Mechanisms Affecting the Gut of Preterm Infants in Enteral feeding trials (MAGPIE)

An EME funded add-on mechanistic study to existing HTA funded trials

Contents

Introduction. The NIHR Health Technology Assessment programme has funded two large interventional randomised controlled trials (RCTs) in preterm infants born <32 weeks gestation. These trials (SIFT and ELFIN) are the largest interventional trials in preterm infants conducted in the UK with almost 5000 proposed recruits from 50 large neonatal units. SIFT (Speed of Increasing milk Feeds Trial) randomizes infants to one of two different rates of increase in milk feeds, commenced recruiting in August 2013 and completed recruitment in June 2015. Follow up will complete in 2018. ELFIN (Enteral Lactoferrin in Neonates) randomises infants to supplemental enteral bovine lactoferrin or placebo and commenced recruiting in June 2014, and is anticipated to finish in 2017. These are pragmatic trials with primary outcomes of survival without disability at 2 years of age (SIFT) and/or sepsis (SIFT and ELFIN).

This protocol is a proposal for an EME-funded mechanistic evaluation (ME) of the trial interventions and outcomes (**M**echanisms **A**ffecting the **G**ut in **P**reterm **I**nfants in **E**nteral feeding studies, MAGPIE) that will be conducted in a subset of infants recruited to the main trials. Participation in MAGPIE will only be possible for infants in the ELFIN study, though REC permissions exist to conduct similar analyses on samples from around 200 babies in the SIFT study. Participation will not affect the main trial protocols or conduct, and will use safe non-invasive collection of specimens (urine and stool) from 480 infants. In addition, we will retrieve gut tissue where infants undergo gut surgery after routine tests are complete. Additional ethics and R&D permissions will be sought and separate informed consent for MAGPIE will be obtained from parents.

Scientific Background. Prematurity is a major cause of mortality and serious long-term

morbidity with an enormous burden on health care and educational systems (£3bn/year in the UK). Necrotising enterocolitis (NEC, a serious inflammatory bowel disease) and late onset sepsis (LOS) are responsible for more deaths after the first week of life in extremely preterm infants than any other single pathology (Berrington et al. 2013.) NEC is associated with significant mortality (<20%); it affects <10% of infants born <32 weeks gestation. In the UK of the 6000+ births per year <32 weeks, NEC will affect <600 of whom approximately 30% will require major surgery, and over 100 will die. LOS affects <20%, of whom 1 in 10 may die. There are more infant deaths from NEC and LOS, than deaths from all childhood leukemia but mechanistic research in this area is limited. NEC and LOS are both associated with significant morbidity in survivors (including worse cognitive outcome and a two-fold increase in the risk of cerebral palsy), and very high healthcare costs (costs of surgery for NEC and prolonged intensive care are >£100,000 per case). The long-term costs to society, the individual and their families due to lifelong physical and mental impairment are substantial, and over £1Million for those most severely affected.

The existing large NIHR trials (SIFT and ELFIN) aim to resolve two key uncertainties in current clinical practice but do not include any ME. Both interventions act via effects on the gut and with interactions on microbes. MAGPIE will use this opportunity to explore actions of the interventions and disease mechanisms. We will do this by collecting non-invasive samples of stool and urine from the infants, and use emerging technologies (next generation sequencing of gut microbes, and mass spectrometry). We will explore how the trial interventions affect gut bacteria (microbiota) and their functional metabolic effects (metabolomics) by detecting chemical compounds in the stool and urine. The aim is to understand the mechanisms of the interventions and diseases, and provide new data in the areas of diagnosis, monitoring and therapeutics.

Brief description of NIHR trials (SIFT and ELFIN). SIFT (www.npeu.ox.ac.uk/sift NCT01727609) and ELFIN (www.npeu.ox.ac.uk/elfin EudraCt No. 2012-004260-22) trials are both funded by the HTA, managed by the National Perinatal Epidemiology Unit (NPEU, Oxford) as CTU and involve collaboration from 30 UK hospitals. Both involve randomization of preterm infants (<32 weeks gestation) in the first few days of life whilst receiving care on a neonatal unit. The trial interventions complete prior to hospital discharge, although follow up for SIFT continues until 2 years corrected age. The SIFT trial will recruit 2800 infants, is powered to detect a significant difference in disability free survival at 2 years, and is also powered to detect a difference in LOS. Infants are randomised to increases in milk feeds of either 18ml/kg/day or 30ml/kg/day meaning full feeds (150ml/kg/day) will be achieved about 4 days later in the slower arm. This will therefore affect duration of central venous access and use of parenteral nutrition, as well as affecting the amount of breastmilk infants receive. The ELFIN trial is powered to detect a significant difference in LOS and will recruit 2200 infants. Infants are randomised to receive either supplemental bovine lactoferrin (150mg/kg/day) or blinded placebo (sucrose) added to milk feeds. The trial pilot started in June 2014, and the main trial will commence in July 2015 and finish in 2017.

Existing research. Mechanistic evaluation of NEC and sepsis in RCT settings. There are very few large clinical studies in preterm neonates exploring biological mechanisms of LOS or NEC. This is partly because of the challenges faced by adequately powered interventional studies which require sample sizes of >1000 infants to detect realistic effects on NEC or LOS. Our research group has considerable expertise in successfully undertaking large trials. Combining large clinical studies with ME in vulnerable preterm neonates presents many

challenges practically, logistically and ethically. There are particular challenges with biological sampling from small infants: a 500g infant has only 40mls of circulating blood. Few RCTs powered to explore differences in NEC or LOS in preterm infants have involved biological sampling on a large scale. We have piloted, refined, and embedded within our neonatal units methodology for collecting and analysing biological 'waste' samples (stool and urine) that can be used to provide a unique insight into the biology of NEC and LOS (Embleton et al. 2013). We have shown daily sampling success in busy units over prolonged time periods. Using this methodology we will use the unique opportunity of SIFT and ELFIN to conduct high quality ME of two trial interventions, and provide diagnostic and therapeutic insights into life threatening diseases.

Existing research. Mechanistic evaluations: Feeding Rates and Lactoferrin. We have conducted relevant Cochrane reviews of feeding (McGuire et al. 2013), but have not identified any large studies incorporating ME. Alterations of gut microbes are one of the key mechanisms through which reduction in NEC or LOS may occur. Slower feeds expose the babies to less breastmilk that stimulates gut cell growth and prolonged use of central venous lines. Early gut microbes are associated with those causing later invasive sepsis. The only existing published RCT (n=450) using lactoferrin did not include any ME (Manzoni et al. 2009). This MAGPIE proposal would represent the largest ME of feeding or lactoferrin in premature infants. We have recently reviewed potential mechanisms of action for lactoferrin (Embleton et al. 2013). Lactoferrin, a member of the transferrin family, is a key component of the mammalian innate response to infection. It is the major whey protein in human colostrum, and also present in tears, and other secretions. However, preterm infants ingest little milk in the first few days and thus have low lactoferrin intake. Lactoferrin has broad microbiocidal activity by mechanisms such as cell membrane disruption, iron sequestration, inhibition of microbial adhesion to host cells, and prevention of biofilm formation. Development of resistance to lactoferrin is improbable as it would require multiple simultaneous mutations. Lactoferrin remains a potent inhibitor of viruses, bacteria, fungi, and protozoa after millions of years of mammalian evolution. Lactoferrin has prebiotic properties, creating an enteric environment promoting growth of beneficial bacteria and reducing colonisation with pathogens. It has direct intestinal immunomodulatory and anti-inflammatory actions mediated by modulating cytokine expression, mobilising leucocytes into the circulation, and activating T-lymphocytes. Lactoferrin enhances proliferation and differentiation of enterocytes, closure of enteric gap junctions, and suppresses free radical activity when iron is added to milk.

