The Effect of Glutamine Supplementation on Microbial Invasion in Surgical Infants Requiring Parenteral Nutrition – Results of a Randomised Controlled Trial

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Funding: We are very grateful to Sparks for a project grant supporting this work. Sparks had no involvement in study design, data collection, data analysis, manuscript preparation or publication decisions.
Registration no. ISRCTN54742344; http://www.controlled-trials.com

Category: Randomised clinical trial

Main findings previously presented at: American Pediatric Surgical Association (APSA) Annual Meeting, Marco Island, FL, USA, May 2013. 'The effect of glutamine supplementation on microbial invasion in surgical infants requiring parenteral nutrition – results of a randomised controlled trial'

Background: To determine whether parenteral plus enteral glutamine supplementation influences microbial invasion in surgical infants requiring parenteral nutrition.

Methods: An ethically-approved prospective double-blind randomised controlled trial studying surgical infants receiving parenteral nutrition for at least five days for congenital or acquired intestinal anomalies (July 2009 – March 2012). Infants were randomised to receive either glutamine supplementation (parenteral plus enteral; total 400mg/kg/day) or isonitrogenous control. The primary endpoint was microbial invasion evaluated after five days of supplementation and defined as either: i) positive conventional blood culture; ii) evidence of microbial DNA in blood (PCR); iii) plasma endotoxin level ≥50 pg/mL; or iv) plasma level of lipopolysaccharide-binding protein (LBP) ≥50 ng/mL. Data are given as median (range) and compared by binary logistic regression.

Results: Sixty infants were randomised and reached the primary endpoint. 25 patients had congenital/neonatal intestinal obstruction, 19 had anterior abdominal wall defects, 13 had necrotising enterocolitis. Thirty six infants showed some evidence of microbial invasion during the study: 17 of these were not detected by conventional blood culture. There was no significant difference between the two groups in the primary outcome: evidence of microbial invasion after five days was found in 9/31 in the control group and 8/29 in the glutamine group: odds ratio 0.83 (0.24 – 2.86; p=0.77).

Conclusion: More than half of surgical infants requiring parenteral nutrition showed evidence of microbial invasion. Approximately half of this was not detectable by conventional blood cultures. Parenteral plus enteral glutamine supplementation had no effect on the incidence of microbial invasion.

Keywords: Bacterial translocation, glutamine, necrotizing enterocolitis, neonatal intestinal obstruction, gastroschisis, neonates

Clinical relevancy statement:

Infants receiving parenteral nutrition following gastrointestinal surgery are at high risk of bloodstream infections, and possibly microbial invasion. The role of glutamine supplementation in these infants is so far unclear. This randomised controlled trial found no benefit of parenteral and enteral glutamine supplementation on microbial invasion (primary outcome). Glutamine supplementation did improve post operative recovery of monocyte function (planned secondary outcome analysis). Furthermore, in the subgroup of infants with low baseline monocyte function, glutamine supplementation was protective against microbial invasion. These findings are relevant for guiding clinicians treating these vulnerable infants.

Introduction:

Infants requiring gastrointestinal surgery for conditions including gastroschisis, congenital intestinal obstruction and necrotizing enterocolitis are unable to absorb enteral nutrition for a variable period postoperatively and thus depend on parenteral nutrition (PN). However, during PN, these infants are at high risk of infection with bloodstream infections found in 27% of surgical infants requiring PN[1], and 61% of surgical infants requiring PN for 28 days or more[2]. These infections may arise from the central venous catheter[3], or by bacterial translocation i.e. migration of micro-organisms from the intestinal lumen across the gut wall[4]. The high rate of these infections may be related to various factors, such as a deficiency of nutritional components which are found in enteral feeds but not supplied in parenteral nutrition, leading to worse immune function or loss of gut mucosal integrity[5].

The amino acid glutamine is not routinely provided in PN, but as it may become conditionally essential, various studies have investigated whether glutamine supplementation is of benefit to patients requiring PN. We have previously shown, in a multicentre randomised controlled trial of intravenous glutamine supplementation in surgical infants[1], that the incidence of sepsis was significantly lower in the glutamine group during the period of *total* parenteral nutrition (i.e. before the introduction of enteral feeds). In that trial, glutamine was only supplemented intravenously, so during the period of mixed enteral/parenteral feeding, the difference in glutamine intake between the two groups was decreasing as enteral feed increased (all enteral feeds contain significant amounts of glutamine).

The aim of this randomised controlled trial was to determine whether glutamine supplementation of both parenteral and enteral nutrition in surgical

newborn infants leads to a reduction in microbial invasion. In this trial the difference in glutamine intake was maintained during the entire study including the period of combined parenteral and enteral feeding.

Methods:

This was a prospective double-blind randomised controlled trial studying surgical infants receiving parenteral nutrition for at least five days for congenital or acquired intestinal anomalies at a single UK paediatric surgical centre and an associated neonatal unit.

Inclusion and exclusion criteria

Infants aged less than 3 months (corrected gestational age), admitted under the care of a paediatric surgeon for congenital or acquired gastrointestinal anomalies requiring abdominal surgery and parenteral nutrition, were eligible for the trial. Parenteral nutrition was indicated in all surgical infants who were not expected to tolerate enteral feeds for at least five days. These would include infants with gastroschisis, meconium ileus, necrotizing enterocolitis, bowel atresia or intestinal surgery for other reasons.

Infants who had already been receiving parenteral nutrition for 5 days or more before enrolment were excluded. Patients enrolled in another trial, or suffering from renal failure, inborn errors of metabolism or immune deficiency, were also excluded.

Ethics

Written informed consent was obtained from parents or guardians of all participants. The trial was registered with Current Controlled Trials (registration no. ISRCTN54742344; http://www.controlled-trials.com). Research ethics committee approval was obtained (Reference 08/H0713/31). A Data Monitoring and Ethics

Committee was convened which was independent of both the trial organisers and those providing therapy.

Randomisation

Infants were randomised to glutamine and control groups using balanced minimization[6] with the following criteria: length of functional small bowel (normal small bowel length – no intestinal resection, remaining small bowel length at least 30 cm, remaining small bowel length less than 30 cm); diagnosis (congenital intestinal obstruction, congenital defect of the abdominal wall, necrotizing enterocolitis, other); gestational age (derived from last menstrual period) at the time of enrolment in the study (less than 30 weeks, 30–36 weeks, over 36 weeks); ileocaecal valve in continuity without proximal diversion (yes, no); and weight at the time of enrolment in the study (less than 1 kg, 1–2 kg, over 2 kg). All factors were weighted equally and the randomisation weighting used was 4^6.

Treatment in intervention and control groups

In the glutamine group, parenteral glutamine was given as a chemically stable dipeptide solution (Dipeptiven®, Fresenius-Kabi, Runcorn, Cheshire, UK; *L*-alanyl-*L*-glutamine 200 mg/ml) in an initial dose of 0.4 g/kg/day glutamine equivalent to 0.6 g/kg/day Dipeptiven® which ensures that the nitrogen intake of the intervention and control infants is equal and that no more than 35% of the total nitrogen intake will be provided by Dipeptiven®. The dose is equal to that used in our previous randomised controlled trial which demonstrated that there were no negative effects from administration of glutamine and no abnormal levels of serum ammonia, urea nitrogen and glucose[1]. This level is also based on our published research which has confirmed beneficial effects on enteral mucosa at this dose[7]. All patients were prescribed not less

than 1.5 g per kg per day of amino acid while receiving total parenteral nutrition. No more than 35 per cent of total amino acids were replaced to prevent essential amino acid deficiency[8-10]. The control group received an isonitrogenous, isocaloric parenteral nutrition solution which contains no glutamine: Vaminolact® (Fresenius-Kabi, Runcorn, Cheshire, UK).

During the period of partial enteral feeding, in which the parenteral intake of glutamine/placebo was reducing, the enteral feeds were supplemented with the balance of glutamine which was no longer being given parenterally to maintain a total dose of 0.4 g/kg/day glutamine. This glutamine was given as Adamin-G® (SHS International Ltd, Liverpool, UK). The control group received isonitrogenous supplementation of enteral feeds using Complete Amino Acid Mix (SHS International Ltd, Liverpool, UK; contains 0.7% glutamine). Enteral feeds were standardised as far as possible. The following enteral feeds were used in the study protocol as clinically indicated: maternal expressed breast milk; SMA First Infant Milk or SMA Gold Prem 1 (SMA Nutrition, Maidenhead, Berkshire, UK) for infants not requiring modified protein / fat / carbohydrate. If cow's milk protein allergy was suspected Neocate LCP (Nutricia, Trowbridge, Wiltshire, UK) was used as first line treatment with Pepti-Junior (Cow & Gate, Trowbridge, Wiltshire, UK) as an alternative if a medium-chain triglyceride diet was clinically indicated. Due to the unstable nature of glutamine when heated, powdered feeds were made aseptically using sterile water at room temperature and were not pasteurised. The glutamine/complete amino acid supplement was weighed using scales accurate to 0.1g. Feeds were mixed well for 2 minutes before bottling to ensure that all the powder dissolved. Feeds were made for each infant once every 24hrs.

Blinding

Following patient recruitment, randomisation was performed by pharmacy. Parenteral nutrition prescriptions and the labels used for the parenteral nutrition bags specified the amount of nitrogen and not its source (whether control or glutamine). The total nitrogen content was specified on the bags to maintain clinical standards but, as the two groups received the same amount of nitrogen, this did not unblind care staff. All parents and staff (nurses, physicians, surgeons, etc.) involved in the care of the patients were blinded to allocation group, except for the parenteral nutrition manufacturing pharmacists and the dieticians preparing the enteral supplementation. The trial coordinator, who was responsible for patient recruitment, sample and data collection, was similarly blinded throughout and did not have access to the randomisation lists.

Endpoints

The primary end point was evidence of microbial invasion evaluated after five days of supplementation and defined as one or more of the following: i) positive conventional blood culture (blood cultures were read automatically using the BioMérieux BactAlert system [BioMérieux, Marcy L'Etoile, France]); ii) evidence of microbial DNA in blood by polymerase chain reaction (PCR); iii) plasma endotoxin level ≥0.5 Endotoxin Units/mL (50pg/mL)[11] ; or iv) plasma level of lipopolysaccharide-binding protein (LBP) ≥50 μg/ml[12-15]. Blood samples were taken at the beginning of the trial, at the introduction of first enteral feeding and when full enteral feeding was achieved (parenteral feeding was stopped when more than 75% of the patient's full nutritional requirement was tolerated enterally for a minimum of 24 hours). Samples were also taken during clinically suspected episodes of sepsis, defined as the clinical state of generalised inflammation manifested by at least 3 of the

following clinical signs: fever, hyperthermia (>38°C) and/or hypothermia (<36°C), lethargy, poor perfusion, age-related tachycardia and tachypnoea, and hypotension[16]. Microbial invasion was analysed both as a discrete event (comparing the number of patients who did or did not experience microbial invasion), and as an incidence rate of episodes of microbial invasion per 100 days (continuous variable). If the blood analysis showed evidence of microbial invasion in more than one way at a single time point, this was regarded as a single episode of microbial invasion.

