eProtocol. Interactions between the diet and gut microbes, metabolism and body composition in preterm infants (INDIGO)

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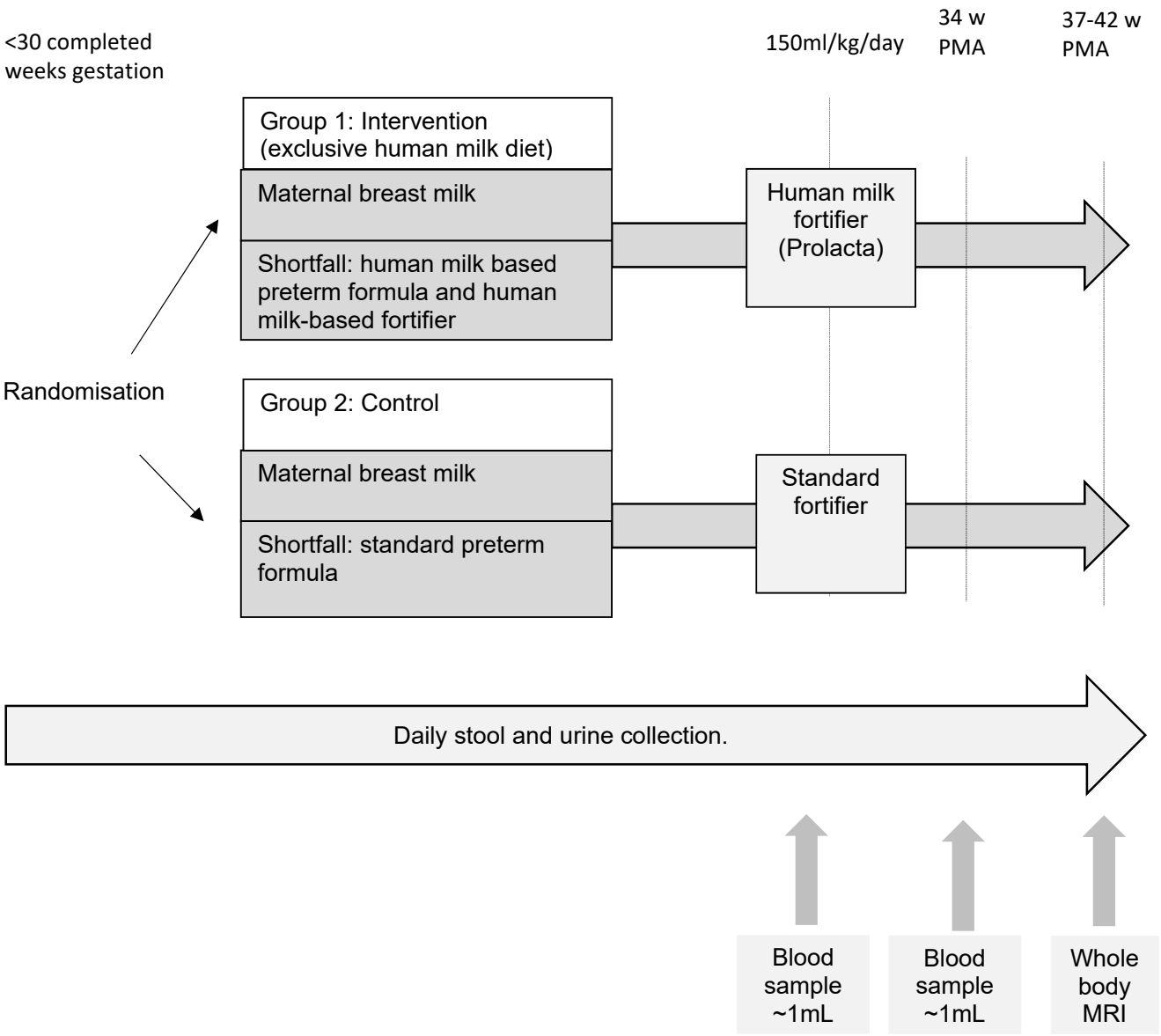
251 # 1. PROTOCOL SUMMARY

Title (Acronym)	**Interactions between the diet and gut microbes, metabolism and body composition in preterm infants (INDIGO)**
Study centres	Newcastle Royal Infirmary and Chelsea and Westminster NHS Foundation Trust (Imperial College London). Additional sites may be added
Study objectives	To evaluate whether an exclusive human milk diet compared with exposure to cow's milk-based products results in differences in a) gut bacteria (types and diversity, b) stool and urine metabolites and c) body composition
Study design	Randomised open label, controlled trial
Study population	**Inclusion criteria** • Preterm infants born below 30 completed weeks of gestation (≤ 29 weeks and 6 days) • <72 hours age • Written informed consent from parents **Exclusion criteria** • Major congenital or life-threatening abnormalities • Inability to randomise within 48 hours of birth • Exposure to bovine milk product prior to randomisation • Likelihood of transfer to another hospital before 34 weeks postmenstrual age
Interventions	• Exclusive human diet (human donor milk and human milk-based fortifier: 'Prolacta RTF 26' and 'Prolacta 6')
Route of administration	Enteral
Target number of patients	At least 100 patients to provide ~80 evaluable infants in total with stool samples for microbiome analysis at 34w PMA
Randomisation	• Using minimisation incorporating the following variables: hospital site, gestation (<25 weeks: ≤24 weeks and 6 days), and multiple birth status • Secured, password protected web-based randomisation using minimisation algorithm (www.sealedenvelope.com or similar)
Primary outcomes	• Microbiota: gut bacterial diversity, proportions of specific taxa, e.g. Bifidobacteria species • Body composition; adipose tissue mass and non-adipose tissue mass assessed using whole body magnetic resonance imaging (MRI) at term (37-42 weeks postmenstrual age)
Secondary outcomes	• Metabolome: Gut derived metabolites and markers of gut function; metabolites include stool Volatile Organic Compounds (VOC, using Gas Chromatography MS), and urine (e.g. using 1H NMR and/or Liquid Chromatography MS); markers of gut health may include those measured in the stool (e.g. calprotectin) and urine (e.g. intestinal fatty acid binding protein, iFABP) • Feed and nutrition related outcomes • Healthcare resource use • Neonatal morbidities and clinical outcomes • Adipose tissue volume and distribution
Duration of trial intervention	To 34 weeks postmenstrual age

Duration of study	• Recruitment period: 21 months • Total trial duration: 24 months
End of Trial	• 37 – 42 weeks at Imperial College. • Discharge from neonatal unit at Newcastle
Safety assessments	• Routine assessments until discharge from Neonatal Unit • Safety tracking during hospitalisation

