PROTOCOL Version 4.0

PROMESA

Promotion of a healthy gut microbiome in elective Caesarean section arrivals

Can exclusive breastfeeding supplemented with a probiotic promote a sustained healthy gut microbiota in babies born by Caesarean section?

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Protocol amendments

Protocol version	Date	Reason for update	Substantial amendment number	Summary of changes
2.0	12DEC2017	Corrections to errors, additional analyses	1.0	Page 10 – added inflammatory markers to Objective 2 Page 11 – added inflammatory markers and RNA analysis to Secondary Endpoints Page 12 – added that a home visit would occur by study staff when the infant is 6-8 weeks Page 13 – added non-smoking as an inclusion and smoking during this pregnancy as an exclusion Page 14 – removed previous breastfeeding history as a minimization factor; replaced with parity and pre-pregnancy BMI Page 18 – added that a home visit would occur by study staff when the infant is 6-8 weeks to monitor supplementation compliance; removed maternal stool sampling (only rectal and vaginal swabs being collected for mums); added inflammatory markers to additional research Page 20 – added RNA analysis and clarified that some analyses will be performed at UC Davis or Evolve
3.0	09APR2018	Addition of sub-study protocol and other minor changes	2.0	Page 1 – added ISRCTN number Pages 2 and 3 – updated contact information Page 12 – added analyses of maternal milk, and analysis of antibiotic usage and additional study supplementation to Secondary Endpoints

				Page 13 – added that pregnant women may be recruited from additional clinics and practices Page 14 – added exclusion for women planning to leave the UK before their baby is 6 months old Page 21 – added shotgun sequencing Page 22 – added sub-study to analyze immune effects of probiotic supplementation on a subset of PROMESA participants Page 31 – added sub-study references Page 34 – added to list of conditions that should not be considered SAEs per study paediatrician Page 36 – clarified that study paediatrician would be contacted by study staff (not participants) for consultation as needed Throughout – corrected capitalisation and spelling of "Caesarean" Throughout – changed name of KCL division to Dept. of Women and Children's Health
4.0	16JAN2019	Increase recruitment for main study, make sub-study compulsory and increase enrolment for sub-study, additional exclusion, removal of some sample and data collection for compulsory sub-study participants, training of research midwives	3.0	Page 14 – increased recruitment to allow for up to 70 randomized subjects in main PROMESA study analysis Page 15 – made sub-study participation compulsory and increased enrolment for a total of 40 randomised in sub-study Page 15 – added exclusion for maternal medication use that may alter infant's gut microbiota Page 21 – removed one timepoint (Hospital Discharge) for infant weight and height/length data collection – was removed from CRF during last amendment and inadvertently left in protocol

		for sub-study blood draw, and corrections		Page 23 – included stool samples for potential biochemical or RNA analysis Page 23 – updated recruitment number to reflect increase in enrolment for sub-study Page 26 – made sub-study participation compulsory and updated recruitment numbers Page 26 – listed changes made to main study participation for subjects newly randomised in compulsory sub-study Page 27 – updated recruitment number to reflect increase in enrolment for sub-study Page 28 – At month 3 of the study timeline: added that blood draws may be performed by a trained research midwife; added that blood draws may be performed at the participant's home by the trained research midwife; changed maximum potential blood draw volume from 1 mL to 2 mL Page 29 – noted that changes were made to the data forms for the main study participation for subjects newly randomised in compulsory sub-study Page 29 – added that transcriptomics and immune/inflammation assays may be performed if sufficient blood samples are available

Protocol approval

Signatures

Prof. Andrew Shennan	_____	_____
Principal Investigator	**Signature**	**Date**
Prof. Rachel Tribe	_____	_____
Chief Investigator	**Signature**	**Date**
Dr Tom Marrs	_____	_____
Paediatrician	**Signature**	**Date**
Mr Paul Seed	_____	_____
Trial Statistician	**Signature**	**Date**
Dr Annette Briley	_____	_____
Trial Manager	**Signature**	**Date**

Table of Abbreviations

BMI	body mass index
CFU	colony forming unit
CI	Chief Investigator
C-section	Caesarean section
CTIMP	Clinical trial of an investigational medicinal product
g	gram
GCP	Good Clinical Practice
HMO	human milk oligosaccharide
HTA	Human Tissue Authority
ID	identification
KCL	King's College London
kg	kilogram
L	Litre
m	Metre
mg	milligram
ml	Millilitre
NGS	Next Generation Sequencing
SAE	serious adverse event
SPSS	Statistical analysis software
STATA	Statistical analysis software
subsp.	subspecies
UK	United Kingdom
US	United States
wks	weeks

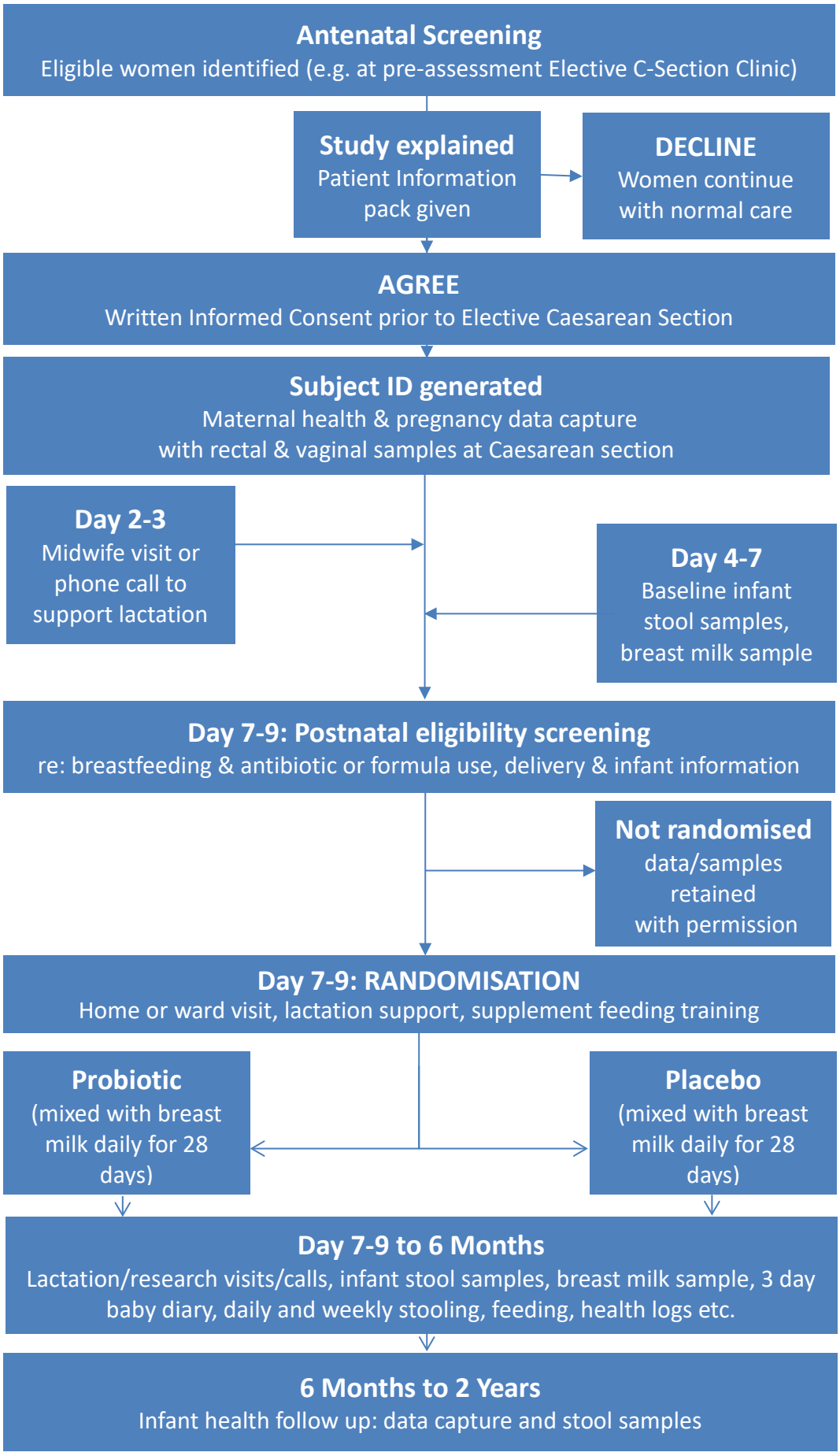
Study Summary

PROMESA is a randomised double-blinded placebo-controlled trial of a dietary (probiotic) supplement added to breast milk for the enhancement of the normal neonatal gut microbiome in term babies delivered by Caesarean section.