Secondary endpoints included clinically suspected episodes of sepsis, time to full feeds, monocyte HLA-DR expression (as a marker of immune function), soluble CD14 (as an additional marker of microbial invasion) and growth (using serial measurements of infants' weight and head circumference). Infants remained in the study they achieved full enteral feeding, or died, or were transferred to a non-participating centre.

Blood sampling and initial processing

Samples of venous blood were taken at the time points described above using a standard aseptic technique. In addition to the conventional blood culture, 1.5 mL of whole blood was collected in EDTA for the other assays. Monocyte HLA-DR expression was determined by flow cytometry as soon as possible after collection (using 300μL of whole blood). 400μL of whole blood was stored at 4°C for subsequent DNA extraction. The remainder of the sample (500-800μL) was centrifuged at 1500rpm for 15 minutes. The supernatant plasma was pipetted into 0.5mL Elkay polypropylene microtubes and frozen at -80°C for endotoxin, LBP and soluble CD14 assays. All distribution and storage was performed using endotoxin-free, DNA-free pipette tips and containers. Original data and samples are stored in the

paediatric surgery department at UCL Great Ormond Street Institute of Child Health. Anonymised data can be provided on request by the corresponding author.

DNA Extraction[17]

DNA was extracted from whole blood, using the QIAmp DNA mini kit (Qiagen), following the manufacturer's protocol. Complete lysis of bacterial cells was ensured by using a Ribolyser cell disrupter (Hybaid) according to the manufacturer's instructions. Briefly, following Proteinase K digestion, Ribolyser resin for bacterial cells was added to the sample and the sample placed in the Ribolyser and run at maximum speed for 40 s. One negative control (200 µl sterile UV-irradiated water) was included in each extraction run. Epstein Barr virus (EBV) is used as internal positive control (10µl of Raji cells, infected with ~50 copies EBV per cell).

PCR assays

The following PCR assays were run as described in the supplementary methods document: 16S rDNA broad-range PCR[17], Staphylococcus aureus (coA) real-time PCR[18], Staphylococcus aureus (mecA/Sa442) real-time PCR[18], Staphylococcal (tuf) real-time PCR[19], Enterobacteriaceae real-time PCR[19]. A positive result for real-time PCR was any target detected with a cycle-threshold (CT) value <38.

Endotoxin assays

Plasma samples were diluted 1 in 10 using endotoxin-free water then heated to 80°C for 15 minutes to inactivate plasma proteins. Plasma endotoxin levels were then measured using a commercially available chromogenic *Limulus* amebocyte lysate assay, according to the manufacturer's protocol (Lonza, Walkersville, MD). Samples were run in duplicate and the background subtracted[11;20].

LBP assays

LBP was measured using a commercially available enzyme-linked immunosorbent assay according to the manufacturer's protocol (Cell Sciences, Canton, MA) using plasma diluted to 1 in 1000.

Monocyte HLA-DR expression

A dual staining technique was used to determine monocyte HLA-DR expression, as described by Allen *et al*[21]. Monocytes were stained by using an R-phycoerythrin (RPE) conjugated antibody to CD14 (TUK4; DAKO, Ely, UK), and HLA-DR expression was determined with fluorescein isothiocyanate (FITC) conjugated antibody to HLA-DR (CR3/43, DAKO). Fifty microliters of whole blood were incubated with 5µL of each of the antibodies for 10 minutes at room temperature. Erythrocytes were removed by lysis with FACS lysing solution (Becton Dickinson) followed by washing and centrifuging at 1500rpm with buffer containing 0.1% sodium azide, and the samples are fixed in 1% formaldehyde (CellFIX, Becton Dickinson). The cells were analysed on a FACScan flow cytometer using Cellquest software (Becton Dickinson). Nonspecific staining was determined with appropriate mouse IgG2a monoclonal antibodies labelled with PE and FITC (DAKO).

Monocytes were identified by their physical characteristics and positive CD14 staining. HLA-DR expression was determined on 5000 gated events and expressed as the percentage monocytes positive for HLA-DR fluorescence and as median fluorescence intensity (MFI).

Soluble CD14

Soluble CD14 was measured using a commercially available enzyme-linked immunosorbent assay according to the manufacturer's protocol (R&D Systems, Minneapolis, MN) using 1 in 200 diluted plasma

Power Calculation

The target recruitment was 60 infants, based on the data from the previous study regarding decreased risk of sepsis during total parenteral nutrition[1]. In our previous trial, 50% of infants had at least one episode of clinical sepsis, and during total PN, glutamine decreased the risk of sepsis by 68%. Assuming that 50% of surgical infants have evidence of microbial invasion at 5 days, using the primary endpoint described above, and that glutamine decreases this risk by 68%, 30 infants in each arm would be required to detect this difference at 80% power, alpha=0.05.

Statistical analysis

Data are given as median (range) and groups are compared using Fisher's exact test for categorical variables, unpaired t test (with Welch correction where appropriate) for normally distributed continuous variables, and Mann Whitney test for non-normally distributed continuous variables. The data for the primary outcome were compared by binary logistic regression. Incidence rates of microbial invasion and clinical sepsis were compared by Poisson regression analysis (Stata Intercooled version 10.0, College Station, TX, USA) adjusting for each of the minimization criteria, with duration of trial supplementation as the exposure variable. Standard deviation scores (Z-scores) for corrected gestational age were calculated for weight and head circumference using the LMSgrowth add-in for Microsoft Excel and UK-WHO 2006 growth charts as a reference. Growth Z-scores were analysed by multi-level modelling using MLWin v2.11, taking into account repeated measures on the same patients, and examining the interaction between glutamine administration and time, with diagnosis as a covariate.

Results:

Between July 2009 and December 2011, 83 infants were assessed for eligibility (see Figure 1 for CONSORT flow chart). 67 infants were enrolled in the study. Two were withdrawn prior to randomisation as they were no longer expected to require parenteral nutrition. One was not randomised due to an oversight in the relevant department. Four infants were randomised but did not receive five days of trial supplementation (and therefore the primary endpoint could not be assessed as they ended their involvement in the study prior to the fifth day and did not have the relevant blood samples taken) due to either achieving full feeds in less than five days (n=2) or being transferred to a non-participating centre (n=2). On the advice of the DMEC, further patients were enrolled to replace these infants. The full target number of sixty infants were enrolled, randomised, reached the primary endpoint and were analysed.

Age at enrolment was 6 days (0-95), gestational age 37 weeks (24-49), and weight 2.3 kg (0.6–4.6). The underlying diagnoses were: 25 patients had congenital/neonatal intestinal obstruction, 19 had anterior abdominal wall defects, 13 had necrotizing enterocolitis, and 3 had other causes of intestinal dysfunction. Baseline demographics were similar between the two groups (Table 1). Markers of microbial invasion at baseline were similar between the groups, except that the glutamine group was found to have slightly lower baseline LBP levels (Table 1). However, the number of infants with an LBP level greater than ≥50 μg/ml was similar between the groups (4 out of 31 infants in the control group, versus 3 out of 29 in the glutamine group). Baseline monocyte function, expressed as both % HLA-DR positive monocytes and as median fluorescence intensity, was also similar between the groups (Table 1). Similar numbers of infants received breast milk in both groups

(18 in the glutamine group compared to 21 in the control group; p=0.76 by Fisher's exact test).

Adverse Events

There were no serious adverse events related to the study, and no patients in whom unblinding of either the clinical team or the investigators took place.

Three infants died during the study. One infant (in the glutamine group) had complex congenital heart disease, which was eventually deemed not amenable to surgical correction, and a decision was made to withdraw care electively thirteen days after laparotomy for duodenal atresia. The second was an infant born at 26 weeks gestation (in the control group) with chronic lung disease, who died of pneumonia, 23 days after laparotomy for necrotizing enterocolitis. The third was an infant born at 25 weeks gestation (in the control group) with chronic lung disease, who also died of pneumonia, 32 days after laparotomy for ileal perforation.

Primary Outcome

There was no difference between the two groups in the primary outcome (Table 2): evidence of microbial invasion after five days was found in 9 of 31 (29%) infants in the control group and 8 of 29 (28%) in the glutamine group. This was not significant either using Fisher's exact test (p=1), or by binary logistic regression analysis adjusting for the minimization criteria (p=0.77, odds ratio 0.83 (95% CI 0.24-2.86). The individual components of the primary outcome are also shown in Table 2.

Secondary outcomes: microbial invasion

Thirty six infants showed evidence of microbial invasion at some time during the study, however the proportion of infants showing evidence of microbial invasion was similar between the glutamine group and the control group (Table 3). There was concordance between blood culture positivity and positive PCR, LBP or endotoxin in 14 infants, 5 infants were blood culture positive only, and 17/36 were not detected by conventional blood culture. There was no difference in the rate of microbial invasion between the two groups, with 4.2 episodes of microbial invasion/100 days in the control group and 2.9 episodes of microbial invasion/100 days in the glutamine group (Table 3). The lack of difference in microbial invasion was confirmed by Poisson regression analysis adjusting for the minimization criteria which showed that the control group has an adjusted incidence rate 0.87 (95% CI 0.48-1.55) times that of the glutamine group (p=0.63). Similarly, there were no differences in concentration of endotoxin or LBP, components of the composite primary endpoint, either at day 5 or at the end of the study (Table 3). There was highly significant correlation between plasma levels of endotoxin and LBP, Spearman r 0.24 (0.11 – 0.36), p = 0.0002.

Secondary outcomes: sepsis

There were 65 clinical episodes of sepsis in 32 patients (53% of patients), of which 25 (in 20 patients) showed evidence of microbial invasion, including 15 episodes (in 13 patients) with positive blood cultures. There was no difference between the two groups in the proportion of infants who developed sepsis, or in the incidence of sepsis, with mean values of 6.0 (median 4.3) episodes of sepsis/100 days in the control group and 4.2 (median 0) episodes of sepsis/100 days in the glutamine group, which was also confirmed by Poisson regression analysis (Table 3, Poisson incidence rate ratio

1.14 [95% CI 0.64-2.02]; p=0.66). Samples taken during episodes of clinically suspected sepsis showed significantly higher levels of endotoxin and LBP compared with samples taken when infants did not have clinical suspicion of sepsis, supporting the use of endotoxin and LBP as markers of microbial invasion (Supplementary Table S2).