2. STUDY FLOW CHART



3. ABBREVIATIONS

MOM: Mother's own milk
NEC: Necrotising enterocolitis
DBM: Donor breast milk
MRI: Magnetic Resonance Imaging
BAPM: British Association of Perinatal Medicine
NNRD: National Neonatal Research Database
GCP: Good Clinical Practice
HTA: Human Tissue Authority
REC: Research Ethics Committee
SAE: Serious Adverse Event
SOP: Standard Operating Procedures
TMG: Trial Management Group
SSC: Study Steering Committee
DMEC: Data Monitoring and Ethics Committee
VOC: Volatile Organic Compounds
iFABP: intestinal Fatty Acid Binding Protein
MS: Mass Spectroscopy
NMR: Nuclear Magnetic Resonance
GC: Gas Chromatography
LS: Liquid Chromatography
PAA: Plasma amino acid
SCFA: Short Chain Fatty Acids
AT: Adipose Tissue
PMA: Postmenstrual age

4. BACKGROUND

4.1. Preterm birth

Prematurity is a major cause of mortality and serious long-term morbidity with a substantial burden on health and educational systems (~£3bn/year in the UK). Diseases such as necrotising enterocolitis (NEC, a serious inflammatory bowel disease) and sepsis are responsible for more deaths after the first postnatal week in every preterm infants than any other single pathology [1]. NEC and sepsis increase the risk of mortality and morbidity. Though widely believed to be related to feeding practices adequately powered and designed trials are lacking. NEC and sepsis are lower in infants receiving their own mother's breast milk, and cognitive outcomes are better, but the impact of pasteurized human donor milk is uncertain.[2]

Maternal milk is strongly promoted in all UK neonatal units and >90% infants now receive some mother's milk. However, the role of donor breast milk (DBM) remains uncertain because data on outcomes are inconsistent and many are smaller studies conducted several years ago when rates of mother's own milk provision were much lower. The most recent published randomized controlled trial[2] shows that the amount of mother's own breast milk remains strongly associated with improved health outcomes. However, there were no differences in the key clinical outcomes of NEC, sepsis or death between the two randomized groups exposed to donor milk compared to supplementation with cow's milk formula in the first 10 days of life. In another recently completed study there was no difference in the primary outcome of longer term neurodevelopment in those who received donor human milk compared to preterm formula used as supplements when there was a shortfall in provision of mother's own milk, but secondary analysis showed a difference in rates of NEC (all grades) [3]. These recent studies mean that there is still considerable uncertainty in the UK as to whether human donor milk should be used and current practices towards using formula or donor milk when there is a 'shortfall' in supply of mother's own milk differ between neonatal units[4]. National data show no clear differences between neonatal networks that do or do not use donor milk. Donor milk is considerably more expensive than formula milk.

4.2. Use of human milk fortifiers

One further issue arises in the use of human milk because the nutrient density both of mother's own and donor human milk, may be insufficient to meet the needs of very preterm infants. Many clinicians therefore fortify human milk with cow milk derived products. These human milk fortifiers increase short term weight gain but long-term evidence of benefit on functional outcomes is lacking. There are also no data examining more important longer-term outcomes. In addition, because fortifiers are of cow's milk origin, it is uncertain what other impacts they might have, for example on the pattern of gut bacteria. Emerging evidence show that the pattern of gut bacteria is associated with a range of health outcomes, but almost all studies to date are observational and subject to the inherent limitations of this type of design.

4.3. Human milk-based fortifiers

A recent new development is the production of commercial donor human milk derived fortifiers that can be used with mother's own or donor human milk enabling clinicians to meet nutrient intake recommendations [5] without exposure to cow's milk components. A recent trial conducted in the US [6] showed a decreased incidence of NEC in infants who received a human milk derived fortifier compared to cow's milk derived products, although not powered for this outcome. A further study supported these benefits in a trial using both donor human milk and human milk derived fortifier compared to preterm-formulated cow's milk formula in infants where maternal breast milk was not available [7]. Observational studies from the US support these data [8] and several US units now use these products. However, these products are not currently available in the UK.

4.4. Rationale for trial

Breast milk is associated with advantages for infants in both the short-term (lower sepsis rate) and longer-term (lower blood pressure, improved cognitive outcome) although the full range of functional benefits, and causal mechanisms are not well defined. Randomisation to breast feeding or formula is not acceptable as the former is recommended for all infants. However, mothers that deliver infants preterm frequently do not produce sufficient expressed breast milk to meet all their infants' milk requirements. In this situation, a cow's milk based infant formula or pasteurized donor human milk are options for use, with evidence for the better

choice remaining uncertain. Human milk (mothers' own or donor) is sometimes fortified with additional nutrients to prevent or treat faltering growth. Currently in the UK this requires the use of a milk 'fortifier' derived from cow's milk, hence very few very infants receive an exclusive diet of human milk. A new human milk fortifier has become available offering opportunity to compare the effect of an exclusive human milk diet, in comparison with a diet containing bovine products, on gut bacteria, metabolites, and body composition.

There are a number of reasons that preclude immediate UK adoption of human milk-based fortifier. Studies in the US have been small and inadequately powered to detect differences in functional health outcomes, and were conducted in hospitals with populations of babies with disease risks that may differ from UK settings. Health service costs applicable to the UK have not been determined so cost-effectiveness is uncertain, and causal mechanisms have yet to be explored.

The proposed trial is a mechanistic study to explore the impact of dietary strategies on gut bacteria, metabolism and body composition. The trial is neither designed nor powered to detect differences in clinical outcomes. However, data on clinical outcomes might contribute to future meta-analyses. Protocol design, study conduct, analyses, and decision to publish are the responsibility of the investigator team completely independent of the funder (Prolacta Bioscience). The Sponsor (Newcastle Hospitals NHS Foundation Trust) will play no role in data analyses but will provide governance oversight for the study as NHS sponsor.

5. HYPOTHESIS AND RESEARCH QUESTION

The hypothesis is that an exclusive human milk diet compared with exposure to cow milk results in differences in a) gut bacteria (types and diversity), b) stool and urine metabolites, and c) body composition. We will address the research question "In infants born <30 weeks completed gestational age (Population), what is the effect of an exclusive human milk diet (Intervention) compared to a current standard diet using cow milk derived products (Control) on gut bacteria, metabolites and body composition at near term/term (Outcomes).

6. TRIAL OBJECTIVES

To evaluate whether an exclusive human milk diet compared with exposure to cow's milk results in differences in:

6.1. Gut microbiota: gut bacterial diversity, proportions of specific taxa, eg Bifidobacteria species

6.2. Metabolomics: Gut derived metabolites and markers of gut function; metabolites include stool Volatile Organic Compounds (VOC, using Gas Chromatography MS), and urine (using 1H NMR and/or Liquid Chromatography MS). Markers of gut health may include those measured in the stool (e.g. calprotectin) and urine (e.g. intestinal fatty acid binding protein, iFABP)

6.3. Body composition: assessed using whole body magnetic resonance imaging (MRI)

6.4. Immune markers in the blood (lymphocyte subsets) and plasma amino acid levels.

7. TRIAL DESIGN

This will be a randomised open label, controlled trial in at least two UK centres. Eligible preterm infants include those where a) mothers intend to provide their own breast milk or b) are medically unable to provide breast milk but are happy for their infant to join the study. Less than 10% of all infants do not receive their mother's milk. Our data show that < 5% of mothers do not wish their baby to receive breast milk. Approximately 5% of mothers are medically not able to provide their own milk: this includes those who are HIV positive, are very sick or receiving chemotherapy (or similar) treatment. Infants will be randomised as soon as possible and no later than 72 hours to receive either standard preterm formula or exclusive human milk to make up any shortfall in mother's own milk (MOM). Supplementation will begin if there is insufficient MOM at any point between 48 - 72 hours after birth to enable feeding according to the trial feed advancement protocol (Appendix 1).