Recent research by our group relevant to MAGPIE proposal. Our data show that NEC and LOS are associated with abnormal gut microbial patterns including lack of diversity, presence of ‘marker’ bacteria and alterations in bacterial community structures (Stewart et al. 2012, 2013 and see figures p15 bottom right.) Differences in the presence of volatile organic compounds (VOCs) are also linked with the development of NEC (Garner et al. 2009), and may relate to the emergence of LOS. We have shown that patterns of VOCs in other settings predict the presence of key pathogens such as Clostridium (Garner et al. 2007 and see figures p15 bottom left), but no current studies combine microbiomic and metabolomic analysis in preterm infants using a systems biology approach. In addition, organisms causing blood culture positive sepsis in preterm infants primarily originate within the gut, typically as an abundant member of the community. Our group has contributed significantly to this data, optimising 16sRNA sequencing techniques and analysis, and showing close correlations between gut microbiota and NEC/LOS. We have shown how the microbiota change in response to other interventions (antibiotics), and

have explored associated changes in metabolites. Our data show a close correlation between a change in key VOCs in the few days before the onset of NEC (Garner et al. 2009), and a correlation between VOCs and microbial patterns in a mouse model (Zomer 2008). We hypothesise that differences in VOCs are driven by changes in the microbiome and may provide an early warning of dysbiosis. The trial interventions used in SIFT and ELFIN will exert effects on NEC and LOS via interaction with the gut microbial community, and can be explored using techniques in which we have expertise. Combined, our multi-disciplinary team collaborations provide us with powerful techniques to explore mechanisms leading to NEC and LOS, and the mechanisms of actions of enteral feeding interventions. We will explore the interaction between microbes and metabolites critical for development of gut immune function. Recent studies (Furusawa et al. 2013, Nature) highlight the importance of host-microbe interactions by demonstrating the pivotal role of short chain fatty acids (SCFA) and other compounds in inducing differentiation of gut regulatory T cells, a pathway of major importance in preterm infant NEC and LOS. Additionally, our expertise will enable exploration of the effects of early gut microbe/host interactions in establishing gut health and immune function. We have optimised techniques to understand gut inflammation in the presence of suppressed or dysregulated immune systems e.g. inflammatory bowel disease. We will utilise specialized immune-histochemical analysis of resected disease and explore causal pathways modulated by the enteral feeding interactions examined in SIFT and ELFIN, by comparing diseased cases versus controls, and examining cell surface markers and cytokines that link microbial and metabolomic changes (See figures p15 top right).

Aims and objectives

The aim of the study is to explore mechanistic differences between trial intervention arms (feed rate, lactoferrin), and dynamic changes in the period preceding disease onset (NEC/LOS). We will explore how the interventions affect relationships between metabolic function and gut microbiota. We will determine effects in both the stool and urinary metabolome because these changes represent mechanistic actions in both bacterial and host metabolism. We will model the dynamic changes to gut microbiota where there is already proof-of-principle that they are associated with disease. Specifically, we will determine changes in community structure i.e. relative abundance of specific taxa e.g. Clostridia (Sim et al. 2014) and overall abundance or bacterial load.

Our specific aims are to test the following hypotheses:

1. Supplemental enteral bovine lactoferrin will result in detectable differences in metabolic function that will be directly related to dynamic changes in the gut microbiota;
2. Faster increases in the rate of milk feeds will result in a different metabolic profile (to slower increases), and these differences will be directly related to changes in the gut microbiota;
3. Infants who develop NEC or LOS will have a different metabolic profile in the period preceding disease onset compared to control infants; metabolic profiles will differ between trial interventions arms; differences will be related to dynamic changes in the gut microbiota;
4. There will be detectable differences in gut tissue inflammatory response (e.g. white cell subsets) between surgically resected gut tissue affected by NEC and control tissue.

Our analytical models will explore the interacting effects of key clinical risk factors already known to be associated with disease (such as gestation, illness severity, and exposure to interventions such as breastmilk and antibiotics), as well as effects on other key outcomes (such

as time to full feeds, age at discharge and pre-discharge weight gain, see figures p15 upper left for simplified model.) We will achieve this by:
1) Recruiting 480 infants from up to 10 neonatal units taking part in ELFIN to an additional study MAGPIE (Mechanisms Affecting the Gut of Preterm Infants in Enteral feeding trials).
2) Collecting a daily stool and urine sample from MAGPIE infants until hospital discharge (average time 40-50 days). Retrieve any residual resected gut tissue of enrolled infants who undergo intestinal surgery. Transport anonymised samples to central labs.
3) Identifying samples which are the most a) **informative** - based on trial intervention and disease presence b) **comprehensive** i.e. consistency of daily sampling and c) **representative** - balanced for trial intervention and other key factors e.g. gestation, breastmilk exposure
4) Samples to be analysed will include a) all diseased cases: MAGPIE infants who meet the SIFT & ELFIN internationally agreed predefined case definitions of confirmed NEC or LOS (expected total n<100 infants); b) non-disease cases: <200 infants who do not develop NEC or LOS, selected using matching algorithms to ensure trial intervention and risk factors coverage.
5) The samples analysed will focus on the early postnatal period when trial intervention differences will be greatest: e.g. day 0-3, day 7, 10, 14 & 21 (+/-1 day) in 25-50 infants per trial intervention arm). We will also analyse at additional time points as necessary to ensure a daily sample is analysed for <7 days before diagnosis in all diseased cases and non-disease controls (n~1,000 total microbiomic samples, and 500-1000 metabolomic samples analysed).
6) Analysing samples a) **gut microbiota**: Next Generation Sequencing using MiSeq to determine gut microbial patterns b) **VOC** stool metabolomic profile using Mass spectrometry (LC-MS) to determine fecal metabolome and 'headspace' GC-MS to determine the presence of gut Volatile Organic Compounds (VOCs) c) **urine metabolomic profile** using LC-MS and assays for inflammatory proteins e.g. intestinal fatty acid binding protein (iFABP).
7) Determining changes due to trial interventions, and changes preceding disease onset. Explore dynamic changes in the gut community structure: proportions of key bacterial operational taxonomy units (OTUs), presence of specific pathogenic strains, diversity and stability of communities and functional metabolomic changes that reflect microbial or host metabolism.
8) Analysing resected gut tissue using optimised immuno-histochemistry to determine gut immune response where NEC develops, and explore how trial interventions (feed rate, lactoferrin) and changes in gut microbiota or metabolome relate to histological findings.
9) Storing residual samples in HTA and REC approved Newcastle Biomedicine Biobank.

Standard Operating Procedures. Samples will be collected, anonymised and analysed according to established SOPs already developed by members of the project team. Frozen samples will be stored at -20^{0}C at the local hospital before transfer to central labs -80^{0}C storage facility. Couriers will transport anonymised frozen samples every 6-8 weeks (duration of local storage 1-8 weeks, average 3 weeks). Disease is sporadic and unpredictable, therefore our SOPs require over-sampling. Any gut tissue resected during surgery for either NEC, or other conditions (e.g. spontaneous intestinal perforation) will be retrieved from paraffin blocks after all clinical tests have been complete.

Methods.

Design and delivery. Mechanistic evaluative study using non-invasive biological sampling in infants already participating in a randomized controlled trial.
Setting: 10 ELFIN approved tertiary level neonatal units (includes at least 5-6 centres where GI surgery occurs, and other tertiary units who refer infants for NEC surgery to those 5-6 units) will

recruit to MAGPIE. The centres who have agreed to participate are represented by the applicants or other sites with a proven track record in neonatal trials: Royal Victoria Infirmary Newcastle, Birmingham Women's Hospital, Leeds General Infirmary, Bradford Infirmary, Sheffield Hospital (Jessop's Wing), James Cook University Hospital (Middlesbrough), Sunderland Royal Infirmary, University Hospital of North Tees, University Hospitals of Leicester and City Hospital Nottingham. If needed, further sites can be selected on the basis of recruitment success to ELFIN. Funding will enable additional sites to be set up if required. 480 infants will be recruited.