Secondary outcomes: Monocyte HLA-DR expression and sCD14 concentration

While there was no difference between the two groups at enrolment (Table 1), monocyte HLA-DR expression was significantly higher among the infants receiving glutamine supplementation, both after five days and at the end of the study (Table 3, Figure 2). There was no difference in monocyte HLA-DR, expressed either as % or as MFI, between samples which showed evidence of microbial invasion compared with those which showed no evidence of microbial invasion (Supplementary Table S1). Although monocyte HLA-DR expression (expressed either as % or MFI) at enrolment did not predict microbial invasion (Supplementary Table S3), low baseline HLA-DR did significantly predict clinical sepsis (Supplementary Table S4). Allen *et al.* calculated that HLA-DR <60% was predictive of subsequent sepsis in infants and children undergoing elective cardiac surgery[21]. Applying this threshold to the current data (exploratory analysis), in patients who had HLA-DR <60% at enrolment, glutamine is protective against microbial invasion, but not against clinical sepsis (Figure 3 and Supplementary Table S5).

Plasma levels of soluble CD14 (exploratory analysis) were not found to be significantly different between the two groups(Table 3). Levels of soluble CD14 were found to correlate significantly with other markers of microbial invasion (plasma endotoxin level and lipopolysaccharide binding protein) and were significantly higher

in samples with evidence of microbial invasion (Supplementary Table S1) although there was no difference in levels of soluble CD14 between samples taken at the time of clinical sepsis and those taken when sepsis was not suspected (Supplementary Table S2).

Secondary outcomes: Nutrition and Growth

The mean weight Z-score at the time of enrolment was -1.34 ± 0.19 (Figure S1). From start of trial to reaching full enteral feeds, there was a mean decrease in weight Z-score of 0.25 (95% confidence interval 0.08 to 0.41), p = 0.004 (unpaired *t* test).

Control infants grew along weight centiles during PN (-0.02 ± 0.01 Z-scores per week of PN, p = 0.1; see Figure S2, Figure S3) but glutamine supplementation led to a clinically small but statistically significant loss in weight Z-score (-0.07 ± 0.02 per week of PN, p = 0.003). Similarly, control infants increased head circumference Z-score (+0.02 ± 0.02 per week of PN, p = 0.003) whereas glutamine supplementation was associated with impaired head growth (-0.098 ± 0.028 per week of PN, p = 0.002).

There was no difference in the estimated enteral energy intake between the groups on day 5 (placebo group median 3 kcal/kg/day, range 0-98, glutamine group median 4, range 0-108), p=0.763).

Intestinal Function and clinical outcomes

The time to first enteral feeding and time to full enteral feeding were not significantly different between the two groups (Table 4). There was no difference in the proportion of infants with intestinal failure (defined as receiving PN for at least 28 days) or intestinal failure associated liver disease (IFALD, defined according to British Society

of Paediatric Gastroenterology, Hepatology and Nutrition [BSPGHAN] guidelines [22] as a persistent elevation of alkaline phosphatase (ALP) 1.5 times the upper limit of reference range for at least 6 weeks) between the groups (Table 4). There were no differences in clinical outcomes between the two groups (Table 4).

Discussion:

This randomised controlled trial did not find any effect of parenteral plus enteral glutamine supplementation in surgical infants on the incidence of microbial invasion. There was a high incidence of microbial invasion in these patients, with 60% showing evidence of microbial invasion at some point, compared with 32% of patients who had a positive conventional blood culture. To our knowledge, this is the first study to apply these techniques in this group of patients. The infants in this study also showed a high incidence (53%) of clinical sepsis, which is in keeping with previous studies[3;23;24].

The limited concordance between positive blood cultures by conventional microbiology and positive biomarkers of microbial invasion (broad range or pathogen specific PCR, LBP and endotoxin) highlights the problems in diagnosis and targeted therapy of infants with clinically suspected sepsis. Five infants with a positive blood culture did not have elevated markers of microbial invasion, whereas 17 infants who were blood culture negative did have raised markers of microbial invasion. The limited ability of conventional microbiology to detect all pathogens has long been known, and these patients are generally treated empirically with broad-spectrum antibiotics, based on clinical findings. The advent of more advanced molecular microbiological techniques such as 16S rDNA broad-range PCR, single species PCR or more recently, pyrosequencing, has improved the ability to detect and treat

infectious organisms. However, these techniques are very sensitive to contamination so may suffer from detection of false positive signals. In addition to the markers of microbial invasion that formed the primary outcome, we also measured levels of plasma soluble CD14, which is an established marker of monocyte response to endotoxin that has been shown to prognosticate mortality in HIV-infected subjects[11;20]. Although soluble CD14 levels correlated with other markers of microbial invasion, plasma levels were not able to differentiate those samples taken during clinical sepsis from those taken at other times, suggesting that soluble CD14 offers no advantage over other markers in this group of patients.

Glutamine supplementation of parenteral and enteral nutrition was not effective in decreasing microbial invasion in the current trial, despite our previous study which showed that glutamine was effective in reducing sepsis during the period of total parenteral nutrition, although when the whole period of parenteral feeding (including partial enteral feeding) was considered, there was no benefit of parenteral glutamine supplementation[1] . In the current study, we hypothesized that maintaining the difference in glutamine content between the two groups during the period of parenteral feeding, by supplementing the enteral feed as well as the parenteral feed, would allow the beneficial effect of glutamine to be extended into the period of partial enteral feeding. However, there was no significant effect of parenteral plus enteral glutamine supplementation on microbial invasion.

Glutamine supplementation was associated with impaired growth in this study. Because the groups were isonitrogenous, the glutamine group in this study received a lower intake of other amino acids. It could be that this lower intake of other amino acids, such as arginine and cysteine, was sub-optimal and may account for the

observed difference in growth. This could also account for the lack of beneficial effect on microbial invasion in the glutamine group. Ideally, we would have liked to compare data on plasma levels of all amino acids in both groups through the study, but unfortunately this was not possible due to the limited blood sampling permissible in these infants (a significant limitation).

Despite the overall negative finding of glutamine supplementation, glutamine did result in significantly higher monocyte HLA-DR expression post-operatively compared with the control group. Monocyte HLA-DR expression has been shown to be reduced following surgery[21] and all of the infants in this study were recruited soon after undergoing surgery. Glutamine supplementation led to a faster recovery of monocyte HLA-DR expression towards normal healthy levels. Glutamine has previously been shown to improve recovery of monocyte HLA-DR following trauma [25;26]. The mechanism of this effect of glutamine on monocyte HLA-DR expression is not completely understood, although glutamine is known to be a preferred nutrient source for monocytes and other cells of the immune system [27;28].

As in previous studies in children undergoing cardiac surgery, in which low monocyte HLA-DR expression was associated with infection and increased length of post-operative intensive care unit stay[21], in the current study low HLA-DR expression at baseline was found to be predictive of clinical sepsis, and low HLA-DR expression was also found at the time of clinical sepsis. Exploratory analysis of the data from the current trial shows that monocyte HLA-DR expression in the early postoperative period can be used to select a subgroup of infants in whom glutamine supplementation appears to be beneficial in preventing microbial invasion. A future study could therefore be designed to prospectively identify those infants with the

lowest HLA-DR expression (i.e. those most at risk of microbial invasion) and specifically target those infants with glutamine and/or other immunomodulatory therapies. Although HLA-DR expression requires flow cytometric measurement, protocols have been suggested for standardisation of HLA-DR measurement that might allow identification of patients most at risk from post-operative sepsis[29]. Such techniques may aid the development of biomarker-guided immunomodulatory therapy, which holds great promise in this context. A number of different cytokine-based immunotherapies have been shown to reverse immunosuppression in sepsis in both animal models and clinical trials [30]. For example, in a randomised controlled trial in adults with immunosuppression following oesophageal or pancreatic resections, patients with low baseline monocyte HLA-DR expression were selected, and it was demonstrated that administration of granulocyte-macrophage colony-stimulating factor (GM-CSF) restored monocyte HLA-DR expression, and led to significantly reduced infections ($p < 0.001$) [31].

In addition to effects on the immune system, as glutamine is also a preferential fuel for enterocytes[32;33], it has been suggested that glutamine supplementation, particularly via the enteral route, may protect mucosal integrity and prevent or reverse gut barrier dysfunction [34]. In the current study, glutamine supplementation was given both parenterally and enterally, in order to maintain the difference between the randomised groups during the period of partial enteral feeding that occurs in all patients during their transition to full enteral feeds. Most studies of glutamine supplementation have used either the parenteral or the enteral route rather than a combination, although there are some studies in critically ill adults that have supplemented with enteral and parenteral glutamine and antioxidants[35]. Despite parenteral and enteral supplementation, in the current study there was no evidence for

a beneficial effect of glutamine in time to full enteral feeds. In addition, plasma endotoxin levels are a proxy measure for gut barrier dysfunction, and glutamine-supplemented infants had similar levels of plasma endotoxin to the control group, suggesting that gut barrier integrity was similar between the groups.

Study in this group of patients has always been challenging due to the relative rarity of the individual conditions, leading to studies such as the present one, which are limited by a degree of heterogeneity. Infants in the current study had a wide range of baseline immune function (Table 1). Application of the laboratory techniques used here can allow the selection of a subgroup of patients who may be similar in terms of their immune status and vulnerability to microbial invasion, allowing us to demonstrate effects which were not apparent in the larger group.

Conclusion

Parenteral plus enteral glutamine supplementation does not prevent microbial invasion in surgical infants. However, glutamine supplementation does assist recovery of post-operative immunoparesis, and glutamine did prevent microbial invasion in those with the most severe immunoparesis, suggesting that a targeted population may benefit from supplementation in the future.

Acknowledgements

We are very grateful to Sparks for a project grant supporting this work. All authors acknowledge support from National Institute for Health Research Great Ormond Street Hospital Biomedical Research Centre.

Registration no. ISRCTN54742344; http://www.controlled-trials.com

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Supplementary Material

Supplementary methods, Tables S1-S5, Figures S1-S3 and full trial protocol are available online at http:/pen.sagepub.com

Authorship statement

A Pierro, S Eaton and NJ Klein equally contributed to the conception and design of the research; M Bishay, V Simchowitz and S Macdonald contributed to the design of the research; M Bishay, KM Harris, S Eaton P De Coppi and the MIGS Trial Group contributed to the acquisition and analysis of the data; A Pierro, S Eaton, M Bishay, N Klein and KM Harris contributed to the interpretation of the data; and M Bishay and S Eaton drafted the manuscript. All authors critically revised the manuscript, agree to be fully accountable for ensuring the integrity and accuracy of the work, and read and approved the final manuscript.