Our data show that almost every infant (>95%) born to mothers who intend to provide their own MOM, actually receive some MOM, but occasionally MOM is not available until after 48 hours of age. Infants will remain eligible for trial inclusion, and remain part of the trial until 34 weeks corrected age regardless of the amount of MOM provided. An infant will therefore be eligible if they have not received any MOM. Our data show that in these infants

MOM will typically provide between 30% and 100% (average around 60-70%) of intake requirements over the first few weeks. The trial will record the duration that an infant receives MOM. The trial is designed to assess the impact of an exclusive human milk diet on gut bacteria and metabolites, therefore the primary analysis will be valid regardless of the proportion of milk intake provided as MOM. Fortifier (human or bovine) will be added to MOM when an enteral intake of at least 150 ml/kg/day is achieved. For practical reasons fortifier will be added when a minimum quantity of 50 ml of MOM is available. Infants will receive the allocated diet until 34 weeks postmenstrual age. At 34 weeks PMA trial intervention will be weaned over a period of approximately four days.

7.1 Exclusive human milk arm: MOM; 'Ready to Feed Human Preterm 26' formula (Prolacta) will be used to make up any shortfall in MOM before 150 ml/kg/day of enteral feeds is reached to allow for nutrient equivalence with preterm formula in the standard arm; once 150 ml/kg/day enteral feed is reached human milk-based fortifier will be added when at least 50 ml of MOM is available. If there is less than 50 ml of MOM available, the balance will be made up with 'Ready to Feed Human Preterm formula 26'. The maximum routine enteral intake will be 165 ml/kg/day providing a protein intake of 3.7- 4.6 g/kg/ day, carbohydrate intake of 8.2 – 12.2 g/kg/day and fat intake of 8.2 – 9.2 g/kg/day.

7.2 Standard arm: MOM; standard preterm formula will be used to make up any shortfall in MOM before 150 ml/kg/day of enteral feeds is reached; once 150ml/kg/day enteral feed is reached standard breast milk fortifier will be added when at least 50 ml of MOM is available. The maximum enteral intake will be 165 ml/kg/day providing a protein intake of 3.7 – 4.6 g/kg/day, carbohydrate intake of 13 – 14.7 g/kg/day and a fat intake of 6.3 – 6.4 g/kg/day. Specific products (i.e. brand and type of preterm formula and bovine fortifier) used will be determined by standard practice in each neonatal unit.

8. TRIAL ENDPOINTS

The outcomes assessed in infants recruited at both sites will be microbiota and metabolomics. Infants recruited at Chelsea and Westminster NHS Foundation Trust will be additionally be assessed for body composition. Infants recruited in Newcastle will have additional blood samples collected and stored for future studies.

8.1. Primary endpoints

8.1.1. Newcastle and Chelsea and Westminster NHS Foundation Trust Hospitals

8.1.1.1. Stool gut microbiota: Next Generation Sequencing using MiSeq

8.1.2. Chelsea and Westminster NHS Foundation Trust (Imperial College London)

8.1.2.1. Body composition at term equivalent age assessed with whole body Magnetic Resonance Imaging (MRI) at 37- 42 weeks postmenstrual age

8.1.3. Newcastle

8.1.3.1. Salvaged blood for growth factors e.g. IGF-1

8.1.3.2. Targeted blood: plasma amino acid (PAA) profiles and lymphocyte subsets.

8.2. Secondary endpoints

8.2.1.1. Stool metabolomic profile using GC-MS for VOCs

8.2.1.2. Urine metabolomic profile using LC-MS or NMR

8.2.1.3. Markers of gut health e.g. urinary intestinal fatty acid binding protein (iFABP) and stool calprotectin, using standard biochemical techniques

8.2.2. Feeding related outcomes: total number of days on which feeds were withheld on any occasion after trial enrolment determined by days of parenteral nutrition (PN); age when enteral feeds ≥150ml/kg/day maintained for at least 3 days (coded as age at last PN); total days exposed to MOM prior to 34 weeks; feeding mode at discharge (breast, formula or mixed)

8.2.3. Healthcare resource use: total length of stay (days); postmenstrual age at discharge; days in intensive, high-dependency and low-dependency care according to national definitions (BAPM)

8.2.4. Neonatal morbidities and clinical outcomes: survival to discharge; Retinopathy of Prematurity requiring intervention; Necrotising Enterocolitis requiring surgery or leading to death; blood-culture positive sepsis; total days when any antibiotic administered; chronic lung disease (oxygen requirement or need for any pressure support at 36 weeks postmenstrual age), peri-ventricular haemorrhage (PVH) and/or presence of parenchymal damage. These will all be determined using established national definitions (National Neonatal Audit Programme), and informed using our recently completed studies on NEC[9] and agreed where necessary by investigator end-point review committee.

8.2.5. Weight, head circumference and length measured on a weekly basis where possible

8.2.6. Residual samples will be stored in the HTA and REC approved Great North Neonatal Biobank (Newcastle Biomedicine Biobank)

9. PATIENT POPULATION

9.1. Inclusion criteria

9.1.1 Preterm infants < 30 completed weeks gestation following signed parent consent [Note **protocol modification April 2018** after 25 recruits to extend eligibility criteria from <29 weeks gestation to <30 weeks gestation]

9.1.2 Have not received any milk other than MOM

9.1.3 Less than 72 hours of postnatal age [Note **protocol modification April 2018** after 25 recruits to extend eligibility criteria <48 hours age to <72 hours age]

9.2. Exclusion criteria

9.2.1 Lack of informed consent, or where the attending medical team feel it is inappropriate for other reasons e.g. social or child protection issues, severely unwell and/or not predicted to survive

9.2.2 Infant who has received any formula or bovine milk product prior to enrolment

9.2.3 Participation in another study will not be an absolute exclusion as this is a mechanistic study, and will be assessed on a study-by-study basis; trials using interventions that are anticipated to have similar mechanisms of action on the key outcomes (gut bacteria and body composition) will be excluded (e.g. infants enrolled to this study will not be eligible to join the trial of enteral lactoferrin (ELFIN) and vice versa). However, where there is no scientific basis to exclude an infant i.e. the trial intervention is not anticipated to either interact with the diet, or impact on the primary outcomes of gut bacteria or body composition, or is purely observational in nature, then study participation will be offered to parents. This view has been developed based on our research experience and feedback from parents; some parents may themselves have been involved in studies or medicines trials during this pregnancy that may impact on neonatal outcomes; it might be considered un-ethical to deny parents the opportunity to participate in a study they view as potentially beneficial; our recently completed in-depth qualitative study funded by the UK

National Institutes of Health Research (PARENT) involved interviewing parents of preterm infants who had been enrolled in more than one study. This showed that parents were aware of and supported the possible need for infants to be enrolled in more than one study.