Recent medical discoveries have found that intestinal levels of *Bifidobacterium infantis* (*B. infantis*) assist with the development of the infant's gut and provide protection against intestinal pathogens. Data from around the world over the past 5 years point to *B. infantis* as universally associated with all newborn babies, and that this culture is naturally introduced to babies who are born vaginally and breastfed. The growth of protective *Bifidobacterium* strains within the intestine of the breastfed infant is supported by human milk oligosaccharides (HMO). Breastfeeding selectively nourishes these bifidobacteria that in turn protect and guide the intestinal health of the developing infant. However, the ever-growing number of deliveries by Caesarean section is resulting in babies with very low levels of intestinal *B. infantis.*

Our principal research question is whether short term daily probiotic supplementation (28 days of *B. infantis*) in breastfed infants delivered by Caesarean section will promote a healthy infant faecal microbiome profile that can be maintained by breastfeeding until weaning at 6 months. Approximately 70 women will be recruited to provide vaginal and rectal swabs for baseline assessment of the maternal microbiome prior to Caesarean section. At postnatal Day 7-9, infants that are eligible to continue in the study (i.e. breastfed and not exposed to antibiotics for > 3 days) will be randomised to receive either a daily supplement of *B. infantis* or placebo in breast milk for 28 days. All women will be contacted regularly by a research midwife trained in lactation support. Infant faecal samples/swabs and accompanying breast milk will be posted or collected, at regular intervals during the study until 6 months of age. There will be up to three follow-up visits until 2 years of age. The infant gut microbiome, and other biomarkers of gut health, will be analysed along with components of the mother's breast milk.

Trial Flow Chart



Background and Rationale

The composition of the initial infant intestinal microbiota influences immune functions and the future developing microbiome. Colonisation of the neonatal gastrointestinal tract is associated with exposure to maternal microbiota (vaginal and faecal) during labour and birth and birth mode is considered an important factor in the establishment of normal intestinal bacterial communities. This is an important issue since the rates of Caesarean section rate are high in many countries including the UK (26.5%, 2014-15) (1) and US (32.8% of all births in 2010) (2).

Differences in this postnatal microbial colonization, for example, have been attributed to the higher incidence of immune mediated diseases such as allergy and asthma in children born by Caesarean section compared with those born vaginally (3-7). Allergic disease has been associated with a low prevalence of *Bacteroides* and *Bifidobacterium* (8, 9), and higher colonization rates with *Clostridium* (10). Administration of probiotics to infants has been found to reduce the incidence of allergy (11, 12).

Infants born to women by vaginal delivery may acquire bacterial communities resembling their own mother's vaginal microbiota whereas Caesarean section infants harbour bacterial communities similar to those found on the skin surfaces (13). The initial intestinal bacterial community established by the mode of delivery influences the microbial succession patterns in the gut that persist over time. For example, it is known that infants delivered by Caesarean section have lower Bacteroidetes phylum and total microbiota diversity during the first 2 years of life (14), with delayed intestinal colonization by *Bifidobacterium*, and significantly lower rates of Bacteroides group (15, 16), and higher *Clostridium difficile* levels (16) compared with vaginally born infants. Daily intake of probiotics from birth until age 6 months has been shown to reduce the incidence of allergy in Caesarean section but not vaginally delivered children (17). Oral delivery of beneficial bacteria, such as lactobacilli and bifidobacteria, has been linked to positive health effects in infants, children and adults including reductions in gut inflammation, diarrhoea, allergic disease and associated metabolic disturbances (18-24).

Using new analytical capabilities to identify bacteria at strain-specific accuracy, our commercial collaborators (Evolve BioSystems, Inc.) have recently discovered that infants in California, born vaginally and breastfed (n = 42) have significantly lower levels of *B. infantis, Bifidobacterium* and Actinobacteria at 4 months of life than babies born in developing countries (25). For example, *B. infantis* represented 30% or higher of the total gut microbiota in only 20% of 4 month-old breastfed infants, yet represented 80-90% of the total gut microbiota in more than 75% of the babies born in the Gambia and Bangladesh. It is proposed that this occurs due to a lack of normal transfer through the faecal (maternal) - oral (fetus) route during Caesarean section. There is also an emerging concern that medicalisation of vaginal birth (increased hygiene) can also contribute to altered patterns of colonisation in some populations.

An abundance of intestinal *B. infantis* is positively associated with immune protection through several proposed mechanisms: 1) through enhanced adherence to intestinal cells and inhibition of gut invasion by undesirable bacterial strains (26); 2) through direct interaction with intestinal cells and dendritic cells resulting in the production of anti-inflammatory cytokines and reduction of pro-inflammatory cytokines and enhanced gut barrier function (27, 28); 3) through the secretion of bioactive factors that directly reduce expression of toll-like receptors 2 and 4, reduce the production

of pro-inflammatory cytokines, enhance release of anti-inflammatory cytokines and enhance gut barrier function (29, 30); 4) through increased activation of Treg cells with concomitant inhibition of chemokine secretion within the mucosa during pathogen infection (31).

The competitive growth of protective *Bifidobacterium* strains within the intestine of the breastfed infant is supported by human milk oligosaccharides (HMO), as natural prebiotics in human milk (~10-20 g/L) (32-45). Specifically, two main *Bifidobacterium* species populate the breastfed infant colon, *Bifidobacterium longum* and *Bifidobacterium breve*. The *B. longum* clade contains two subspecies found in humans, *B. longum* subsp. *longum* (herein termed *B. longum*) and *B. longum* subsp. *infantis* (*B. infantis*). Thus, mammalian lactation has evolved to selectively nourish targeted bifidobacteria that in turn protect and guide the intestinal health of the developing infant.

The reduction in *B. infantis* levels in the infant associated with Caesarean section (and potentially medicalised vaginal delivery) is most likely due to reduced maternal levels and/or limited transfer by the faecal route at birth. This provides a rationale to consider late-pregnancy maternal supplementation or postnatal infant supplementation with *B. infantis* probiotic to promote normal infant gut colonization. Data emerging from the IMPRINT study in the US indicates that *B. infantis* colonization occurs within 28 days of supplementation, but it is not yet known whether this can be maintained in breastfed babies after supplementation has stopped. We also do not know whether this promotes maintained gastrointestinal health as the child develops and whether this influences overall health in terms of allergy and other related conditions known to affect babies born by Caesarean section.

Hypothesis

Daily supplementation of breast milk with *B. infantis* to term infants delivered by Caesarean section for 28 consecutive days combined with exclusive breastfeeding (with lactation support provided) will result in significantly higher levels of faecal *B. infantis* and bifidobacteria compared with matched control infants receiving a lactose placebo. Promoting a normal healthy microbiota with probiotic supplementation may also have longer term benefits to children's health.

Aims and Objectives

The aim of this clinical trial is to determine the effects of supplementing the probiotic *Bifidobacterium longum* subsp. *infantis* from Day 7-9 for 28 days in healthy term breastfed infants delivered by Caesarean section on gut bacteria composition from 1 week until 2 years (with data and samples collected up to 2 years) compared with infants receiving placebo, with both arms receiving lactation support.

Objective 1: To evaluate changes in infant faecal microbiota from baseline, during and after supplementation with *B. infantis* or placebo.

Objective 2: To evaluate differences between *B. infantis* and placebo supplementation in infant stool for levels of HMO, short-chain fatty acids, inflammatory markers, and pH.

Objective 3: To compare maternal faecal and vaginal microbiota prior to Caesarean section to infant faecal microbiota.

Objective 4: To collect and store mothers' breast milk for potential future analysis of human milk oligosaccharide composition and HMO secretor status.

Objective 5: To evaluate changes in infant faecal microbiota related to formula and/or solid foods introduction.

Study Endpoints

Primary Endpoint:

- The change in infant faecal microbiota before, during, and after *B. infantis* or placebo supplementation by molecular methods.

Secondary Endpoints:

- The differences between *B. infantis* and placebo supplementation on levels of HMO excreted in infant stool.
- The differences between *B. infantis* and placebo supplementation on levels of short-chain fatty acids in infant stool.
- The differences between *B. infantis* and placebo supplementation on levels of inflammatory markers in infant stool.
- The differences between *B. infantis* and placebo supplementation on pH of infant stool.
- The response of the infant to the microbial environment of the gut by RNA analysis of infant stool.
- Comparison of maternal faecal and vaginal microbiota composition to initial infant levels.
- Proteomic, biochemical, and microbial analyses of maternal breast milk.
- The relationship between infant weight and gut microbiota**.**
- The relationship between formula use and gut microbiota.
- The relationship between solid foods introduction and gut microbiota.
- The response of the infant gut microbiome to antibiotic usage and additional study supplementation.
- Gastrointestinal and related symptoms before, during and after *B. infantis* supplementation.
- The differences between *B. infantis* and placebo supplementation on adverse events, including occurrence of any illness, health care visits for sickness, and fever.
- The differences between *B. infantis* and placebo supplementation on infant medication usage, including antibiotics.