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Figure Legends

Figure 1

Trial profile (CONSORT flow diagram). PN, parenteral nutrition

Figure 2

Monocyte HLA-DR expression in control and glutamine groups. [A] % monocyte HLA-DR expression, [B] Median fluorescence intensity (MFI). Data plotted as median, range and interquartile range, and compared using Mann-Whitney test. HLA-DR, Human Leukocyte Antigen – DR isotype

Figure 3

Rate of microbial invasion in control and glutamine groups among the subgroup of patients with monocyte HLA-DR <60% at enrolment, compared by Mann Whitney test. Line at median. HLA-DR, Human Leukocyte Antigen – DR isotype

Table 1. Baseline demographics, markers of microbial invasion and monocyte function of glutamine and control groups

Characteristic		Control group n=31	Glutamine group n=29	**Difference glutamine vs. control (95% CI)**	p-value
Males		18 (58%)	17 (59%)	0.006 (-0.24, 0.26)	1.0
Gestational age (weeks) at enrolment		36 (24 – 49)	37 (27 – 46)	1.7 (-0.87, 4.3)	0.23
Gestational age (weeks) at birth		35 (22 – 40)	35 (25 – 42)	1.2 (-1.6, 4.0)	0.51
Postnatal age (days)		6 (1 – 77)	6 (2 – 79)	2.7 (-8.1, 13.5)	0.31
Weight at enrolment (g)		2160 (620 – 3670)	2360 (660 – 4620)	173 (-350, 696)	0.43
Diagnosis	Congenital bowel obstruction	13 (42%)	12 (41%)	0.006 (-0.24, 0.26)	1.0
	Abdominal wall defect	10 (32%)	9 (31%)	0.01 (-0.22, 0.25)	1.0
	NEC	6 (19%)	7 (24%)	0.05 (-0.16, 0.26)	0.76
	Other	2 (6%)	1 (3%)	0.03*	1.0
Patients with intact ileo-caecal valve		31 (100%)	27 (93%)	0.07*	0.23
Patients with ileo-caecal valve in use		19 (61%)	19 (66%)	0.04 (-0.2, 0.29)	0.79
Patients with stoma		14 (45%)	11 (38%)	0.07 (-0.18, 0.32)	0.61
Length of small bowel after resection	no resection	26 (84%)	23 (79%)	0.05*	0.74
	≥30cm remaining	5 (16%)	5 (17%)	0.01*	1.0
	<30cm remaining	0 (0%)	1 (3%)	0.03*	0.48
Bowel perforation prior to enrolment		12 (39%)	9 (31%)	0.08 (-0.16, 0.32)	0.6
Microbial invasion		6 (19%)	5 (17%)	0.02*	1.0
Clinical sepsis		2 (6%)	0 (0%)	0.06*	0.49
Positive blood culture		0 (0%)	0 (0%)		
Positive PCR		2 (6%)	2 (7%)	0*	1.0
Plasma Endotoxin (EU/mL)		0.2 (0 - 0.9)	0.19 (0.01 - 0.3)	-0.03 (-0.09, 0.03)	0.49
Plasma LBP (µg/mL)		31 (0 – 60)	22 (0 – 60)	-8 (-16, 0)	0.04
Plasma sCD14 (pg/mL)		1287 (814 - 2139)	1067 (553 - 2072)	-119 (-306, 69)	0.25
% monocytes positive for HLA-DR		55 (14 - 91)	59 (11 - 96)	3.9 (-9.4, 17.1)	0.59
Monocyte HLA-DR (MFI)		10 (4 - 71)	14 (5 - 43)	0.7 (-5.1, 6.5)	0.22

Values given as median (range) or n (%). Continuous data were compared using Mann- Whitney test and dichotomous data using Fisher's exact test.
EU, endotoxin units; HLA-DR, Human Leukocyte Antigen – DR isotype; LBP, lipopolysaccharide binding protein; MFI, median fluorescence intensity; PCR, polymerase chain reaction; sCD14, soluble CD14.
*95% CIs cannot be calculated as some cells 5 or less.

Table 2 Primary outcome: evidence for microbial invasion on day 5.

Outcome	Control group n=31	Glutamine group n=29	**Difference glutamine vs. control (95% CI)**	p-value
Infants with microbial invasion	9 (29%)	8 (28%)	0.01 (-0.21, 0.24)	1.0
Positive blood culture only	3 (10%)	1 (3%)		
Positive PCR only	3 (10%)	1 (3%)		
Positive blood culture and positive PCR	1 (3%)	1 (3%)		
LBP ≥50 µg/ml	1 (3%)	5 (17%)		
Endotoxin ≥0.5 EU/ml	1 (3%)	0 (0%)		

Values given as n (%). Data compared using Fisher's exact test. EU, endotoxin units; LBP, lipopolysaccharide binding protein; PCR, polymerase chain reaction.

Table 3. Secondary outcomes (microbial invasion and sepsis)

Outcome	Control group n=31	Glutamine group n=29	**Difference glutamine vs. control (95% CI)**	p-value
Infants with microbial invasion at any time	20 (65%)	16 (55%)	0.09 (-0.15, 0.34)	0.60
Episodes of microbial invasion/100 days	4.2 (0 – 33.3)	2.9 (0 – 22.2)	-1.2 (-5.1, 2.7)	0.64
Infants with clinical sepsis on day 5	3 (10%)	2 (7%)	0.03*	1.0
Infants with clinical sepsis at any time	18 (58%)	14 (48%)	0.10 (-0.15, 0.35)	0.6
Episodes of sepsis/100 days	4.3 (0 – 33.3)	0 (0 – 18.2)	-1.8 (-5.2, 1.6)	0.49
Plasma endotoxin on day 5 (EU/mL)	0.24 (0.01 – 0.67)	0.23 (0.09 – 0.32)	-0.03 (-0.08, 0.02)	0.44
Plasma endotoxin at end of study (EU/mL)	0.22 (0.02 – 4.8)	0.21 (0.13 – 0.48)	-0.19 (-0.52, 0.13)	0.16
LBP on day 5 (µg/ml)	23 (5 – 60)	29 (0 – 60)	4 (-4, 12)	0.38
LBP at end of study (µg/ml)	16 (2 – 48)	19 (2 – 60)	3 (-4, 10)	0.56
Infants with positive PCR	8 (26%)	9 (31%)	0.05 (-0.18, 0.28)	0.78
Infants with positive blood culture	11 (35%)	8 (28%)	0.08 (-0.16, 0.31)	0.59
sCD14 on day 5	1206 (640 – 1989)	1385 (625 – 3061)	232 (-9, 473)	0.10
sCD14 at end of study	1209 (590 – 2188)	1196 (456 – 2050)	12 (-196, 219)	0.97
% monocyte HLA-DR on day 5	60 (18 – 95)	72 (48 – 91)	14 (4, 23)	0.01
% monocyte HLA-DR at end	70 (18 – 100)	81 (45 – 98)	13 (3, 24)	0.04
Monocyte HLA-DR (MFI) on day 5	14 (4 - 44)	16 (7 – 56)	5 (-1, 12)	0.11
Monocyte HLA-DR (MFI) at end	15 (5 - 215)	31 (10 – 78)	3 (-15, 21)	0.01

Values given as median (range) or n (%). Continuous data were compared using Mann-Whitney test and dichotomous data using Fisher's exact test.
EU, endotoxin units; HLA-DR, Human Leukocyte Antigen – DR isotype; LBP, lipopolysaccharide binding protein; MFI, median fluorescence intensity; PCR, polymerase chain reaction; sCD14, soluble CD14.

Table 4: Clinical outcomes

Outcome	Control group n=31	Glutamine group n=29	**Difference glutamine vs. control (95% CI)**	p-value
Time to start enteral feeds (days)	6 (0 – 25)	5 (0 – 26)	0.2 (-3.0, 3.3)	0.85
Time to full enteral feeds (days)	12 (7 – 159)	13 (5 – 88)	-5 (-21, 11)	0.85
Time in study	12 (6 – 159)	11 (5 – 88)	-6 (-19, 7)	0.60
Infants with intestinal failure	11 (35%)	9 (31%)	0.04 (-0.19, 0.28)	0.79
Infants with IFALD	6 (19%)	6 (21%)	0.01 (-0.19, 0.22)	1.0
Reached full enteral feeds	25 (81%)	22 (76%)	0.05 (-0.16, 0.26)	0.76
Transferred on mixed parenteral-enteral feed	3 (10%)	5 (17%)	0.08	0.47
Transferred on total parenteral nutrition	1 (3%)	1 (3%)	0.002	1.0
Died	2 (6%)	1 (3%)	0.03	1.0

Values given as median (range) or n (%). Continuous data were compared using Mann-Whitney test and dichotomous data using Fisher's exact test.
IFALD, intestinal failure associated liver disease.

Figure 1. Trial profile

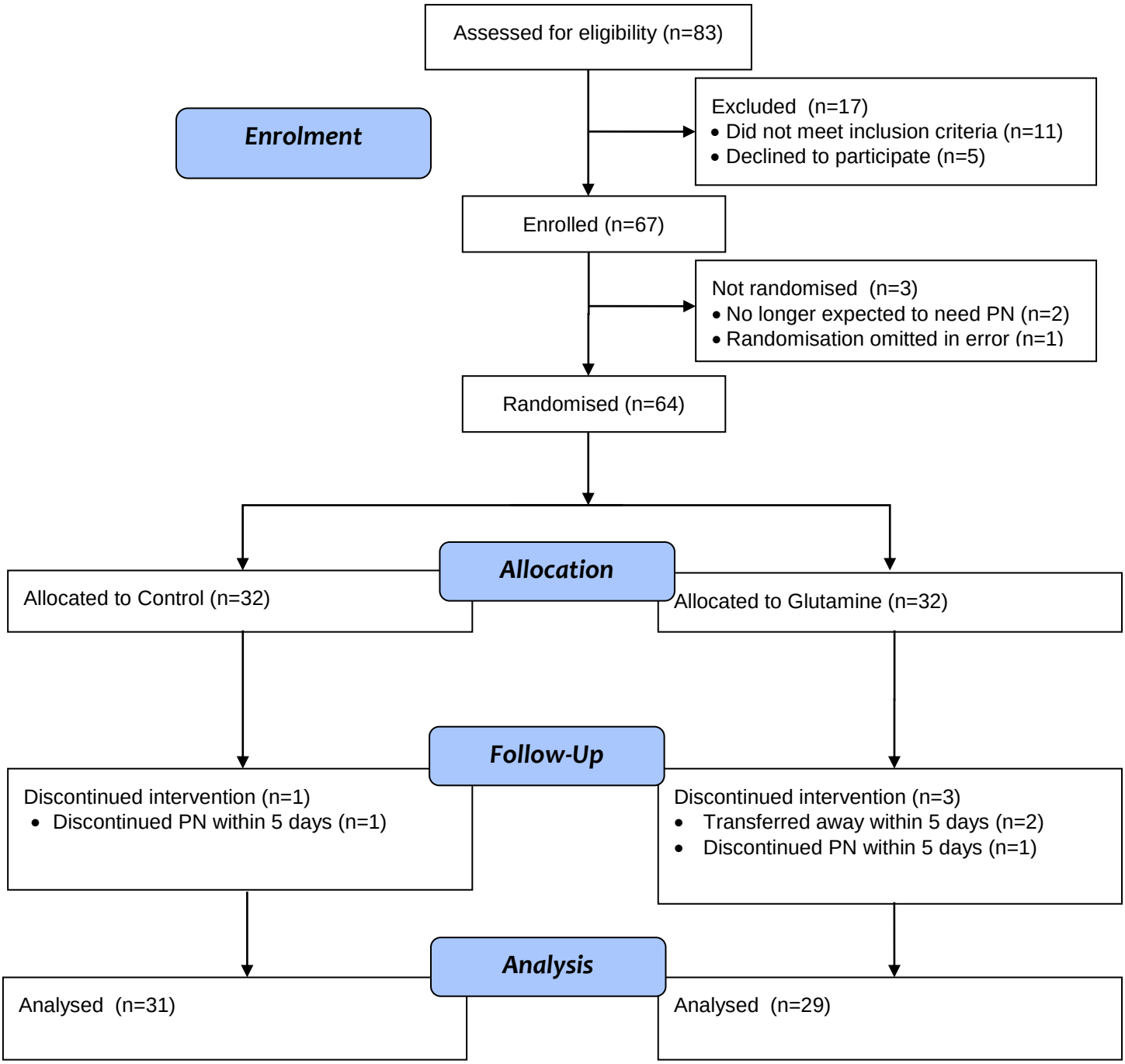


Figure 2.