Eligibility: Preterm infants already consented to SIFT or ELFIN, where additional parental consent for sample collection has been obtained**.**

Exclusions: Lack of informed consent.

Enrolment and consent: Parents can be approached for consent several days after consent for ELFIN is obtained, but usually within the first 3-4 days. An explanation will be given by research staff, followed by written information and an opportunity to ask questions. Parents can have several days to decide. Written consent will be obtained and will explicitly state the intention to share and use data collected for ELFIN.

Case report forms and other data. We will use SIFT and ELFIN data, and supplement this with a small number of additional items for MAGPIE that will include: maternal exposure to antibiotics (including type), antibiotic type, and prophylactic antifungal and probiotic strain/brand. We will record this data on paper forms at individual sites. We will either develop a web-based interface to upload this additional data, and data relating to which daily samples have been collected, or manually update the database. Programming support to develop this will be provided by the NPEU team managing the SIFT and ELFIN trials.

Methods.

Sample collection: Daily stool and urine will be collected by the bedside nurse from admission to hospital discharge. A plan to obtain daily samples is essential because 1) it is more reliably performed 2) it enables more accurate correlation of actions to interventions 3) the gut actions of the interventions may differ as infant development progresses, and 4) onset of NEC and LOS is highly variable and may occur at any time during hospital stay. Samples obtained before consent (days 0-7) have been specifically ethically approved in our existing studies: we will be explicit about this at consent and discard all samples where parents decline to enroll. Stool is collected from the nappy using a clean 'spoon' into a glass vial suitable for both VOC and microbiota analysis. Urine is collected from a cotton wool ball placed in the nappy, aspirated using a sterile syringe and aliquoted into two 2mL cryovials. All samples will be placed in a labelled plastic bag and frozen in a local freezer at -20°C. Samples will be anonymised and labelled with a unique code by research nurses. Gut tissue resected for clinical reasons will be retrieved after standard tests have been completed. Paraffin blocks are routinely made from such tissue and stored indefinitely and we will obtain consent to access these tissues.

Sample transport: Trial manager will liaise with research teams in centres to transfer samples on a 6-8 weekly basis, or more frequently if needed. Commercial courier will transport frozen samples to central laboratory at Northumbria University and stored in a -80°C freezer. Samples will be archived by RA with support from lab technician. Samples for analysis will be identified, and relevant samples sent by courier to Liverpool for VOC analysis. Gut tissue will be salvaged from a) infants with a diagnosis of NEC (diseased and healthy margin tissue) b) infants having resections without a definitive diagnosis of NEC e.g spontaneous perforations. Paraffin blocks will be transported using the same systems to the Newcastle University immunobiology lab. Residual stool and urine samples will be stored according to SOPs in HTA and REC approved

Newcastle Biomedicine Biobank (http://www.ncl.ac.uk/nbb/). HTA licence no. 12534, Ethics approval 15/NE/0334, IRAS 161883

Case definitions of disease: we will use internationally accepted definitions used by SIFT and ELFIN. **Late Onset Sepsis:** Microbiological culture of potentially pathogenic bacteria (including coagulase-negative Staphylococci species but excluding probable skin contaminants) or fungi from fluid sampled aseptically more than 72 hours after birth from blood or CSF **AND** treatment for 5 or more days with intravenous antibiotics. If the infant died, was discharged, or was transferred prior to the completion of 5 days of intravenous antibiotics, this condition would still be met if the intention was to treat for 5 or more days. **NEC:** NEC may be diagnosed at surgery, at post-mortem or clinically and radiologically: At least one of the following clinical signs present: Bilious gastric aspirate or emesis, abdominal distension or occult or gross blood in stool **AND** at least one of the following radiological features: Pneumatosis intestinalis, Hepato-biliary gas or Pneumoperitoneum. Infants who satisfy the definition of NEC but at surgery or post-mortem have a "Focal Gastrointestinal Perforation" will not be coded as having NEC.

Methods. Sample selection for analysis: all diseased cases meeting the SIFT and ELFIN predefined case definition of NEC or sepsis will have samples analysed (expected total n=70-100). Up to 200 non-diseased cases will be selected to ensure sufficient coverage of intervention arms using matching algorithms and coverage of other clinical risk factors and outcomes. Clinical team members will work with the statistical team to develop and refine models of disease. Rushton team will develop analytical models to test the hypotheses (see statistical section below). We will record sample storage duration at -20°C and at -80°C, and conduct analysis throughout the study period in a chronological fashion in order to adjust, where possible, for any confounding introduced by varying storage durations.

Freezing temperature, duration and sample stability. Short-term storage of stool samples does not significantly affect the microbial communities (Lauber et al. 2010, Wu et al. 2010), although measurement of storage time of samples is important to avoid potential bias (Bahl et al. 2012). We will freeze sample at -20°C immediately after collection. Our studies show that freezing for weeks or months at -20°C is suitable for microbiomic and metabolomic tests including VOC analysis. It would not be practically possible in a large multicentre study, to store samples in -80°C immediately after collection. We will transport anonymised samples in dry ice from the local freezers (average 3-4 week storage) for longer term storage at -80°C. This will avoid freeze thaw cycles during transport facilitating optimal preservation (Cardona et al. 2012). **Sample storage quality control**. To determine any storage effects, we will conduct quality control by comparing gold standard immediate DNA extracts from 10 samples to the same number following storage of stool for 12 and 18-24 months, and conduct a similar analysis for VOCs. For experimental samples we will record storage duration and conduct analysis at similar storage times where possible. If there is a loss of specific taxa, species or VOCs over time we will adjust for this where possible in the modelling analysis.

Methods. Laboratory procedures – stool & urine.
Stool Microbiome (Northumbria): We will analyse bacteria DNA extracted from stool samples using our well established protocols (Stewart et al Acta 2012, ADC 2013) and 16S ribosomal RNA methods that are effective tools to explore the diversity of bacterial communities (Stewart et al. Acta 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 <99% coverage of the bacterial community.

Stool & urine metabolome (Northumbria): we will conduct metabolomic profiling of stool and urine. 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 of the stool metabolome reflects changes in gut microbial activity and impacts of host gut function such as changes in permeability. Our non-targeted approach will allow us to compare metabolomic differences between trial enteral feed groups in order to define a metabolite pattern associated with sample groups that can be correlated with information on microbial diversity, health or disease. We have optimized a LCMS method based on C18 reverse phase chromatography coupled to a Q-Exactive high resolution mass spectrometer. This method is validated on 100mg of stool and has demonstrated the robust and reproducible detection of 10^3 metabolites. Identification of significant metabolites will be based on data dependent tandem MS/MS and confirmed using standards. Analysis (see statistics section below) will use modeling techniques to explore relationships between the microbiome and metabolome, and study interventions and disease. 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. To supplement the metabolomic data we will also analyse urine and stool samples using assays for proteins such as calprotectin and iFABP.

Stool metabolomic VOC analysis: VOCs from stool samples will be analysed by GC/MS using well-established protocols for extracting and analysing headspace gases and tested in our recent study (n>1200 DOVE study). We have recently published our methods (Reade et al 2014) which is based on a CARB/PDMS SPME fibre, a Combipal sampler with a Peltier-cooler, and a Perkin Elmer Clarus 600 Gas Chromatograph with Clarus 600T Mass Spectrometer. This is 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.