Study Design

This is a randomised, double-blinded, placebo-controlled trial of a dietary supplement added to breast milk for enhancement of the normal neonatal gut microbiome in babies delivered by Caesarean section. Prior to randomization, women will provide vaginal and rectal swabs for baseline assessment of the maternal microbiome. A baseline infant stool sample will be obtained prior to supplementation between Days 4 and 7-9. Infants of mothers randomised to *B. infantis* will receive a daily feeding for 28 days; controls will receive a daily feeding of placebo for 28 days. All women will be contacted regularly by a research midwife trained in lactation support. Infant faecal samples/swabs will be

collected, alongside data, and stored at regular intervals during the study (6 months) and up to 2 years of age.

Study Population and Setting

Antenatal screening and enrolment will take place predominantly in elective Caesarean pre-assessment clinics in Guy's and St Thomas' NHS Foundation Trust, UK. Eligible patients may also be contacted by the research staff prior to their pre-assessment appointment in other antenatal settings such as External Cephalic Version (ECV), Vaginal Birth After Caesaeran (VBAC) clinic, and tocophobia clinics, private practice offices, as well as over the phone to discuss the trial with them. Postnatal screening will be undertaken by telephone on approximately Day 7, followed by randomisation and home visits on Day 7-9 and Weeks 6-8.

Samples will be collected at the hospital and by participants at their homes. Samples will be transported to King's College London for storage. Analytical procedures will be undertaken locally, outsourced to commercial partners, and in collaboration with researchers at the University of California, Davis. Relevant anonymised data will be released to our collaborators for statistical analysis as needed.

Number of participants

Our recruitment target is approximately 100 women to be enrolled pre-Caesarean section to give us up to 70 women who proceed to randomisation on Day 7-9 postnatal (30% drop out against Day 7-9 exclusion criteria) and 25 women per arm who will potentially breastfeed until 6 months and complete the study for an intention to treat analysis. If needed, depending on drop-out rates, we have capacity to recruit more.

As this will be the first trial to assess likelihood of persistent colonization after supplementation has stopped, detailed power calculations have not been performed. However, we know from preliminary analysis of the related USA based IMPRINT study, that very few infants (~n= 3) were needed to demonstrate significant colonization of *B. infantis* after supplementation.

In our study, we can anticipate that n=19 per group, for example, would provide 80% power to detect a significant difference in colonization if it were reduced to 1/3 of participants in the treatment arm at 6 months. Our recruitment target of 25 allows for 24% failure to provide an end of study sample.

These recruitment targets should provide adequate numbers to determine our primary endpoint as well as observational data to assess a range of interrelated questions about length of time of breastfeeding versus colonization, as well as providing useful information on tolerability and acceptability and ease of use of the supplement by a UK cohort.

Based on a conservative screening of all women at pre-assessment Caesarean section clinic (approximately 15 women) to achieve 6 enrolments per week resulting in 4 women per week passing the Day 7-9 screen, we could comfortably randomise 50 women in 12 months.

After receiving HRA and Ethics approval, recruiting for the PROMESA-IMMUNITY Sub-Study began in July 2018. Fewer women than expected have been recruited for this optional sub-study with only 5 subjects recruited in 3 months. In order to enrol a sufficient number of women into the sub-study to generate meaningful data for analysis, as well as ensure that we have approximately equal numbers of participants in the two treatment arms, we are amending the PROMESA protocol to make participation in the sub-study compulsory. Up to 35 women (or a recruitment period of approximately 9 months, whichever occurs first) may be randomised into the amended protocol for a total of approximately 40 participants in the sub-study (based on current enrolment). Consequently, up to 105 total women may be randomised (70 from original recruitment target plus 35 from amended compulsory sub-study enrollment).

Eligibility criteria

Healthy non-smoking pregnant women who plan to deliver by scheduled Caesarean section at Guy's and St Thomas' NHS Foundation Trust.

Inclusion Criteria at enrolment

- Singleton pregnancies (primip or multip)
- Age of subjects: pregnant women age 18 and above
- Elective Caesarean section ≥37 weeks' gestation
- Maternal pre-pregnancy body mass index (BMI) < 35 kg/m^2
- Resident in UK for 3 years or more
- Intention to exclusively breastfeed for at least 35 days, preferably for 6 months
- Non-smoker (gave up prior to pregnancy)

Exclusion criteria

At antenatal screening

- Multiple pregnancy
- Recent arrival in UK (< 3 years)
- Plan to leave UK in < 6 months
- Vaginal deliveries
- Mothers with another child < 14 months of age at recruitment
- Fetus has a known medical condition that would preclude breastfeeding or alter gut microbiota
- Maternal breast surgery or injury within the past 5 years that would reduce the likelihood of successful exclusive breastfeeding (not exclusionary if mother can evidence successful breastfeeding of a previous infant after the surgery or injury)
- Plan to administer non-study probiotics to infant any time throughout the study
- Plan to apply maternal vaginal swab to infant's mouth
- Maternal infection with HIV or Hepatitis C
- Maternal type 1 or type 2 diabetes (gestational diabetes is not exclusionary)
- Maternal pre-eclampsia
- Maternal medication use that may alter infant's gut microbiota (e.g. daily antibiotics)
- Smoking this pregnancy

At Day 7 postnatal screen, pre-randomisation

- Infants who have taken antibiotics for more than 3 days
- Intake of formula within 24 hours of the Day 7-9 visit
- Infants born with medical complications such as: respiratory distress syndrome, birth defects, and infection
- Mothers who experienced medical complications that would preclude them from breastfeeding
- Infants who had exposure to maternal vaginal microbiome via oral swab

Patient selection and enrolment

Eligible women will be recruited in person from the pre-assessment clinics for elective Caesarean section. The study will be explained to the women in detail and their commitment to breastfeeding determined. They will be given a consent form and patient information leaflet to read prior to the day of elective Caesarean in order to provide the prospective recruit sufficient time to review the consent form and discuss with friends/family/study team prior to providing consent. Trained study personnel will be following Good Clinical Practice (GCP) guidance for taking informed consent. Participants will be made aware that refusal to participate in this study will not affect the medical care that they or their newborn will receive.

Written informed consent can be given on the day or any time prior to transfer to the operating theatre for Caesarean section. Trained study personnel will obtain written documentation of the consent process according to GCP. Three copies of the consent form will be signed by both the subject and clinical coordinator, both parties will keep a copy and one copy will be retained in the hand-held notes. The clinical coordinator will store the signed consent form in a locked cabinet at the Dept. of Women and Children's Health, King's College London.

Following consent, prior to surgery, duplicate rectal and vaginal swabs will be taken for baseline maternal microbiome analysis. Maternal obstetric history (including parity and pre-pregnancy BMI for minimisation) and baseline demographic data will be collected and Subject ID created.

Dependent on when a woman is discharged, research staff will visit the women in hospital or provide telephone lactation support on postnatal Day 2-3 to ensure ongoing participation.

Randomisation

Women will be contacted by telephone (or in person if the mother is still in hospital) prior to the randomisation visit (Day 7-9 postnatal) to ensure that they fulfil the postnatal screening criteria. Women will then be randomised.

Recruitment and trial coordinators will not have access to the randomisation sequence. Randomisation will be carried out online via the MedSciNet web portal (www.medscinet.net) and linked to their initial Subject ID. Allocation will be stored remotely from the main study database, so that members of the study team remain blinded to probiotic/placebo allocation. Minimisation will be based on parity and maternal pre-pregnancy BMI >30.

If a woman does not meet the 'Day 7 postnatal screen, pre-randomisation' criteria, she will be withdrawn from the study and asked for permission to retain her data and samples for analysis. Other women wishing to be withdrawn at a later stage will similarly be asked for permission to retain data and samples. All randomised women will be analysed based on an intention to treat basis.

Treatment Allocations

Intervention

Infants of mothers randomised to *B. infantis* (strain: EVC001) will receive a daily dose of $8x10^9$ CFU added to breast milk for 28 days.

In this study, the *B. infantis* probiotic is being assessed and used as a dietary supplement for healthy infants, not as a drug or biological or used to prevent, treat, improve or manage a disease. The study is therefore designed as a non cTIMP study.

Placebo

Control infants randomised to the placebo arm will receive 625 mg lactose in breast milk daily for 28 days.

Both *B. infantis* and placebo will be supplied by Evolve BioSystems, Inc. prepackaged in blinded sachets.

Study Schedule

Gestation Or Days post-natal	37-42 wks	Day 0 (day of delivery)	Days 1-7	Days 7-28	Days 28-29	Days 29-35	Days 35-63	Days 63-182	Months 12, 18, 24
Eligibility	X								
Consent	X								
Enrolment data capture (demography, maternal health & pregnancy history)	X								
Maternal vaginal & rectal samples at Caesarean section		X							
Lactation support (hospital or home visit or telephone call)		X	X	X	X	X	X	X	
Delivery & lactation data capture			X						
Randomisation			X						
Supplementation training				X					
Daily supplementation and record (probiotic or placebo)				X	X	X			
Infant Weight & Height Measure		X		X				X	X

Baby's Day Diary (3 consecutive days)					X	X			
Antibiotic usage supplementation (if required)							X	X	
Infant stool/swab sampling and record			X	X	X	X	X	X	X
Stool/swab/milk sample pick up or return by post			X		X			X	X
Breast milk samples			X		X				
Infant Feeding Chart (daily)		X	X	X	X	X	X	X	
Infant Stooling & Health Log (daily)		X	X	X	X	X	X		
Infant Health Log (weekly)			X	X	X	X	X	X	
Research team contact & support	X	X	X	X	X	X	X	X	X
Follow up record								X	X

Unblinding study schedule

Study will be unblinded following completion of data collection and sample analysis for the primary 6 Month postnatal endpoints. Mid-trial unblinding would only occur if it was needed for an individual subject for medical reasons. This would be undertaken by the statistician or clinical member of the study team (if out of hours).