Monocyte HLA-DR expression in control and glutamine groups.

[A] % monocyte HLA-DR expression, [B] Median fluorescence intensity (MFI). Data plotted as median, range and interquartile range, and compared using Mann-Whitney test.

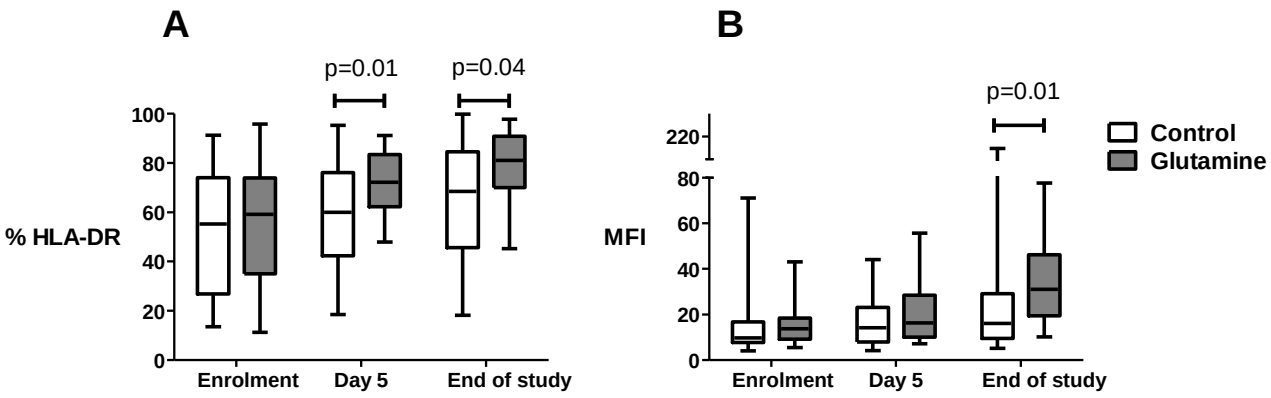
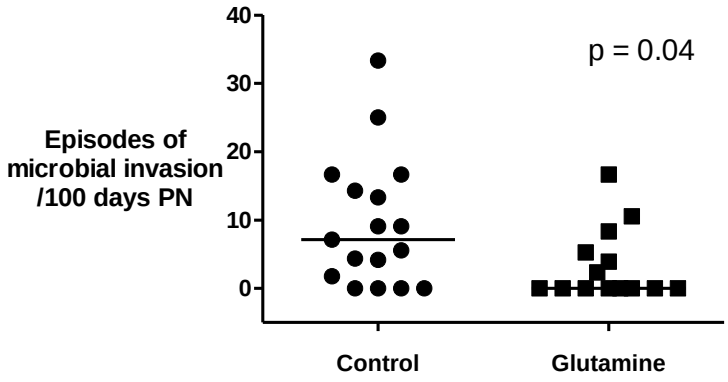


Figure 3.
Rate of microbial invasion in control and glutamine groups among the subgroup of patients with monocyte HLA-DR <60% at enrolment, compared by Mann Whitney test. Line at median.



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Methods

16S rDNA broad-range PCR[17]

The PCR reaction mixture was as follows: 5 µl 10X PCR buffer (Molzym), 1·5 mMMgCl2, 0·1 mM dNTPs (Bioline), 1 U Taq polymerase (Molzym), 0·4 µM of each of the following primers: 16SFa: 5′-GCT CAG ATT GAA CGC TGG-3′, 16SFb: 5′-GCT CAG GAT GAA CGC TGG-3′, 16SR: 5′-TAC TGC TGC CTC CCG TA-3′ and sterile UV-irradiated water to give a final volume of 45µl. Five microlitres of extracted DNA was added to this mixture and the reactions heated to 94°C for 3 min, followed by 32 cycles of 94°C for 30 s, 63°C for 40 s and 72°C for 90 s. A final extension was carried out at 72°C for 10 min. Each PCR run included two positive controls (1 µl each of *Escherichia coli* ~100 c.f.u. μl^{-1} and *Staphylococcus aureus* ~100 c.f.u. μl^{-1}). PCR reactions were electrophoresed through a 2% agarose gel containing 2 µl 500 nM ethidium bromide and bands visualized by UV transillumination.

Staphylococcus aureus (coA) real-time PCR[18]

The PCR reaction mixture was as follows: 14 µl of 1X Quantitec mastermix (Qiagen), 0·7 µl of each of the following primers: Sa-3-F (coA): 5′- GTA GAT TGG GCA ATT ACA TTT TGG AGG -3′, Sa-4-R (coA): 5′-CGC ATC TGC TTT GTT ATC CCA TGT A-3′, Probe coA: 5′(FAM)-CGC TAG GCG CAT TAG CAG TTG CAT C- (TAMRA)3′, 0·3 µl of each of the following primers: EBV-F: 5′-CCG GTG TGT TCG TAT ATG GAG-3′, EBV-R: 5′-GGG AGA CGA CTC AAT GGT GTA-3′, Probe EBV: 5′(JOE)-TGC ACC AGA CCC GGG CTC AGG TAC TCC GA-(TAMRA)3′, and sterile UV-irradiated water to give a final volume of 18 µl. Ten microlitres of extracted DNA was added to this mixture and the reactions heated to 50°C for 2 min, followed by 94°C for 10 min, followed by 45 cycles of

94°C for 15 s, and 60°C for 60 s. Each PCR run included a positive control (methicillin-resistant *Staphylococcus aureus* ~1 c.f.u. μl^{-1}).

Staphylococcus aureus (mecA/Sa442) real-time PCR[18]

The PCR reaction mixture was as follows: 14 µl of 1X Quantitec mastermix (Qiagen), 0·3 µl of each of the following primers: Sa442-F: 5′-TGC GTA CAC GAT ATT CTT CAC-3′, Sa442-R: 5′-ACT CTC GTA TGA CCA GCT TC-3′, Probe Sau3A: 5′(JOE)-TAC TGA AAT CTC ATT ACG TTG CAT CGG AAA CA-(TAMRA)3′, 0·7 µl of each of the following primers: Sa-1-F (mecA): 5′-CGG TAA CAT TGA TCG CAA CGT TCA -3′, Sa-2-R (mecA): 5′-CTT TGG AAC GAT GCC TAA TCT CAT-3′, Probe mecA: 5′(FAM)-TTC CAG GAA TGC AGA AAG ACC AAA GCA- (TAMRA)3′, and sterile UV-irradiated water to give a final volume of 18 µl. Ten microlitres of extracted DNA was added to this mixture and the reactions heated to 50°C for 2 min, followed by 94°C for 10 min, followed by 45 cycles of 94°C for 15 s, and 60°C for 60 s. Each PCR run included a positive control (methicillin-resistant *Staphylococcus aureus* ~1 c.f.u. μl^{-1}).

Real time PCR for Enterobacteriaceae

A novel real time PCR was used to detect *Escherichia coli*, *Klebsiella*, and *Enterobacter* at levels much lower than the broader-range 16S rDNA PCR (as low as ~1 c.f.u. per reaction). The PCR reaction mixture was as follows: 13 µl of 1X QuantiTect mastermix (Qiagen), and 1.0 µl of each of the following primers: Ent-dnaK-F: 5' ACC TGG GTA CWA CCA ACT CTT GTG T-3′, Ent-dnaK-R: 5' GTC ACT GCC TGA CGT TTA GC-3′, Ent-dnaK-probe: 5' FAM-AGG ATG GTG AAA CTC TGG TWG GTC AGC C-BHQ-1-3′, to give a final volume of 16 µl. Ten microlitres of extracted DNA was added to this mixture and the reactions heated to 50°C for 2 min, followed by 94°C for 10 min, followed by 45 cycles of 94°C for 15 s, and 60°C for 60 s. Each PCR run includes a positive control (*Escherichia coli* ~1 c.f.u. μl^{-1}).

Supplementary Table S1

Soluble CD14 and monocyte HLA-DR expression in samples with and without evidence of microbial invasion. Data median (range) compared by Mann-Whitney test.

Marker	No MI (n = 175)	MI (n = 61)	**Difference MI vs. No MI** **(95% CI)**	p-value
sCD14	1149 (456 – 2970)	1301 (590 - 3061)	193 (72, 314)	0.003
% monocyte HLA-DR	66 (12 – 100)	61 (9 – 96)	-4 (-12, 3)	0.28
Monocyte HLA-DR (MFI)	15 (4 – 215)	15 (6 - 100)	-3, (-9, 3)	0.72

HLA-DR, Human Leukocyte Antigen – DR isotype; MFI, median fluorescence intensity; MI, microbial invasion; PCR, polymerase chain reaction; sCD14, soluble CD14.

Supplementary Table S2

Levels of endotoxin, LBP, soluble CD14 and monocyte HLA-DR expression in samples taken during clinically suspected episodes of sepsis, compared to samples taken without clinical signs of sepsis (assessing all samples taken during the study). Data median (range) compared by Mann-Whitney test.

Marker	No sepsis (n = 187)	Sepsis (n = 53)	**Difference Sepsis vs. No sepsis (95% CI)**	p-value
Endotoxin (EU/ml)	0.21 (0 - 4.8)	0.24 (0 - 1.8)	0.002 (-0.09, 0.09)	0.04
LBP (µg/mL)	21 (0 - 60)	29 (9 - 60)	10 (4, 15)	0.0006
sCD14	1178 (456-3061)	1233 (550-2574)	70 (-60, 159)	0.33
% monocyte HLA-DR	68 (11 – 100)	50 (9 – 84)	-13 (-22, -4)	0.005
Monocyte HLA-DR (MFI)	16 (4 – 215)	10 (5 - 34)	-9 (-13, -5)	0.009

EU, endotoxin units; HLA-DR, Human Leukocyte Antigen – DR isotype; LBP, lipopolysaccharide binding protein; MFI, median fluorescence intensity; sCD14, soluble CD14.

Supplementary Table S3

Baseline values and subsequent microbial invasion. Data median (range) compared by Mann-Whitney test.

Marker	No microbial invasion (n = 24)	Microbial invasion (n = 36)	**Difference MI vs. No MI** **(95% CI)**	p-value
Endotoxin (EU/ml)	0.18 (0.01 - 0.31)	0.22 (0 – 0.91)	0.05 (-0.01, 0.1)	0.09
LBP (µg/mL)	21 (0 – 55)	31 (0 – 60)	11 (3, 19)	0.01
sCD14	1167 (580 – 2072)	1166 (553 – 2139)	-12 (-207, 182)	0.90
% monocyte HLA-DR	58 (12 – 96)	56 (11 – 91)	-4 (-18, 9)	0.56
Monocyte HLA-DR (MFI)	14 (5 – 29)	11 (4 – 71)	0 (-6, 5)	0.29

EU, endotoxin units; HLA-DR, Human Leukocyte Antigen – DR isotype; LBP, lipopolysaccharide binding protein; MFI, median fluorescence intensity; MI; microbial invasion; sCD14, soluble CD14.

Supplementary Table S4

Baseline values and subsequent sepsis. Data median (range) compared by Mann-Whitney test.

Marker	No sepsis (n = 28)	Sepsis (n = 32)	Difference Sepsis vs. No sepsis (95% CI)	p-value
Endotoxin (EU/ml)	0.20 (0 – 0.32)	0.19 (0.01 – 0.91)	0.02 (-0.04, 0.08)	0.85
LBP (µg/mL)	22 (0 – 55)	39 (0 – 60)	13 (6 – 21)	0.0018
sCD14	1167 (589 – 2072)	1166 (553 – 2139)	-7 (-198, 184)	0.94
% monocyte HLA-DR	61 (12 – 96)	43 (11 – 91)	-15 (-28, -2)	0.02
Monocyte HLA-DR (MFI)	16 (7 – 43)	9 (4 – 71)	-4 (-9, 2)	0.004

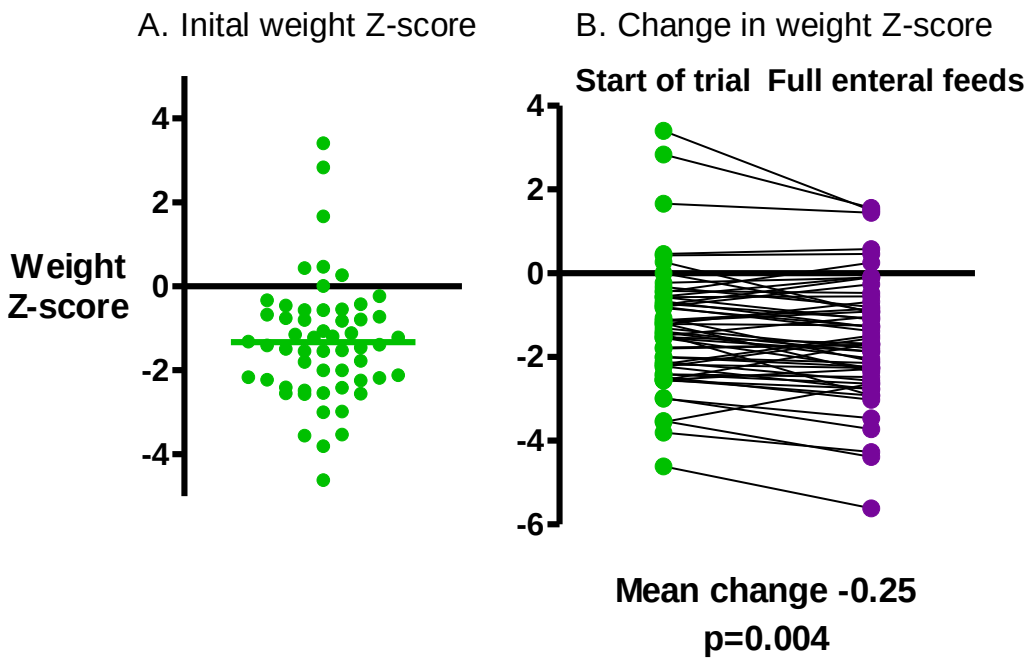
Supplementary Table S5

Microbial invasion in control and glutamine groups among the subgroup of patients with monocyte HLA-DR <60% at enrolment. Data median (range) or n (%) compared by Mann-Whitney test or Fisher's exact test. *The median rate of microbial invasion in the glutamine group is 0 episodes/100 days because less than half of infants in that group experienced any episodes of microbial invasion,

Outcome	Control group n=17	Glutamine group n=14	**Difference glutamine vs. control (95% CI)**	p-value
Infants with microbial invasion at any time	13 (76%)	6 (43%)	0.34	0.075
Episodes of microbial invasion/100 days	7.1 (0 – 33.3)	0 (0 – 16.7)	-6.1 (-11.6, -0.54)	0.04

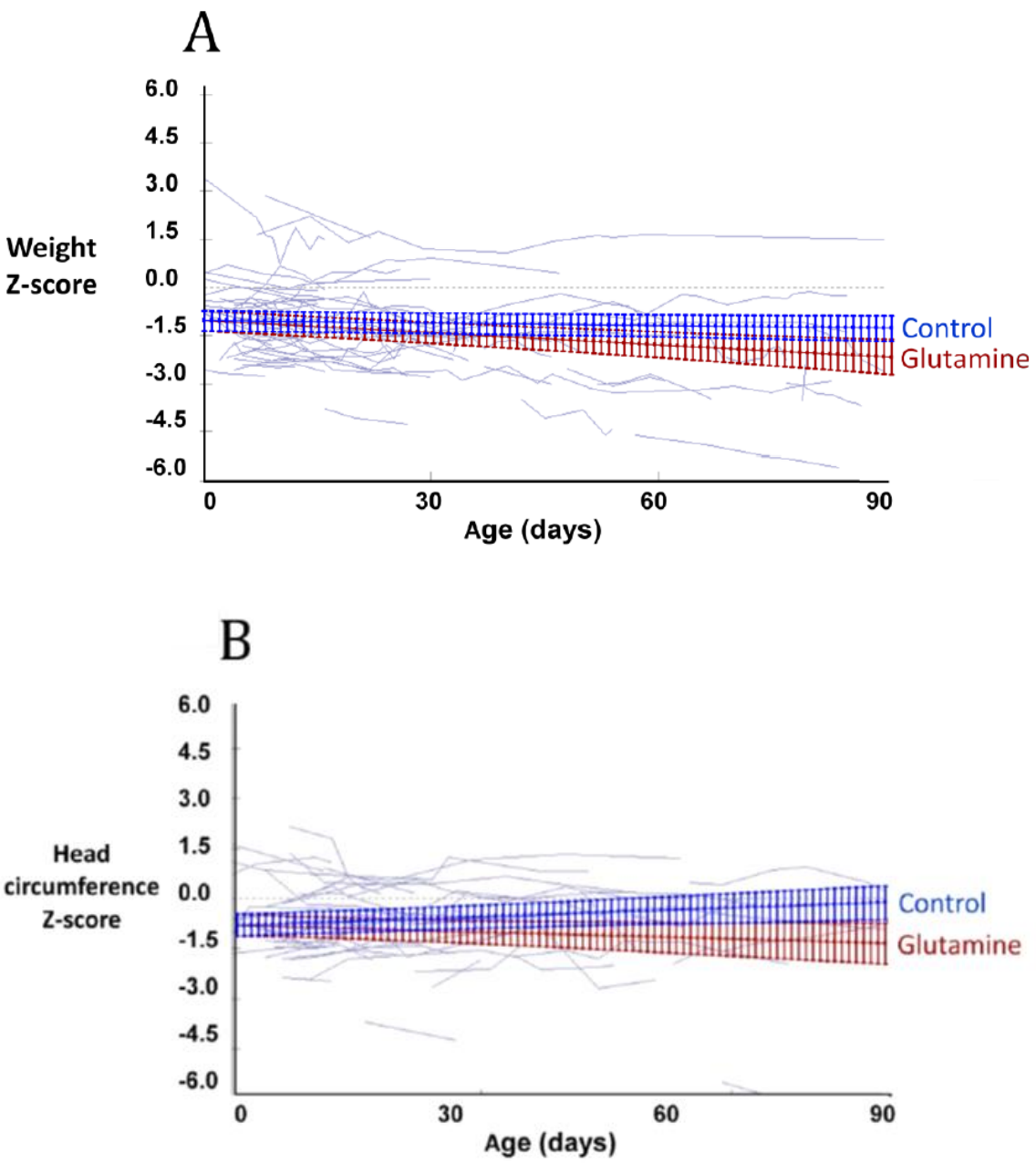
HLA-DR, Human Leukocyte Antigen – DR isotype

Figure S1 Weight Z-scores at baseline and during the study



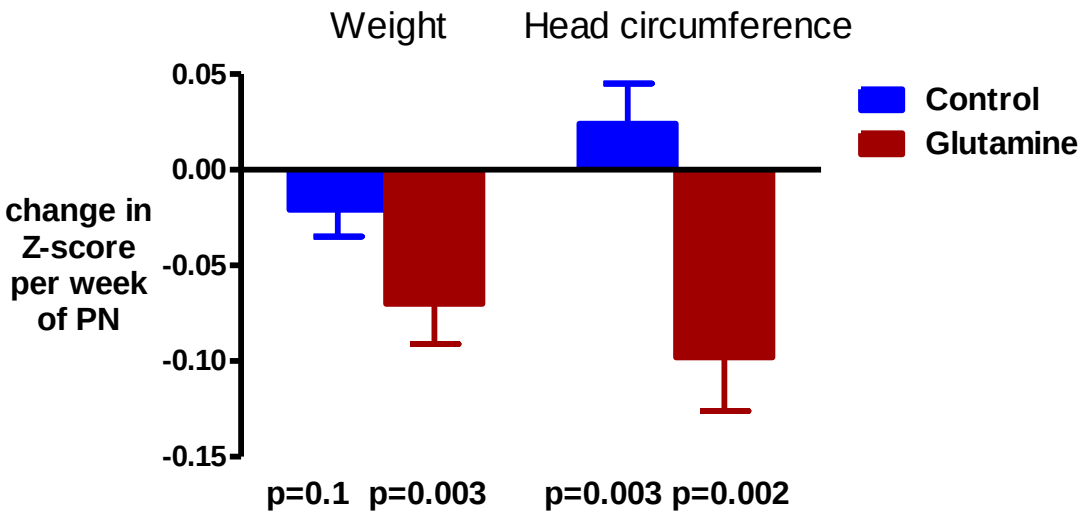
A. Weight Z-score at time of enrolment (green line at mean). B. Change in weight Z-score during study. Mean change -0.25, p = 0.004 (unpaired *t* test).