Methods. Laboratory procedures – Immuno-histochemistry on resected gut specimens: The MAGPIE study provides a unique opportunity to explore gut actions of feeding rate (including age at start and full feed) and lactoferrin: these include immuno-modulatory and anti-inflammatory actions mediated by modulating cytokine expression, mobilising leucocytes into the circulation, and activating T-lymphocytes. Breastmilk and lactoferrin enhance proliferation and differentiation of enterocytes, and closure of enteric gap junctions (Embleton et al. 2013). Tissue-based analyses will take place in two domains: 1) Exploring the aberrant innate and adaptive immune mechanisms; 2) Validating immune pathways or biomarkers identified by microbiomic or metabolomic profiling.

Approximately 5-10% will develop NEC, of whom 20-30% will progress to surgery, meaning that from our 480 MAGPIE infants we expect <10-20 resected specimens to be available. Immunohistochemistry will be performed using paraffin blocks cut into 4 μM sections for staining using a Benchmark XT autostainer (Ventana Medical Systems, Inc., Tucson, AZ, USA). Whole slide images will be acquired using a ScanScope digital slide scanner (Aperio ePathology solutions, Vista CA, USA), using techniques perfected by our group at Newcastle. Antigens of interest will be quantified digitally. Leukocyte infiltrates associated with NEC will be identified using antibodies to several epitopes including: Inflammatory cell subset markers (e.g. CD45, CD3, CD8, CD4, γδTCR, αβTCR etc.)**;** cellular proliferation - Ki67; cytotoxic granule expression - perforin, granzyme B; and Lineage markers/cytokines e.g. FoxP3, Tbet, RORγt, GATA3. These antibodies have been previously optimised for intestinal tissue by the team at Newcastle (Figure 5) therefore there are no optimisation costs. Our group has previously explored intra-epithelial T cell numbers in the GI tract of healthy adults (see figure): this provides data to power our assumptions for any differences we observe between intervention groups or between diseases and control. Staining of 'healthy' resection margins from the same patient will be undertaken in each case and control analyses and by use of matched non-NEC control tissue collected from MAGPIE infants supplemented if needed by further samples from the Newcastle upon Tyne Hospitals NHS archive (e.g. cases of spontaneous neonatal perforation). Targeted exploratory histology will validate immune mechanisms highlighted by metabolomics or microbial assays e.g. dysregulated innate immunity (e.g. IL-8); markers of oxidative stress (e.g, 3-nitrotyrosine); epithelial barrier dysfunction (e.g. claudin 3 and E-cadherin); and specific microbial communities (e.g. adherent-invasive e-coli). In-situ hybridisation may also be performed to identify transcription of relevant immune pathways identified by VOC, microbiotic or metabolomic profiling.

Sample size. Sample size requirements for each element of this study have been carefully evaluated, are based on both published data and practical aspects from our existing work and that from other groups, and include:

1. the size, duration, estimated recruitment rate and feasibility of the SIFT and ELFIN trials
2. **trial efficacy and disease event rate**: the incidence of NEC is expected to be 5-10% and for LOS <20%, and some infants will have both diseases; if the interventions reduce LOS to 15% and NEC to <5% (based on data powering the SIFT/ELFIN trials) the combined incidence is still ~20%. Recruiting n=480 infants will identify ~70-100 'disease' cases, 10-20 cases requiring surgery, and provide well sampled 'non-diseased' infants with varying clinical risk factors, exposures and outcomes.
3. **VOC analysis** may indentify several individual compounds; in our recent paper publishing the methodology (Reade et al. 2014) we show that on average 31.3±10.5 (Mean and SD) VOCs were identified per sample. At a power of 80%, and significance at the 5% level, we would need n=50 infants per trial intervention group to show an increase in 5 VOCs. The SD for each sample was 2.9±1.3 compounds and on average 90% of the VOC abundances showed a coefficient of variation smaller than 30%. Our data also showed in a study of n=13 infants, that VOC number in healthy neonates significantly increased with age (0.49 extra VOCs per day 95% CI 0.12-0.86), a trend not seen in those who developed NEC (Garner et al. 2009.
4. **Microbiomic data complexities** (and see statistical section below) means that the sample size necessary to evaluate the actions of different interventions and the incidence of disease is dependent on effect size, the number of interacting factors and their correlation. For a power of 80% to detect a 50% difference in community profile patterns arising from a categorical descriptor of microbial community variation, using a two-sided test at a

significance level of 5%, the study needs approximately 200 samples. In our previous studies 1) 12 twin pairs analysed (gut microbial profiling) using PLS-DA showed highly significant correlations between pairs (Stewart et al. PLOSOne 2013); 2) Examination of 136 samples from 32 patients (n=20 NEC or LOS) showed significant differences (p=0.002) in microbiomic patterns between diseased and healthy individuals (Stewart et al. ADC 2013).

5. **Immuno-histochemical (IHC) analyses**: Using IHC data from our group (Kirby et al. 2003), assuming that any differences between disease and control will be greater than those in healthy individuals, and using a two-sample t-test, our proposed sample size of 20 would give 80% power to detect a difference of 0.66 (66%) in cells/crypt cross section at a significance level of 5% and an approximated SD of 0.5 (50%).
6. **Models**. Mixed effects models, which we propose for modelling the direct effects of the microbiome on disease risk, are economical with power because the residual variance of these models is smaller since some of it is accounted for in the random effect. Using the method of Cohen (1988 Statistical Power Analysis for the Behavioral Sciences. Psychology Press, New York), we calculate that a GLM would have a power of 0.817 with 10 predictors and n=50 for a 'large' effect size (f^2=0.35, R^2=0.51) at the 5% level of significance, and a power of 0.469 for a 'medium' effect size (f^2=0.15, R^2=0.36) at the 5% level of significance. With n=100 the calculated powers are 0.999 for a large effect size and 0.744 for a medium effect size. Including random effects in the GLM would increase power at the same effect size, or conversely permit smaller effect sizes at the same power. The power analysis of an SEM is altogether more complex because it relies on the goodness of fit criteria selected for the model. Power at the model level may be low when there are few model degrees of freedom even for a reasonably large sample size; requiring greater than 100 samples if there are fewer than 20 model degrees of freedom (Kline 2011 Principles and Practice of Structural Equation Modelling, Guildford Press, New York). We will supplement these models where needed using a Bayesian SEM approach.

Statistical analysis. The analytical approach used in this study will be concerned with modelling the relationship between putative risk factors, trial interventions and incidence of NEC and LOS from longitudinal data capturing variation in both risk factors and disease through time. We will use a progressive modelling strategy based on combining multivariate analyses of micro- and metabolomic data, with mixed effect modelling of outcome in relation to drivers of disease; and finally, structural equation modelling (SEM) to quantify the importance of interacting factors and drivers. Specifically we will:

A) Quantify the impacts of individual risk factors in causing disease;
B) Quantify the effects of direct and indirect risk factors on disease;
C) Identify the impacts of trial interventions and clinical management on the drivers and disease.

There are three major modelling issues that have to be addressed in this analytical pathway.

i) the multivariate nature of the microbiome, metabolome and immunohistochemistry;
ii) the longitudinal/developmental component in the neonate, which will lead to repeated measures of disease state and microbiome on the same individuals and
iii) the interdependence and interactions between different predictors which may have both direct and indirect effects on incidence and progression of disease.

Analysis of microbiome and metabolome. The microbial data may be over-parameterised depending on the taxonomic level at which analysis is carried out. At a strain level we will have

more microbial strains present in samples than there will be samples taken. However, due to current limitations in sequence read length we have shown that at an Operation Taxonomy Unit (OTU) level (that is sequences with >97% similarity which corresponds to species level), we identify considerably less OTUs (138) after normalisation than samples forecast for the proposed study (Stewart et al. 2014).

