustment for covariates including maternal age and prior vaccination history. Multivariable analysis will be performed allowing for various factors and modelling will be performed for the antibody kinetics in the mother following vaccination based on a relationship such as log-titre vs log-time. This will include random effects mixed models to allow for individual declines and to take into account the different timing between the 14 day sample and birth sample.

SO4: To describe the placental transfer of antibody following administration of vaccine at three discrete time points
The transfer ratio will be calculated as the ratio of cord to maternal blood and the geometric mean ratio calculated with 95%CI. This ratio (on a log-scale) will be compared between groups using ANOVA or Kruskal-Wallis test in the event of non-normal log-ratios. Finally, normal errors regression will be used to compare log-ratios for groups 1 and 2 to group 3 with adjustment for covariates including maternal age.

SO5: To explore the impact of repeated vaccination on the antibody response in women who have received a pertussis vaccination in a previous pregnancy
This will be done as described above by inclusion as a covariate in the normal errors regression models. We expect that around 40% will have received a prior pertussis vaccine in pregnancy. Assuming 40% then when combining groups we will have approximately 120 with prior history of vaccination and 180 without; with a log-10 SD of 0.55 this will allow 1.5 fold differences to be detected (80% power, 5% significance). In addition, the interaction term between prior vaccination and group on antibody response will be assessed to see if the effect of prior vaccination depends on timing.

SO6: To evaluate the impact of timing of pertussis vaccination in pregnancy on antibody concentration in the infants following completion of their primary immunisation schedule.
Geometric mean concentrations will be calculated for each group with 95% confidence intervals. Groups will all be compared with one-another for differences using ANOVA on log-concentrations, or Kruskal-Wallis test in the event of non-normal log-concentrations distributions. Finally, normal errors regression will be used to compare groups 1 and 2 to group 3 with adjustment for covariates including time of blood sample.

EO1: To assess the functionality of anti- PT antibody in blood samples from women post vaccination, in the cord blood of infants and in the infant post vaccination
This will be done by describing the measurements using plots. Geometric mean responses with 95% CIs will also be given.

EO2: To investigate functional immunity of colostrum within 48 hours of delivery and of breastmilk at 14 days and 5 months following delivery
This will be done by describing the measurements using plots. Geometric mean responses with 95% CIs will also be given.

Interim analysis
Not planned.

Signature of Statistician (Nick Andrews)

Date 10/03/2021
Signature of Chief Investigator (Paul Heath)

Date 10/3/21

Changes to the plan and reason

Change	Date	Reason
Addition of detail to exploratory endpoints/outcomes (4.3-see below)	1st November 2021	Addition of detail following discussion with labs about which assays would be performed.
Addition of exploratory endpoint analysis	1st November 2021	Addition of detail following discussion with labs about which assays would be performed.
Addition of seropositivity analysis	20th February 2022	To allow comparison with previously published work.
Clarification about analysis performed for SO5	14th February 2022	

4.3 Exploratory endpoints/outcomes

EE1: Antibody concentrations against Pertussis Toxin (PT), Filamentous Haemagglutinin (FHA) and Pertactin (PRN) in colostrum and breastmilk <48 hours after delivery, 14 days following delivery and 28-70 days following delivery.

EE2: Serum bactericidal antibody titres against pertussis specific antigens 14 days following vaccination, at delivery and in the infant following infant vaccination at around 5-6 months of age.

Exploratory endpoint analysis

EO1: To investigate functional activity of antibody in blood 14 days following vaccination, at delivery and in the infant following infant vaccination at around 5-6 months of age.

This will be done by plotting the serum bactericidal antibody titres

EO2: To assess the antibody concentrations against Pertussis Toxin (PT), Filamentous Haemagglutinin (FHA) and Pertactin (PRN) in colostrum and breastmilk <48 hours after delivery, 14 days following delivery and 28-70 days following delivery

This will be done by describing the measurements using plots. Geometric mean responses with 95% CIs will also be given.

Seropositivity analysis
There is no correlate for protection for any pertussis antigen. Eberhardt described a method of defining infant seropositivity in which it was calculated that infants who were born with an anti-PT concentration of >30 IU/mL would continue to have antibody concentrations of >5 IU/mL until at least 3 months of age (1). Following this method as an additional exploratory analysis, we compared the percentage of infants in each group who had anti-PT concentration of >30 IU/mL at birth, who were considered to be seropositive.

Clarification of analysis for SO5: In addition to inclusion as a covariate in analyses of maternal antibody levels univariable analyses were done using normal errors regression on logged titres to assess the effect of prior vaccination and geometric means by prior vaccination calculated.

References
1. Eberhardt CS, Blanchard-Rohner G, Lemaitre B, Boukrid M, Combescure C, Othenin-Girard V, et al. Maternal Immunization Earlier in Pregnancy Maximizes Antibody Transfer and Expected Infant Seropositivity Against Pertussis. Clin Infect Dis. 2016;62(7):829-36.