Figure S2 Modelling growth in control and glutamine groups during the study



Individual lines showing serial data for individuals, with superimposed mean ± SEM for groups. A. Weight Z-scores. B. Head circumference Z-scores.

Figure S3 Growth in control and glutamine groups during the study



Growth in the two groups was compared by multilevel modelling adjusting for diagnosis. P-values are for trend over time.

MIGS:
Microbial Invasion and Glutamine Supplementation Study

Protocol Full Title: *Microbial invasion during parenteral nutrition in surgical infants receiving glutamine*

Sponsor's Protocol Number: *07 SG 10*

Version 2.0 11/05/2009

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Contents

1. Brief Summary

The rate of infection in surgical infants requiring parenteral nutrition is very high. In a multicentre randomised controlled trial in surgical infants we demonstrated that glutamine supplementation reduces the risk of developing clinically evident sepsis during total parenteral nutrition but not during the subsequent period of partial parenteral with partial enteral feeding. It is not clear if the effect of glutamine is related to a reduction in bacterial invasion, or whether this effect could be extended to the period of partial parenteral/enteral feeding.

We propose a double blind randomised controlled trial comparing surgical infants receiving glutamine supplementation (study group n=30) with a control group receiving isonitrogenous amino acids (control group n=30). The supplementation will be performed during both total and partial parenteral/enteral nutrition. The primary end point will be evidence of microbial invasion as demonstrated by: positive blood cultures, detection of bacterial DNA by both conventional single-target and broad-range 16S PCR, elevated levels of plasma endotoxin or plasma lipopolysaccharide binding protein.

This trial is highly relevant to a large number of surgical infants in the UK and abroad.

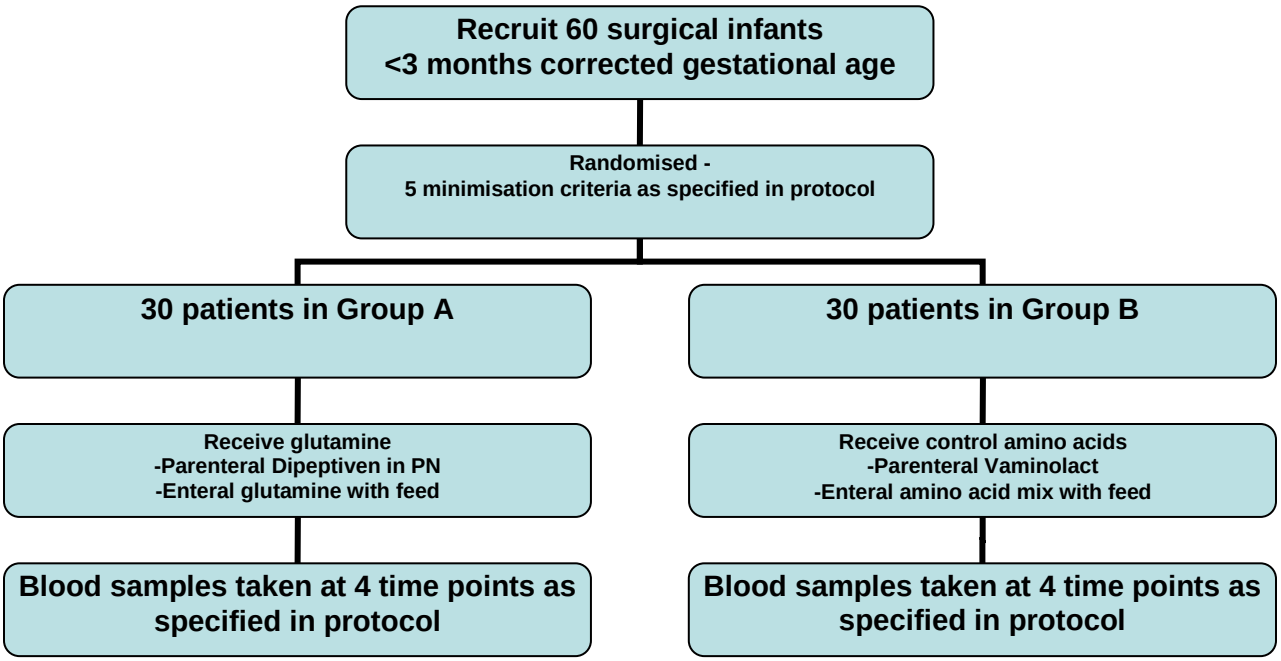

Figure 1. Flowchart of trial design

2. Background

Newborn infants with congenital or acquired gastrointestinal anomalies commonly require parenteral nutrition postoperatively because they are unable to tolerate enteral feeds. Up to one third of these infants may develop sepsis after surgery while receiving parenteral nutrition (PN) (1;2), leading to impaired liver function (2;3), critical illness and removal of central venous catheters.

Microbial invasion
It has always been assumed that the central venous catheter is the major portal of entry for micro-organisms causing septicaemia in these patients (4). However, previous research from our group and others (5) indicates that the gut is another major route for invasion by micro-organisms. The gastrointestinal tract is the principal source of bacteria or microbial products entering the blood because of its massive bacterial load compared to other anatomical sites (6).We have reported that almost half the surgical infants with congenital or acquired gastro-intestinal dysfunction develop abnormal flora and that all cases of septicaemia were preceded by gut colonisation with abnormal flora (2;3). We have also described the migration of micro-organisms from the intestinal lumen to the systemic circulation in surgical neonates with intestinal overgrowth (2). Major difficulties with the investigation of microbial infection in neonates are the small volumes of blood available and the high use of antibiotics. Therefore blood cultures are an insensitive means of detecting microbial invasion. In the last few years, molecular techniques for detecting bacterial DNA and sensitive methods to measure bacterial invasion have been developed. These provide new opportunities for investigating infections in neonates.

Glutamine supplementation
Infants receiving PN may have an increase in gut permeability (7;8) and eventually lose gut mucosal integrity (9). These alterations may lead to increased bacterial translocation (10) and sepsis and are directly related to the duration of parenteral nutrition. These complications may be due to a deficiency in nutritional components which might normally be derived from human breast milk and other enteral feeds.

Glutamine is the most abundant free amino acid in the human body and is found in large quantities in breast milk. It plays key roles in many metabolic pathways, is a preferential substrate for enterocyte (11) and leukocyte metabolism (12), and is a major interorgan nitrogen transporter (13). Endogenous production in organs such as the liver and kidneys is normally sufficient for metabolic demand. However, in conditions of physiological stress such as surgery, critical illness and sepsis, endogenous glutamine stores may dwindle as consumption exceeds production. Thus, it has been considered to be a conditionally essential amino acid (14-16). In surgical neonates who are at risk of glutamine deficiency, this

relative deficiency may be exacerbated due to the lack of enteral feeding as currently PN does not routinely contain glutamine.

Previous Trial
We have completed a prospective double-blind multi-centre randomised controlled trial (SIGN Trial – submitted for publication) in surgical neonates less than 3 months old who required surgery and PN because of gastro-intestinal dysfunction. Infants received either 0.4 g/kg/day glutamine (treatment group) or isonitrogenous isocaloric PN (placebo group) until full enteral feeding was achieved. 174 patients were randomized of which 164 completed the trial and were analysed (glutamine n=82, placebo n=82). The incidence of clinically evident sepsis (43-51%) and proven septicaemia (22-33%) were both very high in this trial. Glutamine had no effect on overall incidence of clinically evident sepsis (51% glutamine vs. 43% control (p=0.27)) or septicaemia (32% vs. 22% (p=0.16)).

However, during total PN, i.e. before the first enteral feed was introduced, glutamine significantly reduced the risk of developing clinically evident sepsis by 68% (relative risk 0.32 [95% confidence intervals 0.15-0.69] p=0.004) but did not appear to have any effect on the risk of having a positive blood culture. In addition, in those patients who did have clinical evidence of sepsis, the time to the first episode of sepsis was significantly longer (12 days) in the glutamine group compared to 4 days in the placebo group (p<0.0001). In this trial we did not supplement glutamine enterally and we did not directly investigate whether the glutamine effect on sepsis during total PN was related to increased microbial invasion.

3. Trial Objectives

The aim of the study is to ascertain whether the addition of glutamine supplementation to both parenteral nutrition and enteral feeds in surgical newborn infants leads to a reduction in bacterial invasion.

Primary end-point: The focus of this trial is to investigate <u>bacterial invasion</u> in surgical infants receiving PN. Evidence of bacterial invasion will be sought by detecting any of the following

(i) positive blood cultures

(ii) presence of bacterial DNA in the blood by both conventional single-target and broad-range 16S PCR (20;21)

(iii) elevated levels of endotoxin (defined as greater than 50 pg/ml (22))

(iv) elevated plasma lipopolysaccharide binding protein (LBP) (defined as greater than 13 µg/mL (23)).

Based on the timing of sepsis in our previous trial (the SIGN Trial), evidence of microbial invasion (primary endpoint) will be sought at 5 days after starting PN. Blood samples will also be taken at the beginning of the trial, at the introduction of first enteral feeding and when full enteral feeding is achieved.

Secondary end-points:

Other parameter indicative of microbial invasion: We will also measure the EndoCAb (also termed endotoxin-core antibodies) which in conditions of acute microbial translocation such as sepsis, bind and clear the LPS from the circulation (24).

Monocyte HLA-DR expression: Decreased monocyte HLA-DR expression is predictive of post-operative infection (25) and is related to outcome and survival. We have recently shown in a small double-blind randomised controlled trial that glutamine administration (in the form of alanyl-glutamine, Dipeptiven) to septic infants and children prevents the fall in monocyte HLA-DR.

Serum amino acid profile (including glutamine level) will also be measured.

Infection*:* Episodes of sepsis and septicaemia, timing of sepsis and septicaemia (days from start of PN). Sepsis is defined (26) as the clinical state of generalised inflammation manifested by at least 3 of the following clinical signs: fever, hyperthermia (>38°C) and/or hypothermia (<36°C), lethargy, poor perfusion, age-related tachycardia and tachypnoea, and hypotension.

At the time of suspected sepsis, blood samples will be taken for culture and tested for other evidence of bacterial invasion as described above. Septicaemia is defined as sepsis combined with a positive blood culture drawn through the catheter and/or at a peripheral site (2;3;27).

Intestinal flora: surveillance samples will be obtained to investigate the status of intestinal flora (2;3). Surveillance cultures of the oropharynx and gut will be obtained at the beginning of the study and thereafter twice each week. These cultures will be processed for all microorganisms in a semiquantitative manner to detect overgrowth (2;3).

Intestinal permeability: this will be measured by 3-*O*-methyl glucose, mannitol and lactulose uptake and recovery in urine (28). This will be undertaken at the same time points illustrated for primary end point.