We will use multivariate ordination techniques to summarise the major trends in variation in microbial community composition of faeces collected from individuals. The analyses will also allow us to identify those taxa most closely associated with microbiome change through time. We will then use canonical ordination to quantify the impacts of other covariates (diet, age, interventions) on the microbiome composition. We will identify key microbial taxa in the ordination space that capture the trend in variation in relation to NEC and use these with the results of the ordination as input variables for the subsequent mixed effect and SEM analyses below. The applicants have extensive experience in use of multivariate techniques in analyzing microbial communities and more pertinently BBSRC funding to investigate successional changes in gut microbiomes in livestock. Similar approaches will be used to analyse the faecal metabolome, but these data will be used as outcomes in the subsequent modelling rather than inputs. The analytical approach will also be used to consider the variation in immunohistochemical data.

Quantifying the direct and indirect impact of risk factors on disease*.* We will use repeated measure mixed effect modelling to quantify the direct effects of microbiome on risk of disease whilst adjusting for age, sex and feed and treatment history. We will use case as the random effect and adjust for autocorrelation in the response using appropriate correlation structures in the model. The applicants have used this approach extensively to investigate the epidemiology of food borne pathogens (Rushton et al. 2009). We will use a SEM approach to quantify direct and indirect effects of risk factors on the incidence of disease. We will develop a set of conceptual models which characterize the relationship between putative predictors; the changing microbial flora, administration of antibiotics, probiotic combinations, developmental stage (postnatal and postmenstrual age) and co-morbidities leading to disease onset and then challenge the model with the data derived from the laboratory and clinical data collection. The applicants have used SEM to analyse complex systems in a range of clinical settings ranging from microbial colonization in intensive treatment units (Rushton et al. 2010), through to life course outcomes with chronic disease and patient survival following hip fracture repair (Khan et al. 2013). This approach is particularly useful in the analysis of disease systems where there are many interacting drivers.

Approach to analysis of blinded samples. The ELFIN study is double blinded so it is important that clinician and trial team blinding is maintained during the main trial. We will conduct analysis on anonymised samples whilst recruitment continues so laboratory and statistical teams are blinded to trial interventions. Due to study timelines, analysis on ELFIN recruits will take place at a stage when recruitment to the main trials is still in progress, so the CTU database team (NPEU) will provide the statistical team with codes that allow ELFIN samples to be grouped. Blinding will also be maintained whilst gut tissue is examined.

Ethics. We will seek additional REC approval for this study and do not consider there are any significant risks. This MAGPIE study will mean that parents who have already consented to an interventional study will receive a second approach for consent. All trial centres are experienced in recruiting infants to multiple trials. Our experience (and that reported in the literature) is that

this can be conducted appropriately and sensitively, and that parents in this situation are capable of making an informed decision about whether they wish to participate. (Beardsall et al. Lancet 2008). We have published our experiences of approaching parents for this type of study (Embleton et al. Acta Paed 2013), and have already successfully recruited babies who were enrolled to the SIFT study, to also join the DOVE study (complete) and our other ongoing SERVIS sampling studies (ongoing). This means that we will already have fully approved stored samples, with permissions for full analysis and ongoing storage available for analysis at the time MAGPIE commences. We will seek explicit parental consent to use and share any SIFT and ELFIN data already collected. Sample storage will continue to follow HTA guidance; information and consent is explicit that residual samples will be archived in the Newcastle Biomedicine Biobank if the parents consent.

Research Governance. MAGPIE will be sponsored by Newcastle Hospitals NHS Foundation Trust, as the employing institution of the lead applicant. The NPEU Oxford acts as the CTU for the main trials. The existing SIFT and ELFIN TSCs have approved MAGPIE, and will be notified of ongoing progress. We will bring experts from both to sit on an oversight Study Steering Committee (SSC) for MAGPIE. This study will not affect any other aspects of research governance for the main trials. As there is no DMEC for the MAGPIE study, EJ/NE will set up clear internal data processes that facilitate transfer of information from the NPEU to the Newcastle team conducting the study. NPEU team will help develop electronic web data entry for the additional MAGPIE data if this is more efficient than hard copy. NE will hold responsibility for research governance of the combined dataset; SIFT/ELFIN data, sample acquisition data, and sample analysis output data. Samples for lab analysis will be fully anonymised (trial number and site) using unique bar coding at the hospital of origin prior to transfer. The funders (HTA) and sponsor (Oxford University) of the main trials have been informed of this proposal. Residual samples stored in the Newcastle Biomedicine Biobank will come under the research governance structures that already exist for the Biobank.

Deliverability.
1) **SIFT Recruitment**: MAGPIE depends on successful recruitment to ELFIN. SIFT is recruiting ahead of schedule and finished recruitment in June 2015. Under existing REC permissions for the SERVIS study we have recruited >100 infants in SIFT prior to MAGPIE commencement, and we will analyse these along with the ELFIN samples.
2) **ELFIN recruitment**. The pilot phase started in June 2014 and succesfully completed in April 2015. The main phase will commence in July 2015.
3) **Sample collection**. We have previously collected, transported and analysed similar numbers of samples in the DOVE and SERVIS studies. We expect 10 centres could each recruit an average of 4 infants/month. We will conduct a project team meeting after 250-300 recruits to determine the range of gestations (the factor most strongly associated with disease) and utilize an adaptive recruitment strategy that focuses on the most vulnerable infants i.e. initially we will recruit all infants <32 weeks, but after that, assuming targets are met, we will aim to primarily recruit those with the greatest disease risk e.g. <28 weeks gestation.
4) **Interdependence** with ELFIN is essential. We will liaise with trial teams and managers to ensure the MAGPIE study does not interfere with the main trials. We will utilise existing governance structures and create an additional oversight TSC.

Futility Analysis: plan for statistical modelling at the end of phase 1
The MAGPIE study funder (EME) and the project team agreed that it would be appropriate to conduct a futility type analysis after approximately n=150 (of the total 480) infants had been

recruited, and prior to commencing ongoing analysis of the microbiome and metabolome. The results of this interim analysis will be presented to the EME board who will determine whether further analysis in phases 2 and 3 should be funded, or whether samples should simply be biobanked pending the results of the main ELFIN trial.

To determine whether mechanistic evaluation is futile, or not, we will quantify the action of Lactoferrin (LF) by measuring changes in gut microbial composition and analyse the two individual trial arms. The main CTU (NPEU, Oxford) will identify cases meeting the necessary criteria and provided blinded group information on these cases to the statistical team at Northumbria University. This means the clinical investigators who are part of the ELFIN project team will remain blinded to group allocation.

1. **select samples taken before disease onset at around 7-10 days after commencing the CTIMP in <60 infants per trial group** so as to compare and assess microbial community changes between trial groups. Because other risk factors are also associated with disease (e.g. gestation, age etc.) we will use progressive model building that investigates microbial community dynamics in relation to these covariates.
2. use multivariate ordination and classification approaches to quantify trial intervention group differences in gut microbial communities. This will (i) quantify the variation in microbial community composition before disease onset and (ii) develop suitable covariates describing microbial community variation for inclusion in statistical analysis as risk factors for disease.
3. use unconstrained ordination to investigate trends in community composition and to identify those microbial taxa from the NGS data which contribute most to community variation. These axes may be used as covariates in the subsequent analysis.
4. use constrained ordination to investigate the impact of trial intervention group on community variation prior to disease onset
5. use divisive classification approaches to identify classes of microbial community amongst the cases. This will create a suite of categorical descriptors of community composition. Higher level community descriptors (diversity; evenness; rate of change in ordination score) will be derived in case the overall structure of the community is an important driver.
6. **determine whether there is any effect on the total microbial community differences or on individual bacterial taxa using MANOVA and consider a difference in the microbial community of p<0.05 to be proof an effect. If the p value is between 0.05 and 0.1 we will consider this to be strong evidence of a likely effect, where statistical significance (p<0.05) might subsequently be achieved in an analysis with larger numbers and/or greater numbers of diseased or high risk cases**