Study Timeline

At enrolment: after antenatal screening and discussion of the study, women will provide written informed consent. The participant will assist the research midwife to complete a clinical record form using the handheld maternal notes to collect study information regarding their demographics, pregnancy, obstetric and breastfeeding history. The participant will be provided with data capture forms (online/paper) regarding their diet, medical and medication history for completion when convenient.

On the day of Caesarean section (postnatal Day 0): Study midwives will collect duplicate vaginal swabs (not requiring a speculum) and duplicate rectal swabs from mothers.

Visit 1 (postnatal Day 1-4): In addition to standard postnatal care, the research midwife will be a point of contact for breastfeeding advice and support to promote and support successful establishment of breastfeeding. Women will have an 'in work hours' mobile number to call. The midwife will informally visit the women in hospital if needed coupled with one formalised point of contact usually between Day 2-4 which will be in person (home or hospital visit) or by telephone; progress with breastfeeding will be recorded on the study database. A Day 7-9 visit will be scheduled at this time.

Baseline Infant stool swabs and samples (postnatal Day 4-7): The mother will be trained on sample collection during enrolment and/or Visit 1. Mothers will collect the baseline stool swabs and samples after meconium has passed but before supplementation at Visit 2.

Breast milk samples (postnatal Day 4-7): The mother will collect a breast milk sample on the day of the Baseline infant stool samples, so that the samples may be transported by courier together.

Randomisation and Visit 2 (postnatal Day 7-9): Prior to the research midwife visiting on Day 7-9, the mother will be telephoned to confirm that her infant is eligible (see postnatal exclusion criteria above) to proceed to the randomisation part of the study. Once this is ascertained, allocation to probiotic supplement or placebo will be determined using internet based randomisation as described above. Mothers may be asked to retain nappies containing infant stool on the day of the randomisation visit so the research midwife can again demonstrate how to take subsequent infant stool swabs and samples. Midwives will weigh and measure the infants at this visit.

For the main supplementation component of the study, mothers will be given 30 probiotic (or placebo) sachets: 28 to feed to their infants and 2 extra doses to account for losses or damage. In addition, mothers will receive 21 sachets for "Supplementation During Antibiotic Usage" (described below), which contain the same treatment (*B. infantis* or placebo) that the infant was randomised to. Sachets will be stored in the home freezer/freezer compartment.

Infants in both groups will consume one sachet (*B. infantis* or placebo) starting on Day 7-9 postnatal for 28 days. Mothers will be trained by the research midwife on administering the supplement/placebo to their infants. Mothers will be instructed to mix the contents of each sachet with ~5 ml of breast milk into a provided bowl and feed the supplement to their infants using a provided syringe.

Antibiotic Usage Supplementation: If an infant requires oral antibiotics between Day 35-37 (end of supplementation) and 6 Months for any reason, the supplementation will be restarted using the 21 sachets designated as "Antibiotic Usage Supplementation". These sachets will be the same allocation (probiotic or placebo) assigned at randomisation. Supplementation will begin on the first day that the infant consumes the antibiotic and will continue once daily for a total of 21 days.

If antibiotics are started while the infant is still within the initial 28 supplementation period, the infant should continue with the initial 28 day supplementation and **should only receive 1 sachet daily**. Additional sachets from the "Antibiotic Usage Supplementation" should then be consumed daily from Day 35-37 until 21 days from the start of the antibiotic.

[Example: An infant is randomised on Day 9, receives 15 days of supplement, and starts an antibiotic on Day 23. The infant should continue with the normal supplementation until Day 36. On Day 37, the infant should start consuming the "Supplementation During Antibiotic Usage" sachets once daily until Day 43, which is the 21st day since the start of antibiotics.]

Ongoing Data and Sample collection: Between Days 7-9 and 6 Months, mothers in both groups will be contacted by text/email or phone (as preferred) for ongoing support in breastfeeding and data recording. This includes lactation support at home.

Daily and weekly infant feeding and infant and maternal health logs will be filled out by parents. Parents will be asked to monitor their infant's stool consistency using a validated infant stool scale (46). Mothers will be asked to collect infant stool swabs biweekly until 6 Months. Additional infant stool samples will be collected at 1 month, 3 months and 6 months.

Mothers will also provide one further breast milk sample during the 28 day supplementation period at 1 month postnatal.

Starting on any day between Day 28 and Day 32, mothers will keep a validated Baby's Day Diary for 3 consecutive days to track their baby's activities, including cry and fuss episodes (47).

Mothers will be asked to have their baby weighed and measured for weight and length by a health care practitioner or health visitor at a local baby clinic when their baby is approximately 2, 4, and 6 months old.

Child follow up: Mothers will be sent three child health data capture forms and asked to provide three further infant stool/swab samples at 12 months, 18 months and 24 months. Mothers will be asked to have their baby weighed and measured for weight and length at 12 months, 18 months, and 24 months.

Additional visits: the research midwife may make additional visits at any time during the first 6 months to provide support with breastfeeding, sample collection or data completion.

Compliance

Study personnel will monitor compliance by counting the number of empty sachets and reviewing daily supplementation charts at a home visit occurring when the infant is 6-8 weeks old.

Samples

Maternal samples will be collected in duplicate from vaginal and rectal swabs at the time of Caesarean section plus two breast milk samples as described above.

Infant Stool samples/swabs: Mothers will swab their infant's stool in duplicate from nappies at home using the infant sampling kit at baseline (any day between Day 4 and Visit 2 after meconium has passed, but prior to the first supplement being given), and every two weeks until around 6 months of age. Duplicate stool swabs will be collected on the last day of antibiotic usage if an infant requires antibiotics between Day 7 and 6 Months. Swabs will be stored in provided bags at room temperature and will either be collected from the home address by courier or posted in pre-paid packaging provided. At the time of the Baseline swab (Day 4 to 7), on Day 28 during the supplementation period, and when their child is 3, 6, 12, 18 and 24 months of age, mothers will also collect their infant stool using a collection utensil and place into sterile collection containers. Mothers will be instructed to store these samples inside two resealable bags. The double-bagged samples will be placed inside the subject's freezer until courier pickup. Mothers will be instructed to collect up to three stool samples into the same container within a 24 hour period when individual stool samples are smaller than 10 ml.

Samples collected during this study will be transported and stored as per commercial swab instruction or in a locked -80°C alarmed freezer (with remote 24 hour temperature monitoring) at King's College London. The samples will be stored until analysis and the study objectives have been met and data published. Unused samples will be retained for up to 10 years from the study start date and destroyed according to the Human Tissue Act. After study objectives have been successfully completed, banked samples and data from subjects may be used for additional research by the study team or shared with other academic researchers and commercial partners (with prospective informed written consent from recruits) for up to 10 years. This will be centred on further determining the relationship between probiotic intake and faecal metabolites, markers of inflammation, peptides, proteins, lipids, yeast and fungus; and breast milk metabolites, peptides, proteins, lipids, microbiota, yeast and fungus. Access to the samples will be governed by the study coordinator and CI, Dr. Rachel Tribe. All samples will be anonymised and barcoded with minimal written labelling (e.g. subject ID numbers, date/time of collection and sample type, etc.). Research personnel and collaborators listed in the study site file who will carry out the study objectives will be able to obtain anonymized data and specimens through approval by Dr. Tribe. Subjects may withdraw their samples from future analyses by submitting a request by email or orally via phone to the clinical coordinator. If a subject withdraws consent, no further information will be collected, but all information collected up until that point may still be used for research purpose.

Data Collected

All data collected with be entered onto a study specific, secure, password protected, internet based database. All individuals who are enrolled will be allocated a Subject ID prior to randomisation. In addition to basic demographic and maternal health and current and previous pregnancy details, all mothers will fill out logs from birth until 6 months postnatal regarding their infant's intake of: breast milk, infant formula, non-study probiotics, and medications (including antibiotics). The participant will assist the research midwife to complete clinical record forms regarding their pregnancy, obstetric history, and breastfeeding history. The participant will be provided with data capture forms (online/paper) regarding their diet, medical and medication history for completion when convenient.