Intestinal function*:* Time to full enteral feeding and time on PN (days). Time to full enteral feeding is defined as the time (days) required to reach adequate calorie intake orally and /or enterally (when at least 75% of expected average requirement (29) is given enterally).

Survival/mortality will also be noted.

4. Trial Design

This study will be a double blind randomised controlled trial, carried out at Great Ormond Street Hospital for Children NHS Trust. Infants (corrected gestational age <3 months) requiring parenteral nutrition (PN) and surgery for congenital or acquired gastrointestinal anomalies will be eligible. These would include infants with gastroschisis, necrotising enterocolitis, bowel atresia or intestinal surgery for other reasons.

These infants will initially receive a period of exclusive intravenous feeding (total PN), followed by a gradual increase in enteral feeding and a decrease in PN (mixed PN and enteral) until full enteral feeding is achieved.

The decrease in PN and concomitant increase in enteral feeding will be carried out as per the usual clinical practice. Parenteral feeding will be stopped when more than 75% of the patient's full nutritional requirement is tolerated enterally for a minimum of 24 hours. Those with inborn errors of metabolism, immune deficiency, renal failure, PN already given for more than 5 days or enrolled in another trial at the same time will be excluded.

Group allocation: Infants will be randomised to receive glutamine supplementation (study group) or isonitrogenous amino acid supplementation (control group).

Group A will receive glutamine supplementation using parenteral Dipeptiven® and enteral glutamine.
Group B will receive isonitrogenous amino acids, using parenteral Vaminolact® and amino acid mix as enteral supplement.

Allocation to the groups will be made using minimisation(17) based on the following 5 criteria:

- *i)* length of functional small bowel (no intestinal resection [i.e. normal small bowel length] / remaining small bowel length ≥30 cm / remaining small bowel length <30 cm);
- *ii)* diagnosis (congenital intestinal obstruction / congenital defect of the abdominal wall / necrotizing enterocolitis / other);
- *iii)* gestational age (derived from last menstrual period) at the time of enrolment in the study (<30 weeks/ 30 - 36 weeks / >36 weeks);
- *iv)* ileo-caecal valve in continuity (yes/no);
- *v)* weight at the time of enrolment in the study (<1 kg / 1-2kg / >2 kg).

The clinical team will be blind to the child's allocation but the hospital pharmacist who prepares the PN solution and the dietician preparing the enteral glutamine supplementation will be unblinded. Written informed

consent for randomisation will be obtained from the parents at the time of enrolment. Parents will have at least 24 hours to make an informed decision.

Intervention: Parenteral glutamine will be given as a chemically stable dipeptide solution (Dipeptiven®, Fresenius-Kabi, Runcorn, Cheshire, UK; L-alanyl-L-glutamine 200 mg/ml) in a dose of 0.4 g/kg/day glutamine equivalent to 0.6 g/kg/day Dipeptiven® which ensures that the nitrogen intake of the intervention and control infants is equal and that no more than 35% of the total nitrogen intake will be provided by Dipeptiven®. The dose proposed is equal to that used in our previous randomised controlled trial which demonstrated that there were no negative effects from administration of glutamine and no abnormal levels of serum ammonia, urea nitrogen and glucose. This level is also based on our published research which has confirmed beneficial effects on enteral mucosa at this dose (18).

There are no reported adverse effects of glutamine intravenous administration. The amount of glutamine that we intend to administer in the intervention group has been safely administered in infants and children (18) and higher amounts have been administered to low birth weight infants with no adverse effects (19).

The control group will receive isonitrogenous Vaminolact® (Fresenius-Kabi, Runcorn, Cheshire, UK; this contains no glutamine). PN solutions will be prepared by the hospital pharmacy and enclosed in identically coloured external bags. Prescriptions and labels will specify the amount of nitrogen and not its source. Supplementation of PN with glutamine will continue for the entire duration of PN.

The doses of both solutions used will provide the same amount of nitrogen equivalent to 0.116g of nitrogen/kg/day. The PN provided for the duration of the study for both groups will have the appropriate amount of nitrogen reduced in their PN for that day.

It is difficult to maintain parenteral glutamine supplementation during the period of partial enteral feeding, as the intake of other parenteral amino acids would have to be reduced, and stability issues could also become a problem. During the period of partial enteral feeding, in which the parenteral intake of glutamine/placebo is reducing, we will supplement the enteral diet with the balance which is no longer being given parenterally. This glutamine will be given as Adamin-G® (SHS International Ltd, Liverpool, UK). The control group will receive Complete Amino Acid Mix (SHS International Ltd, Liverpool, UK; contains 0.7% glutamine).

We will restrict the enteral diet to: breast milk, SMA Gold, SMA LBW Gold, Pepti-Junior, Neocate and modular feeds, and we will measure the glutamine content of each of these.

5. Randomisation

Patients will be randomised using the Simin software. This will be carried out by Dr Simon Eaton (or other trained persons), thereby leaving the investigators fully blinded. This software has been validated as a randomisation program following its use in three large randomised international studies. The patient allocation will be recorded, placed in a sealed envelope, and given to pharmacy for preparation of parenteral solutions. The patient allocation will thus be recorded in pharmacy.

Procedure for breaking randomisation codes – It would be unlikely that breaking randomisation would be needed out of hours, given that there is no restriction on medical management of patients and that there are no known adverse effects of the supplements used. In any event, in the case of emergency, the PI would be contacted via hospital switchboard and he will contact Resident Pharmacist to break the code when necessary.

6. Sampling

Blood samples will be taken at the start of the study, at day 5, once enteral feeds commence and at the completion of the study for the primary endpoint. These samples will be taken from the indwelling central line at the same time as the line is accessed for change of parenteral nutrition.

Intestinal permeability will be assessed using a stable isotope method at the start of enteral feeds and once the study is finished. This will be done by giving 1 mL/kg via feeding tube of 30 mg/mL 3-*O*-methyl glucose, 20 mg/mL mannitol and 30 mg/mL lactulose (osmolarity 352). Urine will be collected for 8 hours from the nappy of patients enrolled.

Follow-up samples and data may be collected at University College London Hospitals NHS Trust if patients are transferred there before enteral feeding is established.

Samples will be stored in the freezers at the Paediatric Surgery Department /Department of Infectious Diseases and Microbiology at the Institute of Child Health which comply with the Human Tissue Act 2006.

7. Data Monitoring and Trial Steering Committees

A Data Monitoring and Ethics Committee will be convened. This will be independent of both the trial organisers and those providing therapy. This

committee will perform interim analyses to: a) review assumptions underlying sample size considerations; b) modify or close intake to trial.

The membership of the Committee will be:

1) Consultant Surgeon not involved in trial (Chairman)
2) Consultant Paediatrician not involved in trial
3) Statistician

The first interim analysis will be done after 30 patients are recruited. The criteria for stopping the trial will be:

1) A significant difference between the two arms of the study in the primary endpoint $p<0.01$
2) Excess episodes of sepsis or mortality in one arm of the trial

A Trial Steering Committee will also be convened, whose members include:

1) The trial co-ordinators (A Pierro, S Eaton, N Klein, M Bishay)
2) A representative of the Data Monitoring and Ethics Committee.

The role of this Committee is to provide overall supervision of the trial and ensure that the trial is conducted to rigorous scientific, clinical and ethical standards. It will particularly concentrate on progress of the trial, adherence to the protocol, data collection and maximise the chances of completion within the agreed timetable. This committee will meet six monthly or more frequently if required by either the Trial co-ordinator or the Data Monitoring Committee representative.

8. Data management

All patient data will be recorded onto an electronic record and we will use direct transcription to Case Report Forms.

Trial Subjects will be coded for anonymity to comply with the data protection act. We will use our Sponsor Protocol number followed by a logical sequence of numbering e.g. 07SG10:01, 07SG10:02 etc.

All clinical observations and laboratory results will be recorded on a secure database, to be stored on secure ICH computers. The Principal Investigator shall be responsible for recording and entering all data in this study.

The investigators as previously detailed will permit regular data review by the Data Monitoring Committee. The data will be freely available for audit, and it is planned that regular reports will be filed with the local REC.

9. Selection and Withdrawal of Subjects

Identification of possible candidates for the study will be made when admitted as inpatients to Great Ormond Street Hospital. Parents or legal guardians will be approached and offered informed consent.

Inclusion criteria: Infants (corrected gestational age <3 months) requiring PN and surgery for congenital or acquired gastrointestinal anomalies will be eligible. These would include infants with gastroschisis, necrotising enterocolitis, bowel atresia or intestinal surgery for other reasons.

Exclusion criteria: Those with inborn errors of metabolism, immune deficiency, renal failure, PN already given for more than 5 days or enrolled in another trial at the same time will be excluded.

Withdrawal criteria: patients will be withdrawn if there are thought to be adverse effects directly related to the study. Patients may also be withdrawn by parental request. Laboratory data would still be gathered if the withdrawn patients still required parenteral fluids. Subjects would be replaced to ensure statistical power.

10. Statistics

In our previous trial (the SIGN Trial), 50% of infants had at least one episode of clinical sepsis, and during total parenteral nutrition, glutamine decreased the risk of sepsis by 68% using a Cox proportional hazards model. Assuming that 50% of surgical infants have some evidence of microbial invasion at 5 days, using the primary endpoints described above, and that glutamine decreases this risk by 68%, 30 infants in each arm would be required to detect this difference at 80% power, alpha = 0.05. We are confident that we will recruit 60 patients in the time frame of the study.

30 patients in each group are to be recruited over 30 months.

Termination of the trial will occur once 60 patients have been recruited or if the stopping criteria are reached at interim analysis as described above (page 10).

Any deviations from the initial statistical plan will be documented in a revised version of this protocol and distributed to REC, Research and development, and all other involved groups.

Patients will be allocated to the two study groups using minimisation to ensure that the groups are comparable with respect to the 5 criteria outlined in Trial Design above (page 7).

Glutamine and isonitrogenous solutions will be labelled identically and there will be no means by which the investigators or clinical carers can know to which group the patients will be allocated. Comparisons will be made between patients in Group A and B before and after glutamine or isonitrogenous amino acids administration. SPSS software will be used for the statistical analysis.

11. Ethics

The trial will be conducted in compliance with the ethical principles enunciated in the declaration of Helsinki 1996, and in accordance with Good Clinical Practice.

12. Financing and insurance

This study is supported by a grant from the children's charity SPARKS. All financial aspects of the grant are managed through the R&D department of GOSH/ICH.

Negligent fault is covered by the NHS CNS. Indemnity and insurance has been applied for under the No Fault Compensation Policy Held by UCL.

13. Publication Policy

Data will be published in medical journals and at relevant conferences. All of the applicants on the grant application are likely to appear as co-authors of work, along with any number of surgical consultants involved in the routine care of children enrolled. Patients and their GPs will have access to a summary of the results of this trial. A full report will be submitted to the REC, and R&D department at ICH.

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