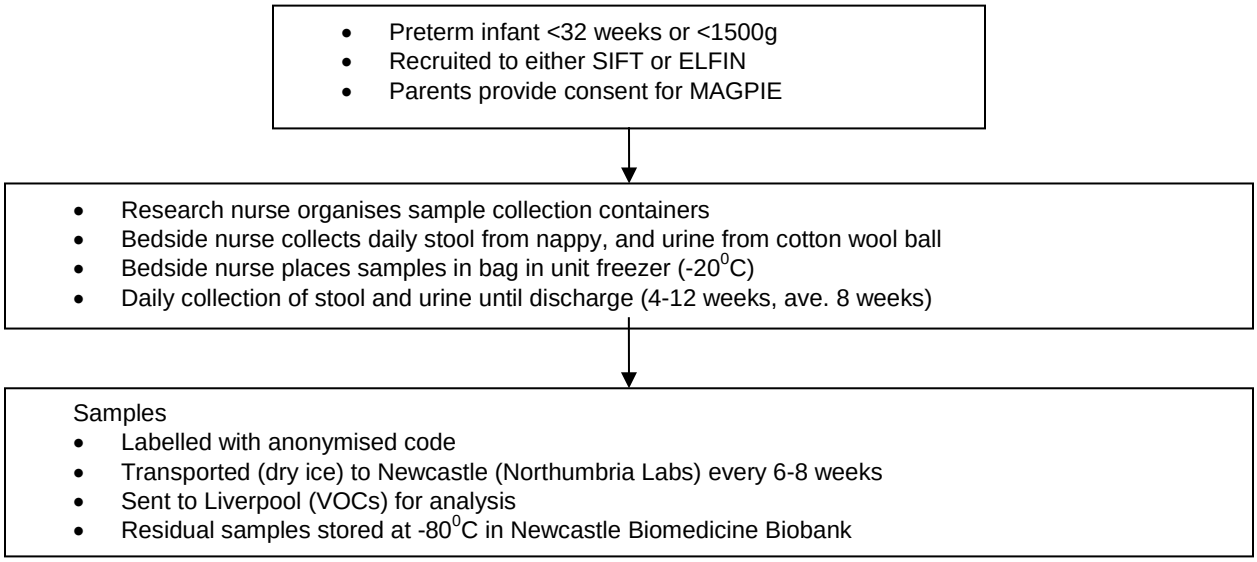
Expertise. NE/JB/JD/AE/WM: neonatologists with extensive clinical/research experience in neonates especially infection, nutrition, follow up, multicentre trials, necrotising enterocolitis; lead microbiomic/VOC studies in infants; PIs for ELFIN/SIFT. NE: chair of UK Neonatal Nutrition Network; nutrition committee ESPGHAN. JD/WM: CI for SIFT/ELFIN. JB&AE: MRCN CSG members. JB: CI for SERVIS study (microbiomic analysis). AE: CI for NIHR PulseOx study (n>20000); CI for DOVE study (n>1300). NE will coordinate study & sites, develop materials, organise sample ID/analysis, organise project meetings & report writing. JB&AE: refine collection SOPs; ID samples with trials manager(TM)/NE & coordinate transport. EJ: Head of Trials NPEU & experienced trials/statistical expert. JD/EJ: liaise with TM/NE, facilitate

identification of trial intervention arm when trials complete, facilitate data transfer from NPEU to Newcastle. SC/CL/CS: experienced researchers; published track record in microbiomic analysis including NEC/LOS. CL: coordinate statistical analysis; SC/CS: supervise RA for micro/metabolomic analysis. CP: academic gastroenterologist with track record in VOC analysis & relationship to infective & inflammatory diseases in adults; CP will supervise VOC analysis. JK/ChrL: experienced gastro-intestinal biologists and will lead immuno-histochemical analysis. CP/JK/ChrL are internationally recognised experts in the basic science of inflammatory bowel & other disorders in adults. SR/MS: extensive experience in modelling biological systems, & application of modelling techniques in biology & medical systems. SR utilises strategic modelling (where objective is to investigate general biological principles) and tactical modelling (where objective is to use modelling to solve specific applied problems). MS has specific interest in process-based models, network analysis & other analytical/statistical modelling; extensive collaborations involving ecological systems, invasive species & human disease.

Service Users. The main trials (SIFT and ELFIN) were both developed with the help of service users and members of the public. Alison Baum (CEO BestBeginings www.bestbeginnings.org.uk), a national charity campaigning for equalities in health care in early life is a member of the TSC for the SIFT study. The Head of Programmes at BLISS (Jane Abbot, www.bliss.org.uk), a national charity advocating on behalf of sick newborn infants, is a member of the TSC for ELFIN. Our group has previously collected stool samples in >350 infants (SERVIS) and n>1200 (DOVE). Parents have been very supportive, and many expressed a clear desire to help by contributing non-invasive samples. As part of the research governance processes for this trial, we would use the existing governance structures to support this study, which already include service user representatives, and work with Bliss to identify a suitable parent representative to join the oversight TSC.

Flow diagram

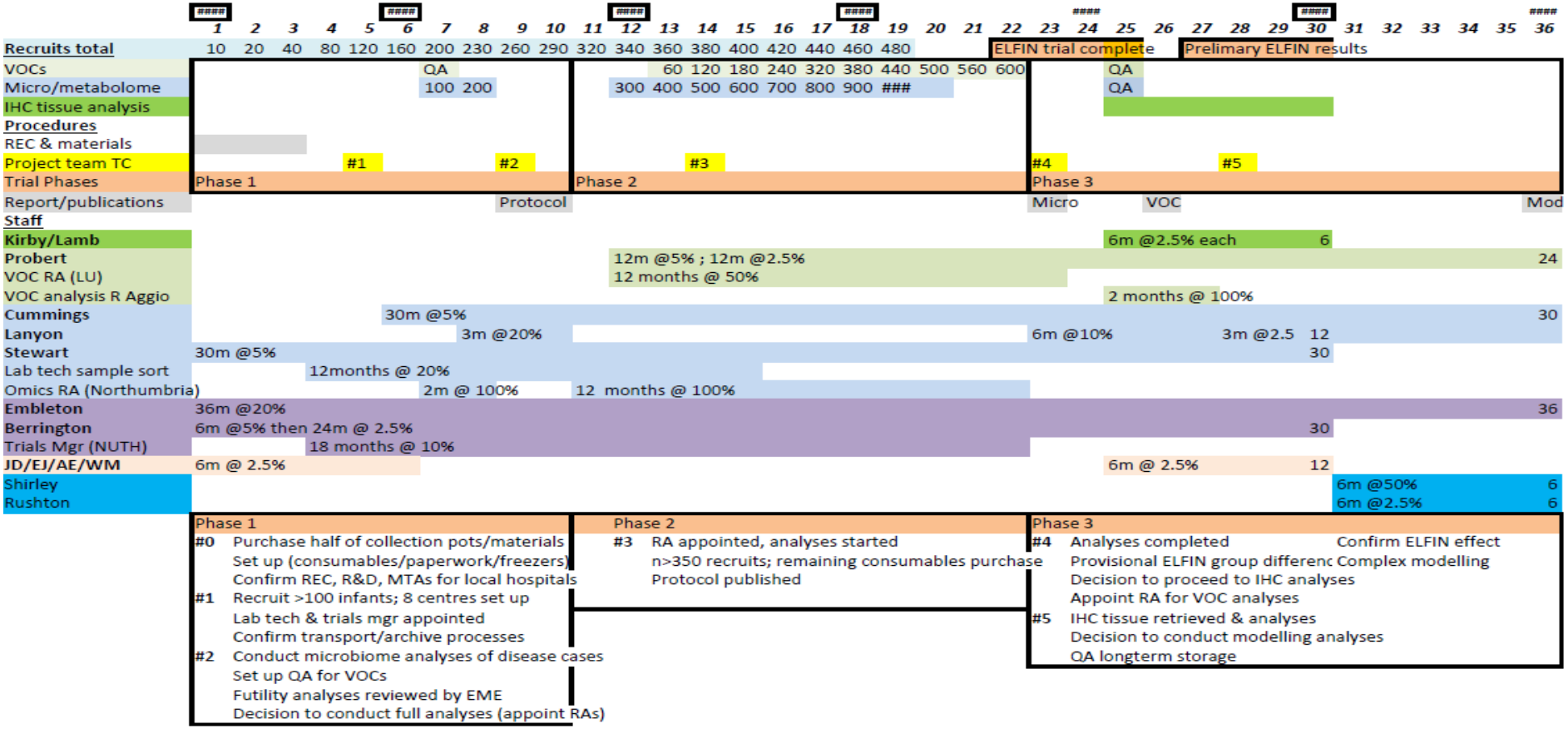
Mechanisms Affecting the Gut of Preterm Infants in Enteral feeding trials (MAGPIE)
Chief Investigator. Dr ND Embleton, Royal Victoria Infirmary, Newcastle upon Tyne UK