9.2.4 Infants likely to be discharged or transferred to another hospital before 34 weeks postmenstrual age.

9.3. Screening and enrolment

Parents can be approached for consent at any time in the first few days after birth as long as babies remain eligible. Additionally, women in threatened preterm labour or with conditions likely to result in preterm delivery before 30 weeks of gestation will be approached antenatally to give them information about the study. An explanation will be given by GCP trained research staff, followed by written information and an opportunity to ask questions. Parents will have as much time as they require provided the infant still remains eligible.

The study will only recruit preterm infants born at a gestational age of <30 weeks. We will approach parents for consent in the first 3 days after delivery but recognise that many mothers of potential infants may be in-patients on antenatal wards or attending fetal medicine clinics, and it is considered good practice to inform these families of potential research studies in advance. Use of a gestational age cut off facilitates this process.

9.4. Informed consent

"Informed consent" requires individual discussion with the infant's parents about the nature of the research to be conducted in a language that is easy to comprehend. The parents will have the study verbally explained to them and also be given a written information sheet about the study. The parents will understand that the infant can be allocated to one of two groups and that the trial is comparing two nutritional regimens. The parents will also understand that refusal to participate in the study will not affect the quality of subsequent medical care and if they do consent to participate, they may withdraw at any point without affecting their infant's care.

Before any trial-related procedures may be performed, informed consent must be obtained from the infant's parents by a trained member of the investigator team by means of a signed declaration. The investigator must record in the medical notes that informed consent was obtained and store the original signed declaration of consent in the patient's notes. A copy should be given to the infant's parents and a copy filed in the investigator file.

Written consent will be obtained once the baby is born and will explicitly state the intention to collect biological samples and the approach to randomisation. Parents may subsequently decline specific aspects of the study e.g. the collection of the targeted blood sample or MRI scan but still continue to participate in other aspects of the trial.

9.5. Randomisation

Infants will be randomised using minimisation incorporating the following variables: hospital site, gestation (<25 weeks: ≤24 weeks and 6 days), and multiple birth status; this will be held and coordinated by the Newcastle research team using an internet based secured randomisation system, and will include strata for hospital site, gestation (<25 weeks and ≥ 25 weeks gestation), and multiple birth status. Contact and allocation will be by password protected internet access. Infants will be allocated to either standard diet (control) or the exclusive human milk-based diet (intervention). Multiples (twins and higher order) will be randomised independently.

9.6. Patient withdrawal

9.6.1. Temporary discontinuation of intervention

There should be no reason to discontinue trial intervention before the infant achieves milk feeds of at least 150 ml /kg /day. In exceptional circumstances however, a temporary discontinuation of the intervention could occur at any point during the intervention period for e.g. feed intolerance. It is unlikely that there will be problems with compliance with the interventions.

9.6.2. Permanent discontinuation of intervention

Parents may permanently withdraw their infant from treatment with the trial intervention at any time and for any reason.

9.6.3. Withdrawal of infant from trial procedures and incomplete follow-up

It is possible that parents may choose to withdraw their infant from trial procedures resulting in incomplete patient follow-up and failure to capture outcome data. Likewise, if an infant dies before discharge they will not have their MRI scan. In these cases data already obtained will be included in all analyses.

10. TRIAL PRODUCTS

10.1. Description

- Ready to Feed Human Preterm 26 formula (Prolacta): Constituted from term human donor milk, each 100 ml provides 90 kcal of energy, 2.6 g of protein, 8.1 g of carbohydrate and 5.2 g of fat. Appendix 2.
- Human milk based fortifier, Prolact+6 (Prolacta): Appendix 3
- Standard preterm formula: SMA Gold Prem 1 or Nutricia Nutriprem 1 (Appendix 4 and 5)
- Standard fortifier Nutriprem (Nutricia) (Appendix 6)

10.2 Labelling

This is an open label study. The Prolacta products will be specifically labelled as a food for special medical purposes (i.e., preterm infants) for shipment and courier according Department of Health specifications. Labelling on the bottles will include batch number and nutrient density. Batch numbers will be recorded in infant case records.

10.3 Storage

The milk products provided by Prolacta are shipped frozen according to carefully established procedures and are stored on or near the neonatal units. Standard Operating Procedures will be used for checking milk on arrival and storage. Bedside clinical nurses will receive training to use the trial products.

11. TRIAL OBSERVATIONS, TESTS AND INVESTIGATIONS

11.1 Trial data

We will obtain routinely recorded clinical data from the National Neonatal Research Database (NNRD), held at Imperial College London, supplemented by the use of standard case report forms where items are not recorded in the NNRD.

11.2 Time scale for trial evaluations

11.2.1 Daily stool and urine samples: These will be collected by the bedside nurse from admission to 34 weeks postmenstrual age.

11.2.2 Salvaged blood samples (Newcastle only): We will salvage leftover blood samples (blood that remains in the laboratories after all routine analyses have been complete and would ordinarily be discarded). These will be stored for biobanking [10].

11.2.3 Targeted blood samples (Newcastle only): With specific parent consent (that would not otherwise preclude ongoing trial participation) we will also take two targeted blood samples: 1) after feeds of 150mls/kg/day have been established for at least 3 days and 2) prior to trial cessation i.e. around 33-34 weeks postmenstrual age. Targeted blood samples will be taken at the same time as any blood samples required for clinical reasons so will not involve additional venepuncture.

11.2.4 Body Composition (Imperial only): at 37-42 weeks postmenstrual age assessed with whole body MRI

11.3 Data

11.3.1 At randomisation

- Date and time of birth
- Sex
- Confirmation of eligibility (full eligibility check)
- Date of parental consent
- Name of person taking consent
- Gestational age (in weeks and days)
- Recruiting hospital
- Study allocation

11.3.2 Baseline evaluation

- Birth weight (kg)

- Birth length (cm)
- Head circumference (cm)
- Ethnicity (NHS Ethnicity)
- Mode of delivery
- Use of antenatal steroids
- Date and time first trial intervention administered

11.3.3 Daily evaluations

- Record nutritional intake (type of feed, parenteral nutrition or formula as applicable)
- Level of care (BAPM 2010)
- If infant is nil by mouth and reason
- Check for SAE
- Record of withdrawal information if relevant

11.3.4 Weekly evaluations

- Weight (kg)
- Length (cm)
- Head circumference (cm)

11.3.5 End of study evaluation

- Weight (kg)
- Length (cm)
- Head circumference (cm)
- Whole body MRI

11.4 Investigations

11.4.1 Stool microbiome

We will analyse bacteria DNA extracted from stool samples using our well-established protocols and 16S ribosomal RNA methods that are effective tools to explore the diversity of bacterial communities (Stewart et al. 2012). Using the MiSeq high throughput analyzer we will incorporate 188 samples as well as necessary controls, which we have found to be the optimum number of samples to obtain coverage of the bacterial community.

11.4.2 Urine and stool metabolome and biochemical assays

We will conduct metabolomic profiling of urine and/or stool. Extraction of samples will be optimised for detection of SCFAs and samples processed using non-targeted and targeted high-resolution liquid chromatography-mass spectroscopy (LCMS) to generate metabolomic profiles that may indicate functional changes in the host and the gut microbiota. Our targeted approach will investigate known gut flora fermented products of complex carbohydrates, including SCFAs, acetates, amino acids and CHO fragments, which will be present in stool samples. Analysis (see statistics section below) will use modeling techniques to explore relationships between the microbiome and metabolome, and diet. Examination of the urinary metabolome is more reflective of changes to the overall host (infant) metabolic state but may also reflect differences in absorption of compounds from the gut. Determining the metabolomic profiles between and within patients to complement the NGS sequencing data will provide significant opportunities to develop quantitative models that show how the host, gut microbes, trial interventions and other clinical factors interact, and any downstream functional effects.

We will also analyse some urine and stool samples using assays for proteins that may indicate gut health including fecal calprotectin and urinary intestinal fatty acid binding protein. These will provide additional insight into any gut inflammatory processes and the integrity of gut epithelium.

11.4.3 Stool metabolomics and VOC analysis

VOCs from stool samples will be analysed by GC/MS using well-established protocols for extracting and analysing headspace gases. These are validated on as little as 50mg of sample: sufficient to analyse ~40 compounds, which includes 8 different acids, particularly SCFAs, branched and linear, alcohols and esters. Interpretation of fragment patterns will be undertaken against the current mass spectral NIST library, followed by manual visual inspection. Standards will be purchased for retention time matching. Summary data of the abundance of acids, alcohols and esters will be determined and prepared for statistical analysis

11.4.4 Blood analyses (Newcastle site only)

We will store salvaged blood with parental permission to analyse in future studies examining immune (e.g. specific antibody) and inflammatory pathways, and to conduct e.g. epigenetic analysis e.g. DNA methylation. Targeted blood samples will be analysed for

- Flow cytometry to determine lymphocyte subset proportions.
- Plasma amino acid profiles
- If sample volume allows we will consider analyses of additional markers of gut health include for example cytokines

11.4.5 Body composition (Chelsea & Westminster site only)

We will use whole-body MR imaging to obtain serial axial images (5mm slice and inter-slice thickness) to quantify total adipose tissue (AT) volume as the sum of 6 discrete depots: superficial subcutaneous abdominal, superficial subcutaneous non-abdominal, deep subcutaneous abdominal, deep subcutaneous non-abdominal, internal abdominal (IA), and internal non-abdominal, as previously described (Modi et al. 2009).[11] Image analysis with the use of Slice-OMatic (Tomovision) will be undertaken independently blinded to participant identity and group allocation. AT volume in litres will be converted to AT mass in kg, assuming a value for the density of AT of 0.90 kg/L. Non-AT mass is calculated by subtracting AT mass from the weight of the infant on the day of the MR scan using the following formula: [body weight (g) - [AT volume (cm^3) × 0.9]]. MR scanning will be carried out in natural sleep between 37- 42 weeks postmenstrual age.

12 PHARMACOVIGILANCE

As this is not a clinical trial of a medicinal product and the intervention, and its delivery, are considered safe and already in universal use we do not anticipate any adverse reactions due to the trial intervention (use of donor breast milk products). However, this is a very high risk patient group and some infants may develop serious complications e.g. sepsis, and some may die. We will record all serious adverse events (SAE). Serious adverse events that may occur include:

- Outcomes recorded and reported as part of the study: necrotising enterocolitis (NEC), sepsis, intraventricular haemorrhage, periventricular leukomalacia, retinopathy of

prematurity, chronic lung disease, feeding intolerance (number of days when nil by mouth i.e. no enteral intake and need for parenteral nutrition), antibiotic exposure (number of days on which an antibiotic was administered) and death.

- Outcomes that commonly occur in this population but that will not be specifically recorded or reported as part of this study include: abnormal electrolytes, bone chemistry, liver functions tests or other routine biochemistry or haematology; transfusion of blood or related products; complications related to use of intravenous catheters (e.g. misplacement or malfunction); presence of and/or treatment for a patent ductus arteriosus; requirement for surgery and/or provision of an intestinal or airway stoma, intraventricular shunt; vomiting, gastro-oesophageal reflux, constipation, diarrhoea, skin or nappy rash.

13 STATISTICAL CONSIDERATIONS

13.1 Sample size calculation

13.1.1 Gut bacteria

Using 16S rRNA bacterial profiling data we will conduct alpha and beta diversity analysis. Based on previously published data by our group[12,13] a Shannon Diversity Index alpha diversity of <1 is representative of infants in the initial weeks of life where the microbiome is undeveloped; a Shannon Diversity Index of >1.5 is representative of infants immediately prior to neonatal unit discharge where the gut microbiome is more developed and risk of gut microbiome mediated disease is significantly reduced. Based on these findings a sample size of 24 in each group (48 in total) is required to achieve over 95% power detect a 0.5SD difference in diversity index. By processing 40 samples in each group we will reach this power for true significance of alpha diversity analysis and note from previous analysis that that this number is also sufficient for beta diversity analyses, including but not limited to UniFrac distance, clustering (PAM and DMM), correlation (CCLasso), ordination analysis (PCA and PCoA), and determining significant differences between the relative abundance of different genera.

We will compare proportions of key taxa e.g. proteobacteria, firmicutes etc., as well as numbers and presence of key species including lactobacilli, bifidobacteria and staphylococci.

13.1.2 Body composition

In our most recent work in preterm infants born at <31 weeks gestational age, the mean (SD) of non-adipose tissue mass was 2.41 (0.46) kg (Uthaya et al. 2016)[14]. A sample size of 20 babies per group will have 80% power at the 5% significance level (2-sided) to detect a 0.42 kg between-group difference in non-adipose tissue mass assessed by whole body Magnetic Resonance Imaging using protocols previously described[15,14].

13.1.3 Metabolome

Stool VOC analysis identifies several individual compounds; our collaborators show an average 31.3±10.5 (Mean and SD) VOCs were identified per sample[16]. With n=40 infants per group we will have 85% power to detect an increase in 5 VOCs between intervention and standard groups. Urinary 1H NMR studies are limited in preterm infants. However, highly significant differences in spectra ($p<0.005$) were detected between adults born preterm (n=19) and term (n=17). The differences observed were in key metabolites such as trimethylamine, an end product of choline metabolism by gut bacteria[17].

13.1.4 Urinary and stool markers of gut integrity or inflammation

Intestinal fatty acid binding protein (iFABP) is highly correlated with gut inflammation, represents damage and release of this protein from gut enterocytes, and has been proposed as a biomarker of gut health and NEC. In a recent case control study (with n=14 pairs) significant differences were detected in infants (compared to well controls) up to 7 days before early NEC stage 1 ($p=0.005$). Because the data are skewed it is not possible to use them for a traditional power calculation but typical values were 4ng/ml (range 2-7ng/ml) in control infants and 15ng/ml (range 6-42ng/ml) who subsequently developed early NEC. Assuming gut inflammation in infants receiving cow's milk derived products was only increased slightly (to 6ng/ml) and that the SD in control infants was 1.5ng/dl, the study would have 85% power to detect such a small increase with just n=20 infants per trial group[18]. We will also explore the use of stool calprotectin as an additional marker of gut inflammation but there are insufficient data to perform a power calculation.

13.1.5 Plasma amino acids

Standard analyses of 9 essential and 9 non-essential amino acids. Plasma levels of threonine, valine, phenylalanine, methionine and tryptophan are typically higher when infants are fed whey based hydrolysed cow's milk protein, which is the source of protein in commercially available cow's milk based breast milk fortifiers. We will compare total essential and non-

778 essential levels. Although reference data in this group of infants are not available for total
779 levels, typical tryptophan levels are 30±12umol/l. 20 infants per group, would therefore give
780 a power of 83% to detect a clinically important increase from 30umol/l to 40umol/l.
781
782 **13.2 Analysis plan**
783 **13.2.1 Microbiome**
784 The microbial data may be over-parameterised depending on the taxonomic level at which
785 analysis is carried out. At a strain level we will have more microbial strains present in
786 samples than there will be samples taken. However, due to current limitations in sequence
787 read length we have shown that at an Operation Taxonomy Unit (OTU) level (that is
788 sequences with >97% similarity which corresponds to species level), we identify
789 considerably less OTU (138) after normalisation than samples forecast for the proposed
790 study[19]. We will use multivariate ordination techniques to summarise the major trends in
791 variation in bacterial community composition of infant stool and will allow us to identify
792 those taxa most closely associated with changes in the pattern of bacteria over time. We will
793 then use canonical ordination to quantify the impacts of the diet on the microbiome
794 composition.

795 **13.2.2 Body Composition**

796 Continuous variables that follow an approximately normal distribution will be summarised
797 using the mean and standard deviation. Skewed continuous variables will be summarised
798 using the median and inter-quartile range. Categorical variables (binary, ordered and
799 multinomial) will be presented in terms of frequencies and percentages. This will be an
800 intention to treat analysis, infants will be analysed in accordance with the treatment to which
801 they are randomised. For outcomes measured on a single occasion, a multiple regression
802 model containing the treatment variable, stratifying variables (gestational age and birth
803 weight), sex and postmenstrual age at time of measurement will be used to estimate the effect
804 of the intervention.

805 **13.2.3 Data queries, missing data**

Owing to the nature of this study, it is expected that a number of infants will not have blood sampling conducted or attend the end-of study MR scan (primarily due to the death, ill-health, or withdrawal of the subject). As far as possible, reasons for non-attendance will be recorded. Where infants are transferred to other hospitals before the end of the study intervention (34 weeks corrected age) or before discharge home, the investigators will make contact with that hospital and arrange to collect data. The data collected will already form part of the infants' medical record and can be extracted from clinical records or clinical databases e.g. the National Neonatal Research Database that holds data from the Badger system. The data will include feeding information (e.g. type of milk and supplements), growth (e.g. weight, length and head circumference), any important medical outcomes (e.g. infection episodes, requirement for respiratory support) and duration of hospital stay.

14 REGULATORY AND ETHICAL CONSIDERATIONS

The trial will require approval by a Research Ethics Committee. We do not consider the trial poses any significant risks or any additional risk or discomfort over and above that already associated with neonatal care. The control arm represents standard UK practice and involves a range of products and milks (MOM, preterm-formulated formula milk and fortifier). The trial intervention involves MOM, donor human milk and human milk derived fortifier produced commercially. This study is an exploratory trial of the mechanisms of action of two milk-based dietary regimes on the pattern of gut bacteria, and is not a Clinical Trial of an Investigational Medicinal Product (CTIMP) according to MHRA definitions because it is a food product and/or supplement that is not presented as a medicine. (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/317952/Algothrim.pdf)

The preterm formulae and cow's milk derived breast milk fortifiers used will be the same as used clinically on UK neonatal units (SMA Gold Prem 1, Nutricia Nutriprem 1, Nutriprem breast milk fortifier).

The trial will be supported by experienced, GCP trained research teams at both sites with substantial expertise in the care of newborn infants and neonatal nutrition research. There is

minimal discomfort associated with obtaining blood samples, but these will only be taken when blood is required for clinical reasons. Parents will have the opportunity to decline blood sampling and MRI. A parent information leaflet will be provided, and signed parental consent will be obtained. Biological sample collection and storage will be conducted according to existing SOP of the research team and sponsor.

15 TRIAL ORGANISATIONAL STRUCTURE AND RESPONSIBILITIES

15.1 Trial sponsor

The institution employing the chief investigator (Newcastle Hospitals NHS Foundation Trust) will act as sponsor

15.2 Trial steering committee

A Study Steering Committee (SSC) will be established to oversee the conduct of the study. It is anticipated that the SSC will comprise the lead investigators, an independent chair, additional independent members and a user representative. The SSC will meet thrice during the course of the study, once shortly after the start of the study and then annually or as required throughout the course of the study.

15.3 Data monitoring and ethics committee

This is a mechanistic study in which trial outcomes will not be analysed until study end. In addition, the dietary regimens used are un-blinded. We therefore do not plan to have a Data Monitoring and Ethics Committee

15.4 Trial management

The trial will be reviewed by the trial management group (TMG) at the end of an internal pilot phase (n=5 per trial arm at each site) and key data reviewed**.** The TMG will consist of the key investigators NE, NM, JB, SU along with trial managers, and research nurses, and will meet by teleconference every 3 months, with more frequent meetings if needed in the set up and initial period of recruitment.

15.5 Trial registration

The trial will be registered on the International Standard Randomised Clinical Trial Number registry.

15.6 Trial sites

Two large tertiary neonatal units with considerable experience of intervention trials in high risk neonates: Royal Victoria Infirmary, Newcastle upon Tyne, UK and Chelsea & Westminster Hospital (Imperial College), London, UK. Further sites with necessary research infrastructure may be added later. **Protocol modification August 2018**: addition of two additional NICU sites at James Cook University Hospital, Middlesbrough, UK and William Harvey Hospital, East Kent, UK.

15.7 Investigator responsibilities

Investigators will be responsible for ensuring that institutional (site specific) approval has been obtained as well as Agreements signed off by their Institution prior to the start of the study. Investigators are required to ensure compliance to the Clinical Trial Protocol, Investigators File and any other study instructions as required by the Sponsor or its representatives. Investigators are required to ensure the accuracy of the trial data according to the instructions provided. Investigators are required to allow access to study documentation or source data on request for monitoring visits and audits performed by, the Sponsor or any regulatory authorities. The Investigator may appoint co-investigators to assist with conduct of the study locally. All co-investigators must be listed as members of the research team and appropriately trained. The Investigator has overall responsibility for ensuring the conduct of the study locally.

16 END OF TRIAL

The end of trial will be declared when the last infant recruited completes the last follow-up visit i.e. MRI scan at 37-42 weeks postmenstrual age (Imperial College) and discharge from the neonatal unit (Newcastle).

17 PUBLICATION POLICY AND DISSEMINATION OF RESULTS

The results from the trial will be submitted for publication irrespective of the outcome. The Study Steering Committee will be responsible for approval of the manuscripts prior to submission for publication. At the end of the study, infants' parents will be able to request a copy of the results of the study from the investigator at that site.

Authorship of presentations and reports related to the study will be in the name of the collaborative group. The final follow-up study results paper will name local co-ordinators as well as those involved in central co-ordination and trial management.

A contract will be created and signed by all parties (Prolacta Bioscience, Newcastle/NuTH, Imperial College London) that clearly identifies the foreground intellectual property IP on the design of the human milk products belonging to Prolacta, and the ownership of any IP created as a result of this study belonging to the clinical teams and institutions. There will be no funder or sponsor role in data analysis, presentation or publication. Newcastle Hospitals NHS Foundation Trust will act as sponsor and will require access to study resources and documents in order to provide oversight of governance issues relating to trial conduct. Anonymised, summary data will be provided in publications and may be shared with the funder if not included in published outputs. Individual patient data will be held according to current NHS permissions to enable their use in any future meta-analysis

18 REFERENCES

1 Berrington JE, Hearn RI, Bythell M, *et al.* Deaths in preterm infants: changing pathology over 2 decades. *J Pediatr* 2012;**160**:49–53.e1. doi:10.1016/j.jpeds.2011.06.046

2 Corpeleijn WE, de Waard M, Christmann V, *et al.* Effect of Donor Milk on Severe Infections and Mortality in Very Low-Birth-Weight Infants. *JAMA Pediatr* 2016;:1–8. doi:10.1001/jamapediatrics.2016.0183

3 O'Connor DL, Gibbins S, Kiss A, *et al.* Effect of Supplemental Donor Human Milk Compared With Preterm Formula on Neurodevelopment of Very Low-Birth-Weight Infants at 18 Months. *Jama* 2016;**316**:1897. doi:10.1001/jama.2016.16144

4 Zipitis CS, Ward J, Bajaj R. Use of donor breast milk in neonatal units in the UK. *Arch Dis Child Fetal Neonatal Ed* 2014;**0**:24–6. doi:10.1136/archdischild-2014-307606

5 Agostoni C, Buonocore G, Carnielli VP, *et al.* Enteral nutrient supply for preterm infants: commentary from the European Society of Paediatric Gastroenterology, Hepatology and Nutrition Committee on Nutrition. *J Pediatr Gastroenterol Nutr* 2010;**50**:85–91. doi:10.1097/MPG.0b013e3181adaee0

6 Sullivan S, Schanler RJ, Kim JH, *et al.* An exclusively human milk-based diet is associated with a lower rate of necrotizing enterocolitis than a diet of human milk and bovine milk-based products. *J Pediatr* 2010;**156**:562–7.e1. doi:10.1016/j.jpeds.2009.10.040

7 Cristofalo E a., Schanler RJ, Blanco CL, *et al.* Randomized trial of exclusive human milk versus preterm formula diets in extremely premature infants. *J Pediatr* 2013;**163**:1592–1595.e1. doi:10.1016/j.jpeds.2013.07.011

8 Hair AB, Peluso AM, Hawthorne KM, *et al.* Beyond Necrotizing Enterocolitis Prevention: Improving Outcomes with an Exclusive Human Milk–Based Diet. *Breastfeed Med* 2016;**0**:bfm.2015.0134. doi:10.1089/bfm.2015.0134

9 Battersby C, Longford N, Costeloe K, *et al.* Development of a Gestational Age–Specific Case Definition for Neonatal Necrotizing Enterocolitis. *JAMA Pediatr* 2017;:1–8. doi:10.1001/jamapediatrics.2016.3633

10 Embleton ND, Turnbull E, Turner S, *et al.* Successful blood salvaging from preterm infants: maximizing opportunities, minimizing interventions. *Acta Paediatr* 2013;**102**:e527-9. doi:10.1111/apa.12373

11 Modi N, Thomas EL, Uthaya SN, *et al.* Whole Body Magnetic Resonance Imaging of Healthy Newborn Infants Demonstrates Increased Central Adiposity in Asian Indians. *Pediatr Res* 2009;**65**:584–7. doi:10.1203/PDR.0b013e3182291b13

12 Stewart CJ, Skeath T, Nelson A, *et al.* Preterm gut microbiota and metabolome following discharge from intensive care. *Sci Rep* 2015;:1–9. doi:10.1038/srep17141

13 Stewart CJ, Marrs ECL, Nelson A, *et al.* Development of the preterm gut microbiome in twins at risk of necrotising enterocolitis and sepsis. *PLoS One* 2013;**8**:e73465. doi:10.1371/journal.pone.0073465

14 Uthaya S, Liu X, Babalis D, *et al.* Nutritional Evaluation and Optimisation in Neonates: a randomized, double-blind controlled trial of amino acid regimen and intravenous lipid composition in preterm parenteral nutrition. *Am J Clin Nutr* 2016;:1443–52. doi:10.3945/ajcn.115.125138

15 Uthaya S, Thomas EL, Hamilton G, *et al.* Altered adiposity after extremely preterm birth. *Pediatr Res* 2005;**57**:211–5. doi:10.1203/01.PDR.0000148284.58934.1C

16 Reade S, Mayor A, Aggio R, *et al.* Optimisation of Sample Preparation for Direct SPME-GC-MS Analysis of Murine and Human Faecal Volatile Organic Compounds for Metabolomic Studies. *J Anal Bioanal Tech* 2014;**5**. doi:10.4172/2155-9872.1000184

17 Thomas EL, Parkinson JR, Hyde MJ, *et al.* Aberrant Adiposity and Ectopic Lipid Deposition Characterize the Adult Phenotype of the Preterm Infant. 2011;**70**:507–12.

18 Gregory KE, Winston AB, Yamamoto HS, *et al.* Urinary intestinal fatty acid binding protein predicts necrotizing enterocolitis. *J Pediatr* 2014;**164**:1486–8. doi:10.1016/j.jpeds.2014.01.057

19 Stewart CJ, Marrs ECL, Magorrian S, *et al.* The preterm gut microbiota: changes associated with necrotizing enterocolitis and infection. *Acta Paediatr* 2012;**101**:1121–7. doi:10.1111/j.1651-2227.2012.02801.x

APPENDICES

1. Feed advancement protocol (Chelsea and Westminster Hospital)

1. Timing of initiation of enteral feeds

Trophic enteral feeds should be commenced within the first 24 hours unless clinically contraindicated. There are very few genuine contraindications to providing a baby with trophic feeds even if there is no intention to advance feeds.

2. Suggested volumes for initiation of enteral feeds

Infants ≤ 31^{+6} completed weeks GA

Start trophic feeds at 15ml/kg/day 1-2 hourly within first 24 hours.

32^{+0} – 33^{+6} completed weeks GA

With reversed/absent EDF: Start trophic feeds at 15ml/kg/day 1-2 hourly within first 24 hours.

Without absent/reversed EDF: Start enteral feeds at 30ml/kg/day 2-3 hourly as soon as possible.

3. Rate of Advancement of enteral feeds

See Figure 1:

Infant ≤ 29^{+6} weeks GA

Increase feeds by 15ml/kg/day once per day (OD).

Infant 30^{+0} – 31^{+6} weeks GA

With absent / reversed EDF: increase by 15ml/kg/day OD

Without absent / reversed EDF: increase by 15 ml/kg twice a day (BD)

Infants 32^{+0} – 33^{+6} weeks GA

With absent / reversed EDF: increase by 15ml/kg/day OD

Without absent / reversed EDF: increase by 30ml/kg/day OD

In all babies, irrespective of gestational age or Dopplers, once 60 ml/kg /day of enteral feeds are tolerated consider going up 15 ml / kg twice a day.

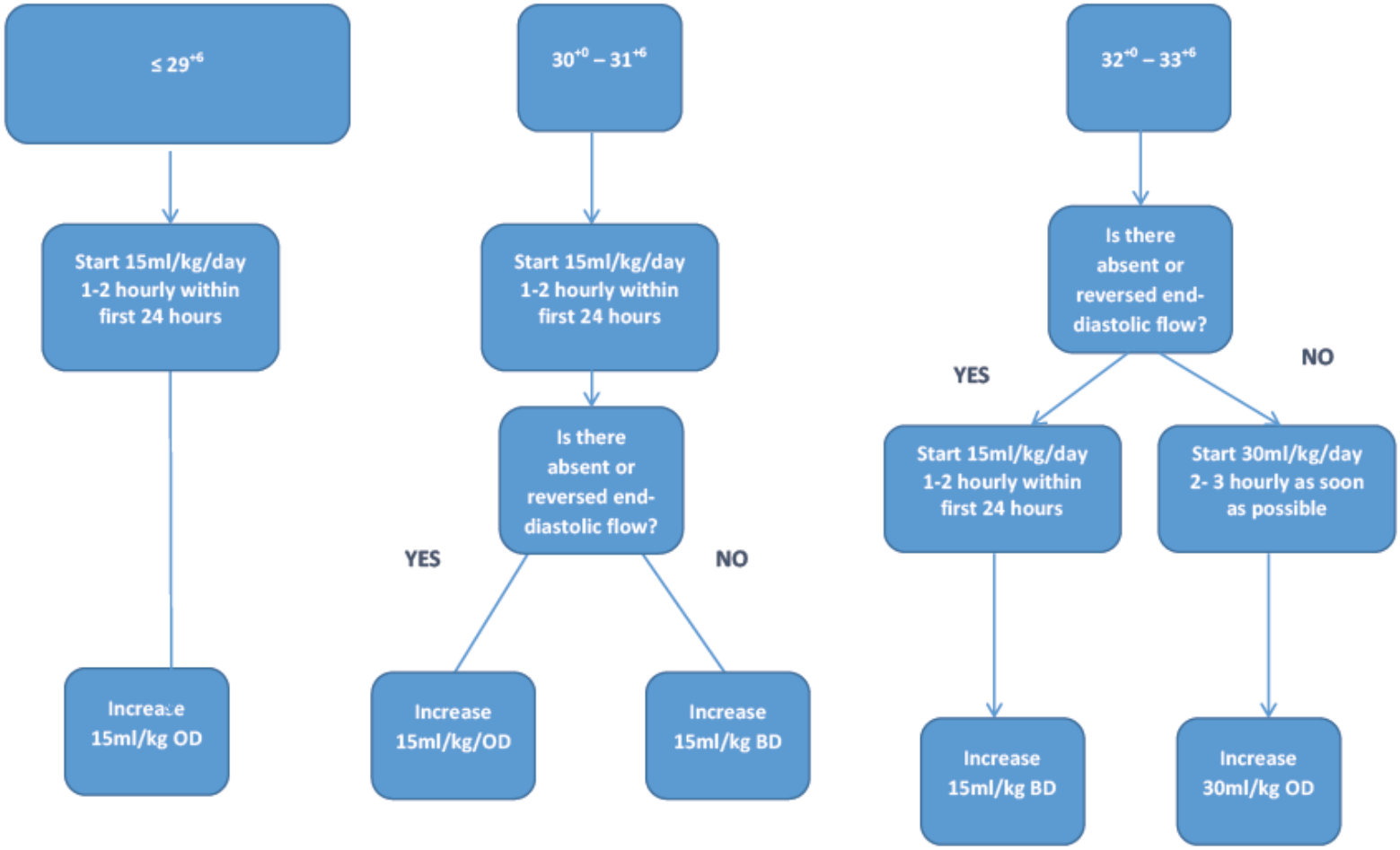
4. Feed Intolerance

Withdrawal of feeds should be considered if:

- the pre-feed gastric aspirate exceeds 4ml/kg
- there are possets or vomits after two consecutive feeds
- there is marked or tender abdominal distension or the presence of visible bowel loops
- there is heavy bile staining of gastric aspirate or bilious vomiting

Figure 1: Summary of Enteral feeding initiation and advancement

In all babies, once 60 ml/kg /day of enteral feeds are tolerated consider going up 15 ml / kg twice a day.



1013

Feed advancement guideline (Newcastle Hospital)

Summary (enteral feed guidelines last updated August 2016)

Summary recommendations relating to feed advancement for infants ≤34 weeks and ≤1750g birthweight

- **Start feeds early** in all infants except where there are particular concerns – if the mother plans to provide EBM but none is available wait at least 48 hours before starting formula
- **Use fresh colostrum as soon as it is available –** use for mouthcare or give as bolus via NG unless specifically contra-indicated
- **Encourage the use of expressed breast milk** (EBM). Speak to all mothers and emphasise the numerous benefits of EBM.
- Babies who are born with Absent or Reversed End Diastolic Flow may also be suitable for early feeds
- Ventilation or the presence of umbilical catheters *per se* are not contraindications.
- Start at a volume of 10-20mls/kg/day
- If feeds tolerated advance at 24mls/kg/day (= increase of 1ml/kg/hour every day or 0.5ml/kg/hour twice a day).
- If there is no EBM use Gold Prem 1 (GP1) or Nutriprem 1 to initiate feeds unless in trial
- Use breast milk (or GP1 or NP1) as the only milk until full enteral feeds are tolerated
- Use oro-gastric tubes in preference to nasogastric tubes
- Use continuous feeds until 28-30 weeks corrected gestation

1044

Supplementary materials Effect of an exclusive human milk diet on the gut microbiome in preterm infants: a randomised clinical trial