Relevant data (e.g. infant feeding, breast fullness) will be collected by the research midwife during lactation support visits. All mothers will be assisted by the research team to complete infant feeding charts and infant and maternal health logs. Mothers will also receive phone calls, texts or emails (whichever they prefer) before sample pick-ups as reminders to collect the stool samples and fill in the logs.

Infant weight and height/length will be collected throughout the study at the following time points:

- **Birth**: as documented in hospital records/handheld notes
- **Day 7-9**: measured by midwife at home visit
- **2 months (Week 10)**: measured at local baby clinic and/or by health visitor or study staff
- **4 months (Week 18)**: measured at local baby clinic and/or by health visitor or study staff
- **6 months (Week 26)**: measured at local baby clinic and/or by health visitor or study staff
- **12 months**: measured at local baby clinic and/or by health visitor or study staff
- **18 months**: measured at local baby clinic and/or by health visitor or study staff
- **24 months**: measured at local baby clinic and/or by health visitor or study staff

The following data capture forms / health logs will be used in the study to report maternal and infant health, diet and any adverse events in response to the supplementation:

- Screening (demographics) Log, Once: Enrolment
- Maternal Health & Pregnancy History, Once: Enrolment
- Onset of Lactogenesis Survey, Daily: Day 3 to Day 7
- Delivery & Infant Information, Once: Randomisation
- Daily Infant Feeding Chart, Daily: Birth to 6 Months
- Daily Supplementation Chart, Daily: Day 7-9 to Day 34-36
- Daily Infant Stooling & Health Log, Daily: Birth to Day 62
- Weekly Infant Health Log, Weekly: Birth to 6 Months
- Sample Collection Chart, as collected: Birth to 6 Months
- Baby's Day Diary, 3 consecutive days: starting between Day 28 and Day 32
- Antibiotic Usage Supplementation Chart, if required: starting on first day of antibiotics usage between Day 15 and 6 Months for 21 days
- Follow up Record, every 6 months up to 2 years of life

Eligibility screening will be done using online electronic forms and recorded in the hand-held patient notes. The signed consent forms and contacts forms with personal information will be stored in a locked filing cabinet separate from all other study files at King's College London, Dept. of Women and Children's Health. Electronic link-anonymised (by Subject ID) information on a secure internet database associated will be password encrypted and only accessible to the study team who will have role specific access. Any paper-based data capture forms and health logs will be stored securely and separately from consent forms in the King's College London, Dept. of Women and Children's Health, in order to ensure documents are protected from loss or adulteration. Only the study team, sponsor, and relevant regulatory bodies will have access to the files with any identifiable private information of the human subjects. Files kept on the computer that contain identifiable information will be encrypted to ensure access only by study personnel.

Data entered electronically or collected from health logs and trackers and sample labels filled out by participants will be checked for completeness by study personnel. Any paper-based data will be transcribed onto the study database and all data will be monitored regularly by the Sponsor of the study. Queries will be raised and data cannot be 'locked' for analysis until queries answered. Some of the data capture may be completed electronically using an online survey tool (some women express a preference for electronic form filling whilst others do not). They will replicate the paper versions provided as attachments to this protocol.

Analyses

Maternal vaginal and rectal samples, breast milk samples, and infant stool samples will be transported and stored in HTA compliant -80° freezers managed by the Dept. of Women and Children's Health, King's College London.

The primary endpoint sample analysis by will be coordinated by the Industry lead at Evolve BioSystems, Inc. in the USA to determine the shift in subjects' gut bacteria in response to supplementation. Stool samples will be analyzed and compared across time points and between

supplement and control groups. DNA will be extracted using developed protocols that ensure Gram-positive bacteria will be represented. To determine a shift in gut microbiota from faecal samples, DNA samples will prepared and used for shotgun sequencing or next generation sequencing (NGS, e.g. Illumina MISeq or HiSeq) (blinded analysis will be outsourced). DNA from select samples may be amplified by PCR and compared to a standard curve with known quantities of *B. infantis* and *Bifidobacterium* DNA, respectively. Breast milk analysis and other biochemical analyses on stool samples including RNA analyses will be performed by Evolve or undertaken in collaboration with researchers at the University of California Davis.

Results will be combined with anonymized metadata and health log information downloaded from the online database and analysed using statistical software such as SPSS or STATA. A detailed analysis plan and publication schedule will be drawn up by the study team and statistician.

To determine if the probiotic is well-tolerated, parents will be instructed to report infant gastrointestinal symptoms, during supplementation and up to a month post-supplementation. We do not anticipate any unexpected intolerability issues due to the consumption of *B. infantis* because this bacterial strain is a dominant strain in the gut of exclusively breastfed infants. The use of this strain and other *Bifidobacterium* strains as probiotics at similar dosages was well-tolerated in premature and term infants (12, 45). Infant health data from the weekly logs will be routinely reviewed by the study coordinator and investigators. Additionally, subjects will be instructed to immediately contact the study coordinator if at any time their infant displays unusual or extreme discomfort.

PROMESA-IMMUNITY Sub-study

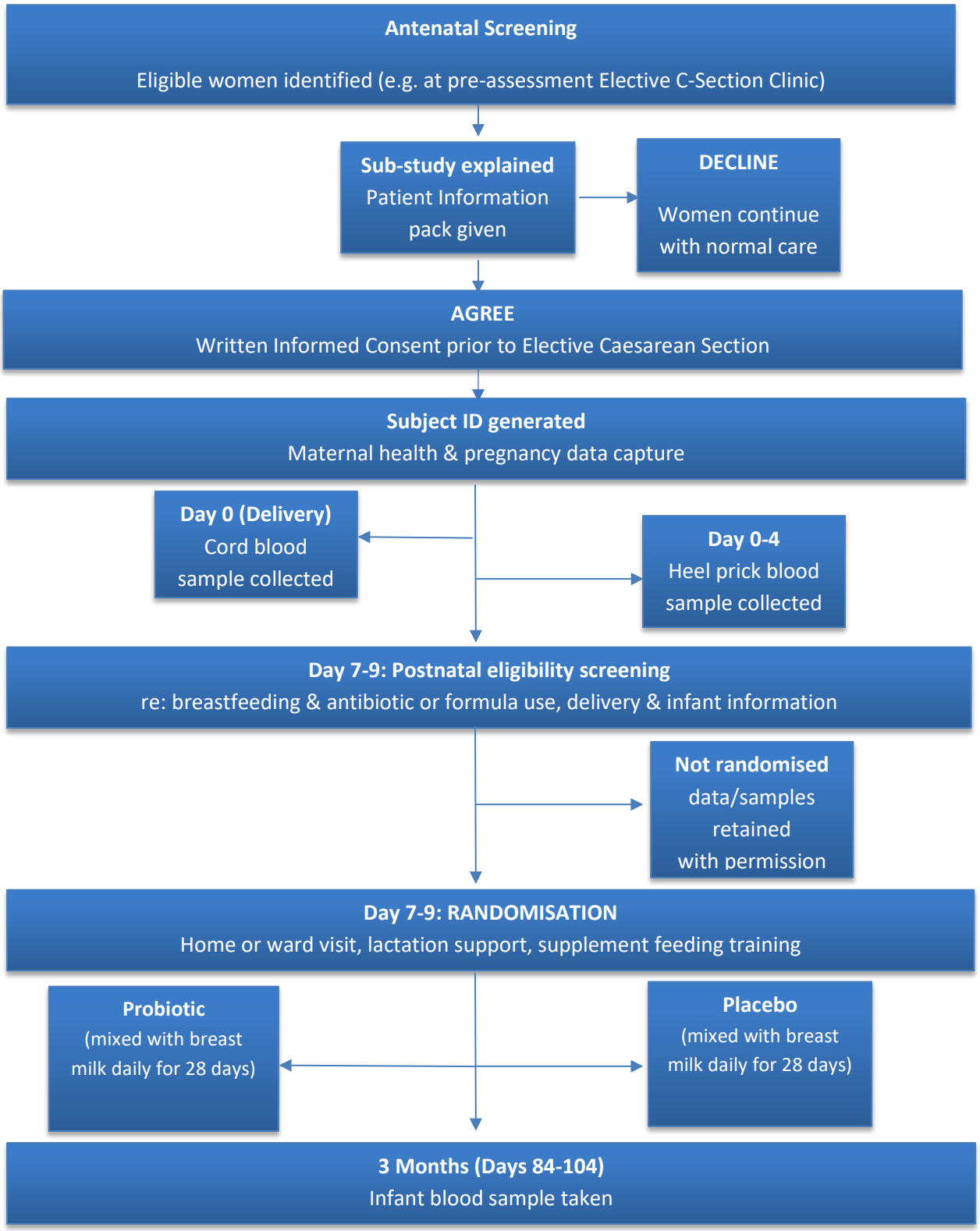
Sub-study summary

This sub-study will examine the development and function of the immune system of a subset of participants from the main PROMESA study.

Infants delivered by Caesarean section have been found to have reduced immune function compared to those delivered vaginally. The hygiene hypothesis highlights the link between the gut microbiota and the development of the immune system, which could provide an explanation for this reduced immune function due to the impaired colonisation of the infant gut associated with Caesarean section. Gut dysbiosis can affect the development of the immune system, increasing the risk of disease, the effects of which can extend into adulthood. Increasing the levels of the protective bacteria *Bifidobacterium subsp. infantis* (*B. infantis)* in the neonatal gut have been highlighted as a potential treatment method to improve priming of the immune system.

Our principal research question is whether promoting a healthy gut by supplementing breastfed infants delivered by Caesarean section with *B. infantis* for 28 days, will improve the development of the infant immune system. Approximately 40 mother infant dyads will be recruited to provide a cord blood sample and two infant blood samples, one on day 0-4 and another at 3 months old (between 84 and 104). Markers of immune function will be analysed and compared to the development of the gut microbiome.

PROMESA-IMMUNITY Sub-study Flow Chart



Background and Rationale

Infants born by Caesarean section have increased risk of developing many immune mediated conditions such as asthma, eczema and food allergies, type 1 diabetes mellitus and coeliac disease, compared to those born vaginally (3, 5, 48, 49). This is thought to be influenced, in part, by the impaired colonization of the infant gut due to lack of contact with maternal microbiota during birth, as the gut microbiota has been shown to play a role in developing the immune system, and is particularly important in early life (14, 50, 51).

Delivery by prelabour elective Caesarean section is associated with significantly lower levels of leukocytes and their precursors in cord blood compared to vaginal delivery (52). Furthermore, lower levels of various cytokines and their receptors (IL-6, IL-1h, sIL2R, sIL-4R, IFN-g, TNF-a and sTNF RI) which play an important role in priming the neonatal immune system, have been observed after elective Caesarean delivery (53). However, it is not known the extent to which lack of labour in elective Caesarean sections plays a role in the development of the immune system.

Modifying the composition of the gut microbiota by means of probiotics have previously been used in the treatment of allergic disease, although the exact mechanism by which probiotics improve allergic outcome remains to be elucidated (54, 55, 56). In particular, having adequate levels of *B. infantis* has been linked to improved immune function, and infants born by Caesarean section have been found to have a particularly low abundance of this protective bacteria (57). Supplementation of *B. infantis* has been successfully used to attenuate inflammation associated with necrotising enterocolitis (58). Similarly, feeding *B. infantis* reduces chemokine secretion in response to Salmonella infection (31). This link between the gut microbiota and the function of the immune system (59), provides a rationale that modifying the composition of the gut microbiota could improve the development of the immune system.

Hypothesis

The promotion of a healthy gut via daily *B. infantis* supplementation will improve the development and function of the immune system in term breastfed infants born via Caesarean section.

Aims and Objectives

The aim of this sub-study is to examine the development and function of the infant immune system and its relationship with the gut microbiome.

Objective 1: To evaluate changes in immune function from baseline to three months

Objective 2: To evaluate the difference in immune function between infants supplemented with *B. infantis* or the placebo

Objective 3: To compare immune function with gut microbiota composition

Sub-study Endpoints

Primary Endpoint:

- The change in levels of immune cells and markers from baseline (Day 0 – Day 4) to three months (Day 84 - Day 104)

Secondary Endpoints:

- The correlation between gut microbiota composition and abundance and levels of immune cells and markers
- The differences between *B. infantis* and placebo supplementation on levels of immune cells and markers
- The differences between *B. infantis* and placebo supplementation on vaccine response (antibody titres)

Sub-study Design

This is a sub-study carried out as part of the PROMESA trial, examining the development of the infant immune system and its relationship with the gut microbiota. To develop a baseline of immune function, a cord blood sample will be obtained after delivery. Following this, a heel prick will be carried out to collect an infant blood sample in the first few days after birth. Between days 84 and 104 an infant blood draw or heel prick will be performed.

Sub-study Population and Setting

From the time of amendment approval for this Protocol Version 4.0 [dated 15JAN2019], all women in the PROMESA trial will be enrolled. If women do not consent to providing the required samples for the sub-study, they will not be eligible for participation in PROMESA. Samples will be collected at Guy's and St Thomas' NHS Foundation Trust or at the participant's home.

Changes to PROMESA Study Schedule, Sample Collection, and Data Collection

From the time of amendment approval for this Protocol Version 4.0 [dated 15JAN2019], the following changes to the main protocol will be made for newly enrolled participants (anticipated 35 compulsory sub-study participants):

- Maternal samples (vaginal swabs, rectal swabs, and breast milk) will not be collected
- Antibiotic Usage Supplementation will not be provided for infants requiring antibiotics
- Infant weight and length measurements no longer required at 2, 4, and 6 months

The following modifications were made to data collection forms:

- Daily Infant Stooling Log will no longer be completed
- Weekly Infant Health Log redesigned and to be used from birth through 6 months
- Daily Infant Feeding Chart redesigned and to be completed weekly
- Onset of Lactogenesis Survey will no longer be completed
- Sample Collection Chart redesigned as a guide and no longer returned to study team
- Antibiotic Usage Supplementation Chart will no longer be completed

Number of Participants

Our total sub-study recruitment target is 25 complete sets of samples and data. A higher number (40 randomised) may need to be recruited to ensure this is achieved.

Eligibility criteria

All women enrolled in PROMESA are eligible to take part in the sub-study.

Patient selection and enrolment

Eligible women who have agreed to take part in PROMESA will be recruited from the antenatal pre-assessment clinic, or other antenatal settings such as External Cephalic Version (ECV) clinic, Vaginal Birth After Caesaeran (VBAC) clinic, tocophobia clinics, or private practice offices.

They will be given a consent form and patient information leaflet to read prior to the day of elective Caesarean in order to provide the prospective recruit sufficient time to review the consent form and discuss with friends/family/study team prior to providing consent. Trained study personnel will be following Good Clinical Practice (GCP) guidance for taking informed consent. Participants will be made aware that refusal to participate in the sub-study will not affect their participation in the main study or the medical care that they or their newborn will receive.

Written informed consent can be given on the day or any time prior to transfer to the operating theatre for Caesarean section. Trained study personnel will obtain written documentation of the consent process according to GCP. Three copies of the consent form will be signed by both the subject and clinical coordinator, both parties will keep a copy and one copy will be retained in the hand-held notes. The clinical coordinator will store the signed consent form in a locked cabinet at the Dept. of Women and Children's Health, King's College London.

Following consent, after surgery, a cord blood sample will be taken. During an early postnatal visit, a midwife will collect an infant heel prick blood sample for baseline immune function analysis.

An infant venous blood draw or heel prick will be carried out at month 3, between days 84 and 104.

Sub-study schedule

Below is the schedule for additional study activities as part of the PROMESA sub-study:

Gestation or days post-natal	37-42 weeks	Day 0 (day of delivery)	Days 0-4	Days 84-104
Sub-study consent form signed	X			
Cord blood sample collected		X		
Heel prick blood sample collected			X	
Site visit for infant blood/heel prick sample collection				X

Sub-study timeline

At enrolment: after antenatal screening and discussion of the sub-study, women will provide written informed consent. The informed consent for the sub-study will be a separate consent form from the main PROMESA study.

On the day of Caesarean section (postnatal Day 0): Study midwives will collect a blood sample from the umbilical artery (and vein) after delivery.

Visit 1 (postnatal Day 0-4): The research midwife will collect an infant blood sample into heparinised tubes using a capillary system by standard heel prick.

At month three (Days 84-104): An infant venous blood draw (or heel prick) will be performed. Blood draws will be performed by a paediatric phlebotomist or trained research midwife; study research midwives can perform the heel prick. The blood draw may require a site visit for the sub-study, where the infant is brought to Guy's and St Thomas' NHS Foundation Trust, or may be performed at the participant's home by the trained research midwife. The heel prick can be undertaken at the hospital or during a visit to the participant's home.

Samples

Cord blood samples will be collected after delivery.

Infant blood samples will be collected during the first postnatal visit during Days 0-4 by heel prick. A second blood draw or heel prick will also be collected at 3 months of age. A maximum of 2 mL of blood will be collected at each time point.

Blood samples collected during this sub-study will be taken to King's College London (post anonymisation) where they will be processed prior to freezing and storage in a temperature

monitored liquid nitrogen tank. The samples will be stored until analysis and the study objectives have been met and data published. Unused samples will be retained for up to 10 years from the study start date and destroyed according to the Human Tissue Act. After study objectives have been successfully completed, banked samples and data from subjects may be used for additional research by the study team or shared with other academic researchers and commercial partners (with prospective informed written consent from recruits) for up to 10 years. Analysis will be focused on examining the relationship between the gut microbiome and development of the infant immune system.

Access to the samples will be governed by the study coordinator and CI, Dr. Rachel Tribe**.** All samples will be anonymised and barcoded with minimal written labelling (e.g. subject ID numbers, date/time of collection and sample type, etc.). Research personnel and collaborators listed in the study site file who will carry out the study objectives will be able to obtain anonymized data and specimens through approval by Dr. Tribe. If a subject withdraws consent, no further information will be collected, but all information collected up until that point may still be used for research purpose. Subjects may withdraw their samples from future analyses by submitting a request by email or orally via phone to the clinical coordinator.

Data collected

The data collected as part of PROMESA will be used in the analysis of these samples. Some data collection forms have been modified for the PROMESA-IMMUNITY sub-study to reduce patient burden.

Analyses

Blood samples will be stored in HTA compliant liquid nitrogen tanks at King's College London.

Immunophenotyping of the cell subset composition and function in the different blood samples will be carried out in King's College London. If enough material is available from the blood samples, transcriptomics may be performed.

Diluted plasma isolated from blood samples will be frozen and shipped to Evolve BioSystems, Inc. for analysis of vaccine response (antibody titres) and other immune and inflammatory markers.

Results will be combined with anonymized metadata and health log information downloaded from the online database and analysed using statistical software such as SPSS or STATA. A detailed analysis plan and publication schedule will be drawn up by the study team and statistician. Relevant anonymised data will be released to our collaborators for statistical analysis as needed.

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SAFETY REPORTING (Research other than CTIMPs)

In other research other than CTIMPs, a *serious adverse event* (SAE) is defined as an untoward occurrence that:

(a) results in death;
(b) is life-threatening;
(c) requires hospitalisation or prolongation of existing hospitalisation;
(d) results in persistent or significant disability or incapacity;
(e) consists of a congenital anomaly or birth defect; or
(f) is otherwise considered medically significant by the investigator.

An SAE occurring to a research participant should be reported to the main REC where in the opinion of the Chief Investigator the event was:

- *Related* – that is, it resulted from administration of any of the research procedures, and
- *Unexpected* – that is, the type of event is not listed in the protocol as an expected occurrence.

	Who	What and when	How	Action by REC
SAE	Chief Investigator (CI) or sponsor	Any SAE that is related and unexpected. Within 15 days of the CI becoming aware of the event.	SAE report form for non-CTIMPs, available from NRES website. To main REC only.	Acknowledge by signing and returning copy of form. Review at REC or sub-committee meeting. Write to sponsor and CI following review if appropriate.
Urgent safety measures	Chief Investigator or sponsor	(i) Immediate notification. (ii) Within 3 days, setting out in full the reasons for the urgent safety measures and the plan for further action.	(i) By telephone. (ii) Notice in writing. To main REC only.	Review at REC or sub-committee meeting. Write to sponsor and CI following review.

PROGRESS REPORTING

Type	Who	When	How	Action by REC
Progress reports	May be submitted by sponsor, sponsor's legal representative or Chief Investigator (CI). Must always be signed by CI.	Annually (starting 12 months after the date of the favourable opinion) *Main REC may exceptionally request more frequent reports.* Co-ordinator of main REC to send reminder using SL38 if not received.	Annual progress report form on NRES website. Separate forms to be used for CTIMPs and non-CTIMPs.	Acknowledge using SL37. Review by Chair and/or any member of the REC. Notify REC in Co-ordinators' report.
Declaration of the conclusion or early termination of the research (CTIMPs)	Sponsor, sponsor's legal representative or CI	Within 90 days (conclusion). Within 15 days (early termination). *The end of the trial should be defined in the protocol.*	"Declaration of the end of a clinical trial" form prescribed by the European Commission (Annex 3 to ENTR/CT1), available from EudraCT website.	Acknowledge using SL39. Review by Chair and/or any member of the REC or Scientific Officer. Notify REC in Co-ordinators' report.
Declaration of the conclusion or early termination of the research (non-CTIMPs)	May be submitted by sponsor or CI. Must always be signed by CI.	As for CTIMPs.	End of study declaration form (non-CTIMPs) on NRES website	As for CTIMPs.
Summary of final report	Sponsor, sponsor's legal representative or CI	Within one year of the conclusion of the research. Co-ordinator of main REC to send reminder using SL41 if not received.	No standard format. The summary should include information on whether the study achieved its objectives, the main findings and arrangements for publication or dissemination including feedback to participants.	Acknowledge using SL40. Review by Chair and/or any member of the REC or Scientific Officer. Notify REC in Co-ordinators' report.

Version 2.0 dated April 2007

Detecting SAEs

All SAEs must be recorded from the time a participant is randomised until 6 Months of the infant's life. The investigator should ask about SAEs at each contact point during the study. Open-ended non-leading verbal questioning of the participant should be used to enquire about SAE occurrence. Participants should also be asked if they or their baby have been admitted to hospital, had any accidents, used any new medicines or changed concomitant medication regimes. If there is any doubt as to whether a clinical observation is an AE, the event should be recorded. Information to be collected includes type of type of event, onset date, investigator assessment of severity and causality, date of resolution in addition to treatment required, investigations needed and outcome. All adverse medical events reported by the participant should be documented in the handheld maternity records or the child health record (red book).

A clinician will assess **ALL** reported SAEs. Some adverse events are expected and will not therefore be reported as SAEs, but will be recorded on the database and presented to the data monitoring committee, as part of the ongoing safety review.

For this study the following events will **NOT** be considered SAEs:

- Minor infant gastrointestinal disturbances (including stooling frequency), in the absence of systemic involvement
- Gastro-oesophageal reflux disease of infancy
- Minor respiratory symptoms and illnesses that do not require a visit to Accident and Emergency (including sneezing, running nose and self-limiting viral illnesses effecting breathing)
- Jaundice, unless requires admission overnight
- Tongue tie / difficulty latching / low breast milk production and resulting dehydration
- Immunisation side effects
- Accidental injury; pulled elbow, head bruises, rolling off changer
- Febrile convulsions
- Abnormalities including hip dysplasia, cryptorchidism, absent red retinal reflexes, undescended testis, umbilical hernia or granuloma, and hernia investigations or procedures
- Rashes of infancy (including erythema toxicum, milla and eczema) unless requiring admission overnight
- Infant hospital admission on behalf of health of the mother
- Caesarean section and post-partum complications for the mother
- Any aspect of maternal physical or mental health unrelated to study participation

Evaluation of SAEs

Seriousness, causality, severity and expectedness should be evaluated. The clinician involved should make an assessment of seriousness according to the criteria:

A serious adverse event is any adverse event that:

- Results in death

- Is life threatening
- Requires hospitalization or prolongation of hospital stay
- Results in persistent or significant disability or incapacity
- Is a congenital anomaly or birth defect.

Assessment of Causality

The clinician must make an assessment as to whether the SAE is related to the study according to the following definitions:

1) As this is a non cTIMP in this study, all SAEs will be judged as having a reasonable suspected causal relationship (e.g. possibly, probably, definitely) to the study intervention, and adverse reactions/serious adverse reactions (AR/SAR) will not apply.
2) Similarly there will be no assessment as to whether the SAE is likely to be caused by an interaction between study drugs and rescue/escape drugs.
3) **Unrelated**: where an event is not considered to be related to the intervention.
4) **Possibly**: although a relationship to the intervention cannot be completely ruled out, the nature of the event, the underlying disease, concomitant treatment or temporal relationship make other explanations possible.
5) **Probably**: the temporal relationship and absence of a more likely explanation suggest the event could be related to the intervention.
6) **Definitely:** the known effects of the study intervention or it's consequence, suggest that the intervention should be considered and investigated.

Assessment of Severity

The clinician should make an assessment of severity for each SAE and record this according to one of the following categories:

Mild: an event that is easily tolerated by the participant, causing minimal discomfort and not interfering with every day activities.
Moderate: an event that is sufficiently discomforting interfere with normal every day activities.
Severe: an event that prevents normal every day activities.

Note: the term 'severe', used to describe the intensity, should not be confused with 'serious' which is a regulatory definition based on participant/event outcome or action criteria. For example, a headache may be severe but not serious, while a minor stroke is serious but not severe.

Assessment of Expectedness

This relates to adverse reactions and serious adverse reactions and therefore is not required in this non cTIMP trial.

Reporting SAEs

Once the study investigator becomes aware that an SAE has occurred in a study participant, they must report the information to the Trial Co-ordinating Centre within 24 hours.

The SAE form must be completed as thoroughly as possible with all available details of the event, signed by the investigator or designee. If the investigator does not have all the information regarding the SAE, they should not wait for this additional information before completing the SAE form. The form can be updated when subsequent information becomes available.

The SAE report must provide an assessment of causality and expectedness at the time of the initial report to the Trial Co-ordinating Centre.

The SAE form should be transmitted to the Sponsor and the site R&D Office.

Follow up procedures

After initially reporting an AF or recording and reporting an SAE, the investigator is required to follow each participant until resolution. Follow up information on an SAE should be reported to the trial Co-ordinating Centre.

AEs/SAEs still present in participants at the last study visit should be monitored until resolution of the event or until no longer medically indicated.

Out of Hours Cover

The Trial Management Team, Trial Steering Committee, Trials managers and Co-sponsors do not provide out of hours advice for study participants. Dr Tom Marrs will be the study paediatrician who will be available for consultation by the study staff as required.

Trial Management and Oversight Arrangements

Trial Management Group (TMG)
The trial will be co-ordinated by the TMG comprising the chief investigator, study staff, and co-applicants. The Trial Manager will oversee the study and be responsible to the Chief Investigator. The checking of the database for completeness, plausibility and consistency will be the responsibility of the trial manager and designated members of the midwifery team. Queries will be resolved by the study midwife and/or the Principal Investigator. A delegation list will be prepared, detailing the responsibilities of each member of staff working on the trial.

Division of Responsibilities of the Trial Management Group (TMG)

- Chief Investigator, Tribe: overall responsibility for the design, conduct, analyses and reporting of the trial; assisted by the TMG

- Seed for statistical support.

Central Trial Office
The PI and trial manager will co-ordinate the study based in KCL Dept. of Women and Children's Health, St Thomas' Hospital, London.

Trial Steering Committee (TSC)
A trial steering committee will be established to oversee the conduct and progress of the trial. The terms of reference of the Trial Steering Committee will be developed separately.

Data Monitoring Committee (DMC)
An independent data monitoring committee (DMC) is not required to oversee the safety of subjects in the trial. This is not a cTIMP therefore the TSC will take overall responsibility for the conduct of the trial.

Inspection of Records
Investigators and institutions involved in the study will permit trial related monitoring, audits, REC review and regulatory inspection(s). In the event of an audit the investigator agrees to allow the Sponsor, representatives of the Sponsor or regulatory authorities direct access to all study records and source documentation.

Study Monitoring
The trial will be monitored by the Sponsor or monitors hired by the Sponsor. A study start-up visit will be completed.

Good Clinical Practice
The trial will be conducted in accordance with the principles of Good Clinical Practice (GCP). A favourable ethical opinion will be sought form the appropriate REC and local R and D approvals obtained prior to commencement of the study.

Investigator Responsibilities
The investigator is responsible for the overall conduct of the study at the site and compliance with the protocol and any protocol amendments. In accordance with the principles of GCP, the following areas listed in this section are also the responsibility of the investigator. Responsibilities may be delegated to an appropriate member of the study staff. Delegated tasks must be documented on a Delegation Log and signed by all those named on the list.

Informed Consent
The investigator is responsible for ensuring informed consent is obtained before any protocol specific procedures are carried out. The decision of a participant to take part in clinical research is voluntary and should be based on a clear understanding of what is involved.

Participants must receive adequate oral and written information- appropriate Patient Information and Informed Consent Forms will be provided. The oral explanation to the participant should be performed by the Investigator or designated person, and must cover all the elements specified in the Participant Information Sheet/Informed Consent Form.

The participant must be given every opportunity to clarify points they do not understand and it necessary, ask for more information. The participant must be given sufficient time to consider the information provided. It should be emphasised that the participant may withdraw their consent to participate at any time without loss of benefits to which they otherwise would be entitled.

The participant should be informed and agree to their medical records being inspected by regulatory authorities but understand that their name will not be disclosed outside the hospital.

The Investigator or delegated member of the trial team and the participant should sign and date the Informed Consent Form(s) to confirm that consent has been obtained. The participant should receive a copy of this document and a copy filed in the Investigator Site File.

Study Site Staff
The investigator must be familiar with the protocol and the study requirements. It is the investigator's responsibility to ensure that all staff assisting with study are adequately informed about the protocol and their trial related duties.

Data Recording
The chief investigator is responsible for the quality of data recorded in the database.

Investigator Documentation
Prior to beginning the study, each investigator will be asking to provide particular essential documents to the main trial office, including but not limited to:

- Curriculum vitae (CV) signed and dated by the investigator indicating that it is accurate and current.
- A valid GCP certificate.

The main trial office will ensure all other documents required by GCP are retained in a Trial Master File (TMF) and that appropriate documentation is available in local ISFs.

GCP Training
All UK based study staff must hold evidence of appropriate GCP training or undergo GCP training. The co-sponsors require that GCP is updated every two years throughout the trial.

Confidentiality
All laboratory specimens, evaluation forms, reports, and other records must be identified in a manner designed to maintain participant confidentiality. All records must be kept in a secure storage area with limited access. Clinical information will not be released without the written permission of the participant, except as necessary for monitoring and auditing by the Sponsor, its designee, Regularity Authorities, or the REC. The Investigator and the study site staff involved with this study may not disclose or use for any purpose other than performance of the study, any data, record, or other unpublished, confidential information disclosed to those individuals for the purpose of the study. Prior written agreement from the Sponsor or its designee must be obtained for the disclosure of any said confidential information to other parties.

Data Protection

All investigators and study site staff involved with this study must comply with the requirements of the Data Protection Act 1998 with regard to the collection, storage, processing and disclosure of personal information and will uphold the Act's core principles. Computers used to collate the data will have limited access measures via user names and passwords. Published results will not contain any personal data that could allow identification of individual participants.

Study Conduct Responsibilities

Protocol Amendments

Any changes in research activity, except those necessary to remove an apparent, immediate hazard to the participant, must be reviewed and approved by the Chief Investigator. Amendments to the protocol must be submitted in writing to the appropriate REC, Regulatory Authority and local R&D for approval prior to participants being enrolled into an amended protocol.

Protocol Violations and Deviations

Investigators should not implement any deviation from the protocol without agreement from the Chief Investigator and appropriate REC, Regulatory Authority and R&D approval except where necessary to eliminate an immediate hazard to trial participants. In the event that an investigator needs to deviate from the protocol, the nature of and reasons for the deviation should be recorded in the eCRF. If this necessitates a subsequent protocol amendment, this should be submitted to the REC, Regulatory Authority and R&D for review and approval if appropriate.

Study Record Retention

This is a study involving pregnant women and research records should be retained according to NHS Guidelines for the retention of documentation involving pregnant women. All medical records will be retained for at least 25 years after publication of the final study report. Guidelines on retention of other research related documents are continually under review. We plan to retain all documents for 5 years and then will review according to current guidance at the time.

Serious Breach Requirements

A serious breach is a breach which is likely to effect to a significant degree:

a) The safety or physical or mental integrity of the subjects of the trial (this should be relevant to trial subjects in the UK); or
b) The scientific value of the trial.

If a potential serious breach is identified by the Chief Investigator, Principal Investigator(s) or delegates, the Co-sponsors must be notified within 24 hours. It is the responsibility of the Co-sponsor to assess the impact of the breach on the scientific value of the trial, to determine whether the

incident constitutes a serious breach and take the appropriate action. Not every deviation from the protocol needs to be reported to the regulatory authority as a serious breach, if the Co-sponsors deem the incident to be a minor deviation from the protocol when identified, corrective and preventative actions will be taken where appropriate and they will be recorded in file notes, held within TMF or ISF.

End of Study

The end of the study declaration will be submitted to the relevant authorities after the last baby is born and discharged from the hospital or the end of the postnatal period (28 days after the birth), whichever is sooner. The end of the study will be reported to the REC and Regulatory Authority within 90 days, or 15 days if the study is terminated prematurely. The Investigators will inform participants and ensure that the appropriate follow up is arranged for all involved.

A summary report of the study will be provided to the REC and Regulatory Authority within 1 year of the end of the study.

Insurance and Indemnity

The Co-sponsors are responsible for ensuring proper provision has been made for insurance or indemnity to cover their liability of the Chief Investigator and staff.

The following arrangements are in place to fulfil the Co-sponsors' responsibilities:

- The protocol has been designed by the Chief Investigator and researchers employed by the University and collaborators. The University has insurance in place (which includes no-fault compensation) for negligent harm caused by poor protocol designed by the Chief Investigator and researchers employed by the University.
- Sites participating in the study will be liable for clinical negligence and other negligent harm to individuals taking part in the study and covered by the duty of care owed to them by the Sites concerned. The Co-sponsors require individual sites participating in the study to arrange for their own insurance or indemnity in respect of these liabilities.
- Sites which are part of the United Kingdom's Nation Health Service will have the benefits of NHS Indemnity.
- Sites out of the United Kingdom will be responsible for arranging their own indemnity or insurance for their participation in the study, as well as for compliance with local law applicable to their participation in the study.

Reporting, Publications and Notification of Results

Authorship Policy

Ownership of the data arising from this is set out in the collaborators' agreement and an authorship policy will be developed. On completion of the study, the study data will be analysed and tabulated, and a clinical study report will be prepared in accordance with GCP guidelines.

Publications

The clinical study report will be used for publication and presentation at scientific meetings. The results of the study and any protocol deviations will be published in writing by the team headed by the Chief Investigator, which will report to the Trial Management Committee. Individual investigators may be able to produce oral reports with the permission of the Trial Management Committee and the Sponsor.

Summaries of results will also be made available for dissemination to participants and the public, where appropriate.

Trial Steering Committee

CHAIR: TBA	**MEMBERS:**