Mechanisms Affecting the Gut of Preterm Infants in Enteral feeding trials (MAGPIE)
Chief Investigator. Dr ND Embleton, Royal Victoria Infirmary, Newcastle upon Tyne UK

Project timetable: Start project = 01 February 2016 (month 1). **Month 1-5**: prepare study materials with PPI representatives; develop supplementary case record forms & web access; obtain REC, identify site PIs, R&D & SSI; appoint RA & trials manager; purchase consumables & distribute, set up meetings; liaise with pathology departments to establish SOPs; organise project team meeting #1. **Month 6-11**: recruit 30-40 babies/month; collect daily samples & store; transfer samples to labs or biobank; project team meeting #2 & #1 TSC with main trials; ensure tissue collection operational; write protocol for open-access publication, develop & pilot statistical models. **Month 12-17:** recruit 20-30 babies/month; micro/metabolic/VOC analysis of 5-10 samples from <100 babies **7. Month 18-23**: recruit 20 babies/month; micro/metabolic/VOC analysis of 500 samples; project team meeting #3. **Month 24-29:** recruit 20 babies/month; complete micro/metabolic/VOC analysis of 500 samples; immuno-histochemical analysis of retrieved tissue; appoint PDRA for VOC stats analysis at Liverpool. **Month 30-36**: complete any remaining micro/metabolic/VOC analysis; complete statistical analysis; organise project team meeting #4 & writing committee; complete paper writing of report +/- publications.

Mechanisms Affecting the Gut of Preterm Infants in Enteral feeding trials (MAGPIE)
Chief Investigator. Dr ND Embleton, Royal Victoria Infirmary, Newcastle upon Tyne UK



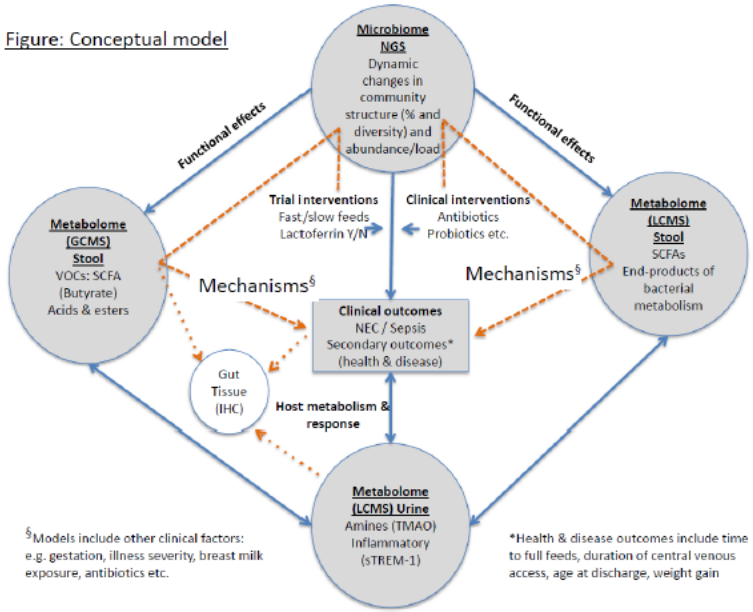
References

1. Arpaia N, Campbell C, Fan X et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature 2013; 504:451–5.
2. Bahl MI, Bergström A, Licht TR. Freezing fecal samples prior to DNA extraction affects the Firmicutes to Bacteroidetes ratio determined by downstream quantitative PCR analysis.FEMS Microbiol Lett. 2012;329:193-7.
3. Beardsall K, Brocklehurst P, Aghluwalia. Should newborn infants be excluded from multiple research studies? Lancet 2008; 372: 503-505
4. Berrington JE, Hearn RI, Bythell M et al. Deaths in preterm infants: changing pathology over decades. J Pediatr 2012; 160:49–53.
5. Cardona S, Eck A, Cassellas M, et al. Storage conditions of intestinal microbiota matter in metagenomic analysis. BMC Microbiology 2012; 12:158 doi:10.1186/1471-2180-12-158
6. Embleton ND, Turnbull E, Turner S and Berrington JE. Successful blood salvaging from preterm infants: maximising opportunities, minimising interventions. Acta Paediatrica 2013; 102: e527-529
7. Embleton ND, Berrington JE, McGuire W. Lactoferrin: Antimicrobial activity and therapeutic potential. Seminars in fetal & neonatal medicine 2013; Mar 15. doi: 10.1016/j.siny.2013.02.001
8. Furusawa Y, Obata Y, Fukuda S et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature 2013; 504:446-450
9. Garner CE, Ewer AK, Elasouad K, Power F, Greenwood R, Ratcliffe NM, Costello BedL, Probert CS. Analysis of faecal volatile organic compounds in preterm infants who develop necrotising enterocolitis: a pilot study. J Pediatr Gastroenterol Nutr. 2009; 49:559-565
10. Kirby JA, Bone M, Robertson H, et al. The number of intraepithelial T cells decreases from ascending colon to rectum. J Clin Pathol 2003;56:158.
11. Khan SK, Rushton SP, Dosani A, et al. Factors influencing length of stay and mortality after first and second hip fractures: an event modelling analysis. Journal of Orthopaedic Trauma. 2013; 27(2):82-86.
12. Lauber CL, Zhou N, Gordon JI, Knight R, Fierer N. Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. FEMS Microbiol Lett. 2010;307:80-6.
13. Manzoni P, Rinaldi M, Cattani S et al. Bovine lactoferrin supplementation for prevention of late-onset sepsis in very low-birth-weight neonates: a randomized trial. JAMA 2009; 302:1421–8.
14. Neu J, Walker A. Necrotising enterocolitis. New Engl J Med 2011; 364:255-264
15. 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 metabolomics studies. Journal of Analytical and Bioanalytical Techniques. 2014; 5: 184. doi:10.4172/2155-9872.1000184
16. Rushton SP, Humphrey TJ, Shirley MDF, et al. Campylobacter in housed broiler chickens: a longitudinal study of risk factors. Epidemiology and Infection. 2009; 137(08):1099-1110.
17. Rushton SP, MDF Shirley, EA Sheridan, et al.The transmission of nosocomial pathogens in an intensive care unit: a space–time clustering and structural equation modelling approach. Epidemiology and infection 2010;138 (06), 915-926
18. Sim K, Shaw A, Randell P et al. Dysbiosis Anticipating Necrotizing Enterocolitis in Very Premature Infants. Clin Infect Dis. 2014 doi: 10.1093/cid/ciu822
19. Stewart CJ, Nielson A, Scribbins D, et al. Bacterial and fungal viability in the preterm gut: NEC and sepsis Arch Dis Child Fetal Neonatal Ed. 2013;98:F298-F303
20. Stewart C, Marrs E, Magorrian S et al. The preterm gut microbiota: changes associated with necrotizing enterocolitis and infection. Acta Paediatr. 2012 ;101:1121–7.
21. 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
22. Zomer S, Dixon SJ, Xu Y, et al. Consensus multivariate methods in gas chromatography mass spectrometry and denaturing gradient gel electrophoresis: MHC-congenic and other strains of mice can be classified according to the profiles of volatiles and microflora in their scent-marks. Analyst. 2009;134:114-23.
23. Wu GD, Lewis JD, Hoffmann C, Ying-Yu C, Knight R, Bittinger K et al. Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. BMC Microbiology 2010;10:206 doi:10.1186/1471-2180-10-206

24. Rushton SP, MDF Shirley, EA Sheridan, CV Lanyon, AG O'Donnell. 2010. The transmission of nosocomial pathogens in an intensive care unit: a space–time clustering and structural equation modelling approach. Epidemiology and infection 138 (06), 915-926

Mechanisms Affecting the Gut of Preterm Infants in Enteral feeding trials (MAGPIE)
Chief Investigator. Dr ND Embleton, Royal Victoria Infirmary, Newcastle upon Tyne UK

Figures



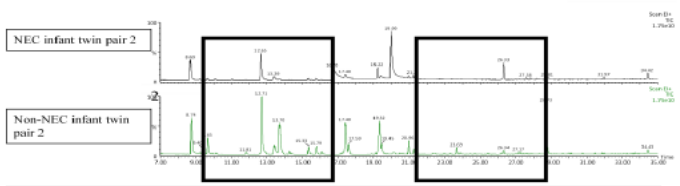


Figure Garner et al. 2009. Chromatograms from twin pairs examined using headspace VOC of faeces by GC-MS. A healthy twin-pair aged 18 days have very similar chromatograms (**left**). A twin-pair discordant for disease exhibit different patterns of VOCs, with some VOC appearing to have been lost from the child who developed NEC (**below**).

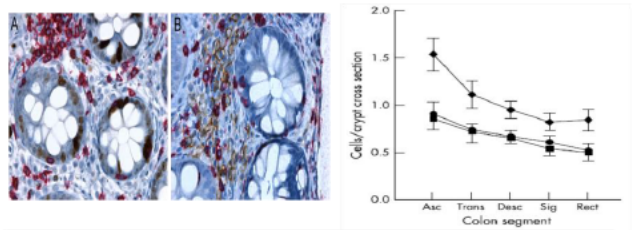


Figure: Intestinal leukocyte immunohistochemistry **A.** CD3+ T lymphocytes (red) and Ki67 (brown) **B.** CD3+ T lymphocytes (red) and CD19+ B lymphocytes (brown) **Figure 6 (right):** number of cells positive for CD3 (closed diamonds), CD8 (closed circles), and CD103 (closed squares) in each crypt from different colonic sections.

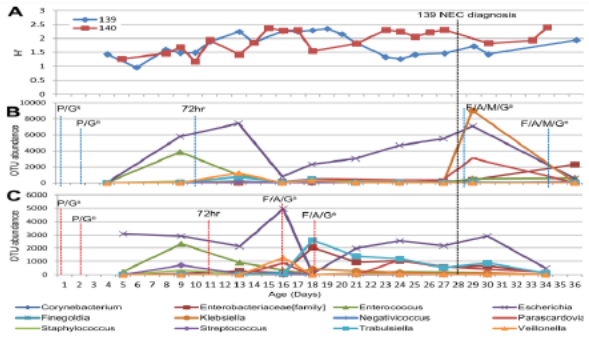


Figure (Stewart, PLoS 2013) – Bacterial analysis of the gut microbiota in preterm twins discordant for NEC. P – Penicillin, G – Gentamicin, F - Flucloxacillin, A - Amoxicillin, M – Metronidazole, s –Start of antibiotics, e – End of antibiotics, 72hr – full enteral feed (at least 150 ml/kg/day) sustained for 72 hours A) DGGE diversity indices. B) 454 pyrosequencing of NEC twin. C) 454 pyrosequencing of healthy twin. Most abundant bacterial genera show increase in Escherichia (later identified to species level as E. coli) and the significant effect of antibiotics.

Appendix: Examplar Standard Operating Procedures

Urine and Stool collection – collect daily samples

1. Use a sterile plastic spoon to retrieve stool from nappy, and place stool in glass bottle. Aim to half fill the bottle, or at least half a teaspoon of stool although smaller amounts are better than none. Collect a stool sample every day from birth until 34 weeks corrected age.
2. Place a clean cotton wool ball in nappy and squeeze into two 2mL cryovials using a syringe. Aim to fill both cryovials. If there is <0.5mLs of urine, replace cotton wool ball and wait for next urine.
3. Place both the urine and stool samples in plastic bag labelled with baby's hospital sticker and the current date to indentify the samples. It is simplest to put the plastic bag in the freezer as soon as complete. However, it is OK to leave at room temperature for several hours if staff are busy. Make sure the samples are clearly labelled before leaving at the bedside.
4. Complete the bedside MAGPIE log so it is clear that the samples were collected

Anonymisation of samples - once a week check freezer and label samples

1. Identify samples and label by using
    a. baby's ELFIN number
    b. collection date
    c. sample type to code the samples
2. E.g. if ELFIN number is D1357 and the stool sample was collected on 1st August 2015, the label is D1357_010815_S
3. E.g. if ELFIN number is E2468 and the urine sample was collected on 21st September 2015, the label is E2468_210915_U
4. If there is more than 1 sample collected then add a number e.g. E2468_210915_U1, E2468_210915_U2
5. Make sure the labels are well fixed and place in plastic bag back in freezer. The samples will all be identifiable but the name and hospital number stickers should be removed.
6. Complete the sample log sheet by writing on the barcode number
7. Photocopy the weekly sheet: store one copy in the baby's notes, and one copy in site file.
8. Put a new sample log at the bedside along with another week's worth of collection bags

Transport samples

1. Approximately every 6-8 weeks contact MAGPIE trial coordinator and arrange shipment of samples
2. Place samples on dry ice and send along with log sheets to Newcastle central labs
3. More frequent shipments can be arranged where there are lots of samples.
4. Defrost freezer

Mechanisms Affecting the Gut of Preterm Infants in Enteral feeding trials (MAGPIE)
Chief Investigator. Dr ND Embleton, Royal Victoria Infirmary, Newcastle upon Tyne UK

This baby is taking part in the MAGPIE study. Please collect a daily stool and urine sample. Place in freezer bag with hospital label

ELFIN study number (e.g. D1357) _____ Baby Name _____

Day of week	Date	Stool sample	Urine sample 1	Urine sample 2	Barcode number (research team)	Barcode number (research team)	Barcode number (research team)
Monday	21/08/15	Yes / No	Yes / No	Yes / No	D1357_210815_S	D1357_210815_U1	D1357_210815_U2

Research team only

Sample barcode date completed _____

ELFIN Gut signs etc, CRF checked _____

Name of person completing this form _____ Photocopy x 2 (original – site file, copy – baby notes, copy – freezer log file)

Mechanisms Affecting the Gut of Preterm Infants in Enteral feeding trials (MAGPIE)
Chief Investigator. Dr ND Embleton, Royal Victoria Infirmary, Newcastle upon Tyne UK

Shipment date _____ Name of person organising shipment _____

Notes: