Cincinnati Children's Hospital Medical Center
Research Protocol

Title: Efficacy of Enteral Glutamine in Reducing Bloodstream Infections in SBS Infants

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I. Abstract

Short bowel syndrome (SBS) following massive bowel resection for various conditions, especially necrotizing entercolitis (NEC), is a devastating complication that affects neonates especially those delivered < 36 weeks gestational age. These children are especially susceptible to recurrent blood stream infections (BSI), repeated hospital admissions and subsequent poor growth. All of these contribute to a very high burden on the primary caretakers and the health care system. Organisms associated with recurrent BSI in these children are predominantly gut related. However, there is still a lack of understanding of the intestinal microbial ecology of these children and whether it has a role in BSI, enteral feeds and ultimately growth. Glutamine (GLN) supplementation has been shown to decrease mucosal damage, lower rates of BSI and associated sepsis and improve positive nitrogen balance in adults with SBS. In children, there is currently insufficient data to determine whether enteral GLN supplementation improves clinical outcomes in young infants with significant gastrointestinal resection leading to SBS. The overall purpose of this pilot clinical study is to obtain needed preliminary data on the efficacy of enteral GLN supplementation in children with SBS as a result of significant intestinal resection due to NEC, omphalocele, gastroschisis and intestinal atresia in the context of a rigorous, double-blind exploratory clinical trial with the following specific aims: **Specific Aim 1:** To perform a double-blind, randomized, placebo controlled, 6-months pilot study of enteral GLN treatment in PN-dependent SBS children due to NEC, omphalocele, gastroschisis and/or atresia evaluating the efficacy of enteral GLN supplementation to decrease BSI and serial serum concentrations of TNF-α, IL1- β, IL-6 and IL-8; **Specific Aim 2:** To assess the efficacy of 6 months of therapy with enteral GLN as a method to improve somatic growth parameters (length velocity, weight velocity, head circumference and mid arm circumference) in Aim 1 subjects and the impact of BSI on growth parameters; **Specific Aim 3:** To comprehensively assess the gut lumenal microbiome (using state-of-the art molecular methods) in Aim 1 pediatric SBS patients versus non-SBS, age-matched controls, and, in the SBS patients to correlate major bacterial populations present in the lumen with BSI due to these microbes and to explore whether the gut lumenal microbiome is altered by enteral GLN treatment. This proposal will facilitate the development of a multicenter, multidisciplinary team from Cincinnati Children's Hospital Medical Center, University of Michigan and University of Colorado to assess new methods for decreasing BSI and improving growth as well as to gain insights into the intestinal microbial ecology of SBS children.

II. Purpose of Study

This is a double-blind, randomized placebo-controlled pilot study to investigate the efficacy of enteral glutamine (GLN) supplementation in 36 infants, ≤ 12 months of age with parenteral nutrition (PN)-dependent short bowel syndrome (SBS) due to massive small bowel resection for NEC, omphalocele, gastroschisis and/or atresia on improving weaning of PN and preventing infections. We intend to evaluate the effect of enteral feeding and GLN supplementation on the gut bacteria. We will also recruit 12 age-matched controls to evaluate the normal gut bacteria. Investigators at the University of Michigan and Cincinnati Children's Hospital Medical Center will recruit the potential subjects (18 patients and 6 controls will be recruited at each site). We will estimate residual small and large bowel length and the presence, or absence of the ileocecal valve and colon by review of surgical records and postoperative intestinal barium studies in the subjects. Estimates of bowel length for gestational age have been previously published[1,2]. Data on parenteral or enteral GLN supplementation (L-GLN or the dipeptide Alanyl-GLN) in infants have not shown consistent effects, potentially because these studies were under powered; the dose of GLN was suboptimal or other factors.

III. Background

Significance

Pediatric SBS is characterized by insufficient bowel length causing inability to maintain protein, energy, fluid, electrolyte or micronutrient balances when the child is on a conventionally accepted enteral diet[3, 4]. The use of PN is often required in SBS to provide nutrition and sustain life; unfortunately PN does not promote gut adaptation and also predisposes to BSI[5, 6]. Appropriate management with PN typically prevents nutritional deficiencies, enhances weight gain and is associated with improved overall survival rates[7]. In infants, NEC is the major cause of surgical SBS; other causes include gastroschisis, midgut volvulus and intestinal atresia[8, 9]. Children with PN-dependent SBS exhibit a high rate of recurrent BSI [10]. The quality of life burden and healthcare costs associated with SBS in affected children is extremely high, in part, due to costs and complications of PN and hospitalizations for BSI[11, 12]. Healthcare costs in the first year of life in pediatric SBS patients routinely exceed $500,000 [12]. Inpatient hospitalization accounted for most (>80%) of this expense, attributed to prolonged NICU stay, surgical procedures and recurrent BSI[12]. The cost of home health care in children with SBS increases during each subsequent year of PN use[8, 12]. **In light of the clinical importance of pediatric SBS, the high healthcare-related costs, and the ineffectiveness of current therapy to prevent BSI, new treatments to improve clinical outcomes are urgently required. Data to increase our understanding of the putative causes of BSI in this orphan disease are also needed.**

Recurrent sepsis and prolonged PN are each predictors of increased morbidity and mortality in children with SBS[13, 14]. The very high rate of recurrent BSI in infants with SBS is presumably due to impaired gut and systemic immunity and gut barrier dysfunction. Translocation of indigenous intestinal microbes has been shown to occur in animal models of SBS, with the translocation being promoted by generalized host immunosuppression, gut-associated immune dysfunction and gut barrier dysfunction[15, 16]. The incidence of catheter sepsis is significantly higher in children with SBS than in those without SBS (7.8 vs. 1.3 per 1000 catheter days;

p<0.05) and enteric organisms being responsible for 62% of catheter sepsis in patients with SBS compared to only 12% in patients without SBS[17].

Preliminary data strongly supports the finding of increased BSI and predominance of enteric organisms in the bloodstream of febrile SBS infants[8, 18]. The SBS infants in our preliminary data also exhibit elevated levels of proinflammatory cytokines (tumor necrosis factor-α, interleukin 1-β, interleukin-6 and interleukin-8) at enrollment vs. control subjects[18]. Previous studies in adults requiring chronic home PN also showed increased blood levels of TNF-α and IL-6[19].

Despite receiving adequate calories, SBS infants enrolled in the NICHD Neonatal Research Network had poor growth indices (length and head circumference) and also exhibited a high rate of BSI[8]. These findings are similar to those previously reported for SBS children requiring long term PN[20]. Catabolic responses due to BSI and elevated systemic cytokines are likely involved in the poor growth exhibited. However, the relationship between BSI and growth failure is conjectural. There are no previous rigorous therapeutic trials designed to decrease BSI and improve growth velocity in infants affected by SBS, as we here propose.

GLN is a central amino acid in major metabolic processes and is also utilized as a major fuel/substrate by intestinal mucosal cells and by immune cells, including the gut-associated immune system[21-24]. During catabolic illness, skeletal muscle exports large amounts of GLN into the blood, and GLN-utilizing tissues, including gut mucosa, immune cells, and kidney markedly increase GLN uptake[25]. If stress persists, GLN requirements may exceed endogenous GLN production, and skeletal muscle and plasma GLN concentrations decline[23]. Numerous studies in animal models show that enteral or parenteral GLN supplementation with either the dipeptide alanyl-GLN or free GLN, enhances gut mucosal growth, repair, and function, decreases gut-origin sepsis and inflammation, and improves nitrogen balance in animal models of intestinal atrophy, injury, and adaptation [21, 22, 26-29]. Several lines of evidence suggest that GLN may stimulate growth and function of gut mucosal cells in humans[27,,30-32]. **In adults, GLN has potent anabolic effects, enhances immune functions and decreases nosocomial infection rates [24, 30, 32, 33]. In small studies, the combination of enteral GLN, subcutaneously administered human growth hormone and a modified enteral diet improved absorptive capacity and facilitated weaning of PN in adults with SBS[34, 35].**

Pediatric studies have shown conflicting results regarding the impact of GLN to decrease morbidity and improve somatic growth. **Studies done in clinically stable preterm infants showed that GLN is well tolerated both enterally and parenterally[36].** Premature infants are susceptible to GLN depletion because placental supply ceases at birth and tolerance to enteral nutrition is limited especially in those with SBS. Current PN in use in the United States does not contain GLN due to solubility and stability issues [37]. Thus, total body GLN depletion may be exacerbated in SBS due to intestinal malabsorption, coupled with inadequate GLN intake, and potentially increased endogenous GLN requirements due to recurrent BSI and other stresses such as surgical trauma[38]. In a group of 69 premature, non-SBS, very low birth weight (VLBW <1500g birth weight) infants between ages 8 and 120 days, enteral GLN supplementation (0.3 g/kg/day) for four months had a significant positive effect on growth indices (weight, length; mid arm, mid thigh and head circumferences)[39]. In a randomized, double-blind, controlled single

center trial in 68 VLBW infants, there were no growth or hospital stay difference between the GLN and placebo groups[38]. However, infants receiving GLN had a decreased incidence of sepsis ($p<0.05$) with risk of infection nearly 4-fold higher in controls[38]. In that study GLN was administered for a median of 27 days. There is limited available data on GLN supplementation in SBS children. In one trial of 20 preterm infants with SBS, due to NEC, intestinal atresia, gastroschisis, omphalocele, intestinal volvulus or malrotation were enrolled. Enteral GLN was administered to only 9 infants at a dose of 0.4g/kg/day up to 120 days, or until full enteral feeds were achieved. GLN was well tolerated, but did not show any benefit on duration of PN use or tolerance of enteral feeds vs. controls[40, 41]. However, given the sample size and diagnosis variability these data should be considered as preliminary.

Although GLN has systemic effects after absorption, the potential effect of this amino acid to decrease BSI and increase protective mucosal immune response in SBS is our goal in this study. *In vitro* studies in human intestinal cell lines show that apical-derived GLN has trophic and cytoprotective effects in both enterocytes and colonocytes, inhibits apoptosis and promotes gut mucosal integrity by markedly attenuating disruption of tight junctions and paracellular permeability. I*n vivo* studies in animal models of SBS show that enteral GLN improves indices of gut barrier function independent of its absorption [26, 30, 42-44]. Investigators have shown that enteral GLN upregulates mucosal and luminal sIgA in rat models[27].

The available data are inconsistent on the clinical efficacy of parenteral or enteral GLN supplementation (L-GLN or the dipeptide alanyl-GLN) in infants (and more limited data on enteral GLN in pediatric SBS). The reasons for this potentially include underpowered sample size, suboptimal dose of GLN (0.3g/kg/day - 0.5g/kg/day) or other factors. The recommended dose of protein administered to infants is 3.0-3.5 g/kg body weight/day. The dose of parenteral GLN administered to critically ill neonates in a previous NICHD-sponsored multicenter trial was 20% of the total amino acid content of the parenteral nutrition and this GLN dose was well tolerated [45]. We propose to administer enteral GLN at a dose of 0.6g/kg/day, which is approximately 20% of the 3.0-3.5g/kg total protein recommended intake. Doses of 0.5 g/kg/day of enterally administered L-GLN have been well tolerated in clinical trials in infants [46, 47].

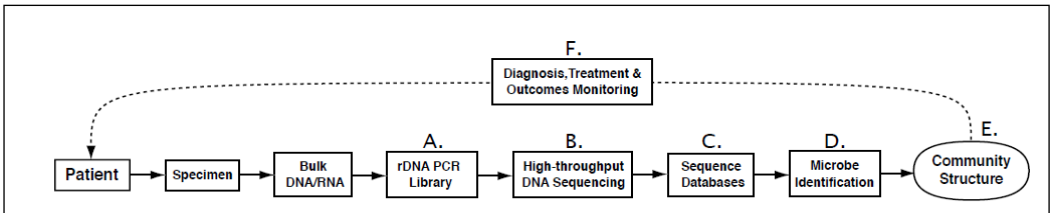
Innovation

The available data on GLN outlined above supports the need for a rigorous, double-blind clinical study to obtain critically needed pilot data on the efficacy of enteral GLN supplementation in pediatric SBS. This proposed study represents collaborative, multidisciplinary, multisite, comparative effectiveness clinical research. The rigorous nature of this trial of GLN supplementation in SBS infants is innovative compared to previous studies; our subject group is also more homogeneous, as we limit enrollment to children with NEC, omphalocele, gastroschisis and those with atresia as the primary cause of SBS. Our proposed design incorporates important clinical outcome measures (incidence of BSI and somatic growth parameters) to provide needed information on GLN efficacy in pediatric SBS. The data, in turn, will inform the design of subsequent definitive multicenter Phase III studies. In addition, to follow-up on our studies of GLN-induced upregulation of stool and mucosal sIgA in rat models of SBS[27], we have measured stool sIgA in SBS children in a previous prospective trial and propose here to evaluate the impact of GLN on stool sIgA as a secondary index of enteral GLN efficacy.

The study of the gut microbiome in pediatric SBS and the response to GLN on these microbial populations is innovative. Commensal microbes may have a role in the immune conditioning of the gut, and may influence subsequent immune responses in neonates[48]. The intestinal microbiome may also be important for physiologic gut absorptive and barrier functions; small bowel bacterial overgrowth and/or altered luminal microbiota (e.g. due to anatomic disruptions after intestinal resection, dysmotility, stasis, etc.) may represent a potentially crucial, but as yet uncharacterized pathopysiologic factor in SBS [49]. The underlying disease (NEC), malnutrition, and illness-associated immunodeficiency have been hypothesized to promote the proliferation of lumenal bacteria. Further, effects of altered nutrient intake in SBS on the microbiome are unknown. **Therefore, in specific aim #3, we will comprehensively assess the gut lumenal microbiome in pediatric SBS patients versus controls to: 1) correlate alterations in lumenal bacteria populations with BSI risk; and 2) determine whether GLN supplementation influences the gut microbiome and concomitant microbial pathogenesis of BSI.**

Collaborators, Drs. Frank and Pace have developed molecular tools with which to study naturally occurring microbial communities without the requirement for preliminary culture [50-53]. **A number of these methods are centered on analysis of bacterial 16S ribosomal RNA gene (rDNA) sequences (see Figure 1, below)** which vary from species to species and thus can be used as molecular 'bar-codes' for species identification[54-58]. rDNA can be PCR amplified with pan-bacterial primers from genomic DNA isolated directly from specimens, without need for prior culture of resident microorganisms (Figure 1, step A). Advances in sequencing technology, such as the massively parallel GS-FLX pyrosequencing platform of Roche 454 Life Sciences, Inc. dramatically increased throughput compared to the previous generation of sequencers (Figure 1, step B).This platform was successfully deployed in a pilot study of the pediatric SBS microbiota.

Figure 1: Analysis of human microbiome through culture-independent rRNA sequence analysis



A. rDNA gene libraries are generated by broad-range PCR with pan-bacterial rDNA primers. **B**. rDNA amplicons are directly pyrosequenced. **C**. Phylogenetic comparison of rDNA sequences identifies individual microorganisms in a specimen **(D)** and thus provides a census of the microbial community **(E)** that can enhance diagnosis, treatment, and outcome monitoring in the treatment of Short Bowel Syndrome.

Quantitative analysis of the human microbiome data will be done as follows: DNA sequences will be phylogenetically compared to rDNA sequence databases (e.g. GenBank which stores >2,000,000 16S entries) to relate newly generated rDNA sequences to those of known organisms and thereby identify the source organisms (Figure 1, steps C and D). The results are a snapshot census of the microbial community within a specimen (Figure 1, step E) which provides a reference point from which physiology and lifestyle can be inferred [59,60]. Many rRNA-based methods demonstrated the merits of monitoring intestinal microbes to gain insights into how commensals contribute to gastrointestinal pathology, including antibiotic

associated diarrhea and inflammatory bowel disease [61-63]. **Analogous to IBD, large-scale changes in the enteric microbiota of SBS patients may exacerbate the breakdown of gut mucosal immune homeostasis and barrier function, and thus contribute to BSI.**

Updated data on glutamine use and new procedures not in original grant
Following the submission and approval of this application, more data regarding the potential benefit of enteral GLN in promoting growth and stimulating immune response against infections has not been published. There were no new relevant trials in humans using enteral GLN in infants. There is follow up data on infants enrolled in earlier trials showing that those who received enteral GLN had decreased for developing gastrointestinal infections and dermatitis likely due to the impact of GLN on the immune system[83]. However, new animal models suggest GLN supplementation may be useful in reducing the severity of weaning-related gastrointestinal infections, by reducing the mucosal cytokine response and altering intestinal barrier function [84, 85]. As a result of this we are proposing to evaluate as part of specific aim 1 the serum levels of anti-flagellin and anti-liposaccharide(LPS) immunoglobulin (Ig) G levels in the infants with SBS. These antibodies are markers of response to bacterial antigen and improve with advancing enteral nutrition [18]. This proposal seeks to evaluate the impact of entral L-GLN on the mucosa's immune response by measuring fecal IgA.

IV. Duration of Study

This anticipated duration to complete the study is 3 years (enrollment, analyses, and report writing). Each subject will be monitored for 6 months (± 1 month).

V. Potential Benefits

1. Duration of PN in patients with surgical short bowel syndrome may be decreased
2. Incidence of central line infections in these patients may be decreased
3. Improving overall nutrition and growth
4. Improving the gut mucosal immunity with oral GLN

VI. Potential Risks

Potential risks for GLN supplementation:

- Stomach pain - 11%
- Nausea - 22%
- Tenesmus - 11%
- Pancreatitis - 11%
- Constipation - 11%
- Fever - 11%
- Gastric Ulcer - 11%
- Increased irritability - 11%
- Mild skin rash or itching - 11%
- Hyperammonimia in patients with renal and hepatic dysfunction - 11%
- Laryngitis/Pharyngitis - 11%

Other adverse events reported in the product information package

- Gastrointestinal fistula in patients with Crohn's disease - 11%

- Vaginal fungal infections – 11%
- Psychiatric disorders – 11%
- Pyelonephritis – 11%
- Vascular disorders- 11%

Potential risks for L-alanine (placebo):

- Nausea and vomiting - 11%
- Hyperammonimia in patients with renal and hepatic dysfunction - 11%

Potential risks for L-Glutamine and L-alanine

- Hyperosmolality of the feeds leading to increased stool output is a potential side effect of L-Glutamine and L-alanine when added to formula. However, this has not been reported in previous trials [37-40, 47, 86]. The osmolality of L-glutamine is 310 mosm/kg of water (data provided by Emmaus Medical Inc).

Potential risks for blood draw:

- Occurrence of discomfort and/or a bruise at the site of puncture
- Less commonly, fainting
- The formation of a clot or swelling in the vein and surrounding tissue
- Bleeding from the puncture site
- On rare occasions an infection may develop at the site where the blood is collected

VII. Overall Risk Assessment

The overall risk assessment is minimal based on previous pediatric and adult studies. The potential long term benefits of reducing the duration of PN, decreasing central line infections and improving growth is greater than the potential risks.

VIII. Risk Category

Minimum

IX. Methods

A. Subjects and controls Selection

Inclusion criteria for age matched controls (n=12: 6 per site)

- Males and females will be recruited as controls for this study if they are ≤ 12 months of age (corrected for gestational age)
- Normal small bowel length without any intestinal resection or primary intestinal disease
- Not currently on TPN and if ever on TPN this should have been discontinued for at least 4 weeks

Exclusion criteria for age matched controls

- Major congenital or chromosomal anomalies
- Inability to tolerate enteral nutrition/ regular cow's milk, breast milk or soy formula

- History of liver/intestinal transplantation
- Active milk protein allergy
- Inflammatory bowel disease
- Multiorgan failure

Inclusion Criteria for glutamine and placebo group of SBS patients (n=36; 18 per site)

- Males and females ≤ 12 months of age (corrected for gestational age)
- Patients who have undergone small bowel resection due to complications of or related to NEC, omphalocele, gastroschisis or intestinal atresia with known small bowel length
- Patients who have been PN dependent for more than 42 consecutive days (6 weeks) and currently on TPN at time of enrollment. This would include patients who were off TPN for < 7 consecutive days.
- Patients who have the ability to take partial enteral nutrition of at least 5ml/hr breast milk or elemental formula (Elecare® is the preferred formula at both clinical sites but use of other elemental formula does not exclude the patient) (via gastric or intestinal tube, or orally) to allow the appropriate dose of GLN (0.6 g/kg/day) or placebo (L-alanine)
- Signed informed consent for the use of GLN or placebo obtained

Exclusion Criteria for glutamine and placebo group of SBS patients

- Major congenital or chromosomal anomalies
- Inability to tolerate enteral nutrition that will preclude treatment with enteral GLN or L-alanine placebo for >2 consecutive weeks
- Liver/intestinal transplantation
- Multiorgan failure

B. Withdrawal Criteria

1. Patient cannot complete evaluation visit assessments
2. Patient develops any of the exclusion criteria detailed above
3. Patient develops significant hepatic dysfunction (defined as INR > 2.5 or direct serum bilirubin level > 10 mg/dL)
4. Parent(s)/legal guardian are unwilling to administer the GLN or placebo at the required dosage
5. Patient is lost to follow-up
6. Patient has 3 missed study visits
7. It is in the patients best interest

C. Dropouts

Data collected from patients who voluntarily withdraw from the study or who are withdrawn from the study will be included in the final analysis using intent to treat. Patient and control enrollment will continue until 32 patients complete all requested study requirements outlined. The reason for withdrawal will be documented.

D. Stopping Rules

An early stopping rule pending further investigation for this study will be invoked if any of the following adverse events occur:

- The rate of BSI in SBS patients in either the GLN supplementation or the placebo is an absolute 20% higher compared to the historical rate in SBS patients. The rate of catheter associated BSI is assessed monthly in all SBS patients at both CCHMC and the University of Michigan as part of the current clinical improvement process, If the rate of the BSI is higher in either group by 20% then study enrollment will be halted until further evaluation by the Data Safety Monitoring Board (DSMB).
- If two subjects develop the same grade-3 adverse event (including liver function tests, coagulation profile) related to the study medication that is irreversible upon de-escalation (does not reverse after 10 days) or upon discontinuation of the study drug
- Any subject develops a related grade-4 adverse event.

Since this is a double blind placebo control design, the data will only be unmasked by the research pharmacy at the request of the DSMB, if any of the above events occur. The statistician on the DSMB will evaluate the data. The unmasked data will not be revealed to the investigators unless the DSMB requests that the study be terminated.

E. Monitoring for toxicity due to GLN

Potential toxicity will be assessed by analyzing clinical and laboratory parameters using the following PN labs (Complete blood count, complete metabolic profile (Renal, Calcium, Phosphorus, Magnesium, Liver panel, Total Protein, Albumin) monthly. Additional blood samples will be done in accordance with current institutional standards for monitoring chronic PN use in infants. Plasma GLN levels will be obtained at baseline and at 6 months.

F. Evaluating compliance

All of the subjects will be instructed to bring to the monthly visits, the containers containing the supplement supplied at the previous visit. The study team will count the remaining bags left over. We will also evaluate the diary provided to the families. Compliance will be evaluated based on the diary and the number of returned containers. Families are expected to be 90% compliant. If the child/family is less than 90% compliant, the family will receive additional counseling to identify reasons and how to attain this level of compliance. Children will remain in the study as long as they receive at least 70% of the expected amount for 3 consecutive months. The level of compliance on enteral GLN effect will be included in the analysis. Children who receive less than 70% or more than 120% of the prescribed glutamine or placebo will considered to be non-compliant. The reason for non-compliance will be documented and included in the final analysis. Average intake/day of glutamine by the patients will be included in the analysis including the response based on dose received.

G. Patient Recruitment and Consenting Process

Patient Recruitment

A total of 36 patients with short bowel syndrome (SBS) ≤ 12 months of age will be recruited for this study. Of the 36 patients, approximately 24 patients will have colon in continuity and 12 will have colon not in continuity. Assuming a 10% dropout rate, it is expected that 32 SBS patients will complete the study.

For Aim 3 of the study, an additional 12 infants without SBS will be enrolled as the non-SBS control patients. Therefore, a total of 48 patients will be enrolled across the two sites, University of Michigan and Cincinnati Children's Hospital Medical Center.

The study will be explained in detail by the study coordinator or other study team member and the consent form will be reviewed per institutional IRB requirements. These subjects will be recruited through the Division of Gastroenterology, Hepatology, and Nutrition, the Department of Surgery, or the Division of Neonatology. The prospective patients will be identified through the clinical staff within all three divisions working collaboratively. The clinical staff will initially inform potential subjects and families about the research study. The research staff will notify and obtain permission from the patient's primary gastroenterologist, surgeon, or neonatologist before contacting the family.

Subjects who drop out prior to drug initiation may be replaced with new subjects until target enrollment is achieved, as these will not have been given study medication.

Consenting Process

For those patients meeting inclusion criteria, the study purpose, procedures, costs, risks, benefits, and alternatives to participation will be thoroughly explained and presented to the patient and their family by the designated study coordinator and/or investigator, and subjects will be screened for willingness to participate. Subjects and parents will be explicitly informed that choosing not to participate will in no way affect the quality of the medical care that they will receive. Once patients and families have had enough time to consider participation and have expressed a willingness to participate, consent will be obtained from the parents or legal guardian.

One copy of the consent will be given to the family and one will be retained in the study records. One copy will also be placed in the child's medical record.

Vulnerable Population

This study will enroll children. To protect this population from coercion or undue influence, consent will be obtained only after a full discussion of the research protocol and of the risks and benefits that may occur from participation. Patients and parents will be explicitly informed that choosing not to participate will in no way affect the quality of the medical care that they will receive.

H. Study Procedures

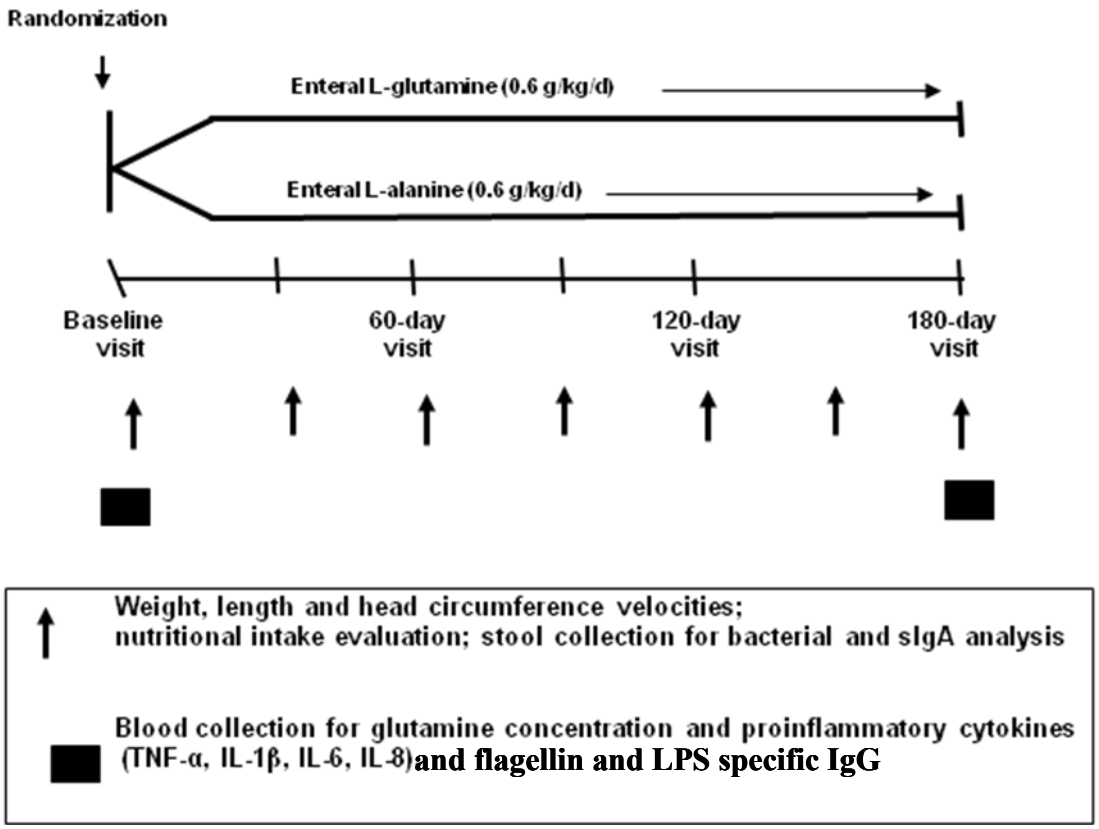
Randomization and study groups

At the baseline visit, SBS subjects will be block randomized in a 1:1 scheme by site and strata (i.e the presence or absence of **colon in continuity)** to receive either L-GLN powder or isonitrogenous, isocaloric placebo [L-alanine (ALA)]. Randomization will be done by the Cincinnati Children's Hospital Medical Center research pharmacist who will not be blinded. Distribution will be done by the site study personnel. The subjects, site investigators and study personnel at all sites will be blinded to the randomization. Since patients will be on breast milk or Elemental formula (Elecare® being the preferred elemental formula at both institutions; however use of other elemental formula does not exclude the patient from the study), the randomization process will take into account whether breast milk or formula is been consumed by the infant. The research pharmacist will make decisions regarding randomization based on the use of formula versus breast milk to ensure adequate distribution within groups.

Glutamine group: Infants randomized to the GLN group will receive L-GLN administered enterally at a dose of 0.6g/kg body weight/day (0.3g/kg/dose) in 2 divided daily doses for 6 months (figure 2). GLN will be dissolved in water, breast milk or formula and administered to the subject orally or through their enterostomy tube approximately every 12 hours (twice a day). This can be administered as a bolus or a continuous infusion lasting a maximum of 2 hours. We will use Nutrestore^{TM} (Emmaus Medical, Inc.), a commercially available, FDA approved pure L-GLN product in the study. In the event that this is not available we will use commercially available 100% USP grade L-GLN. The minimal volume L-GLN will be dissolved in at least 10 ml of water, breastmilk or formula.

Placebo group: Infants in the placebo group will receive ALA at a dose of 0.6g/kg body weight/day in 2 divided doses (0.3g/kg/day twice a day), exactly as outlined above for the GLN group.
ALA is a non-essential amino acid and will be used as the placebo in this study because it is a nutritional supplement without any known impact on gastrointestinal permeability or local effect on the mucosa or infections. It will balance out the administration of an amino acid (L-GLN) to the study population.

Figure 2: Study design



GLN/Placebo groups-Visits to clinical research site /clinic: baseline, days 30, 60, 90, 120, 150 and 180: Infants will be evaluated at the clinical research site at baseline, 30 (± 7 days), 60 (± 7 days), 90 (± 7 days), 120 (± 7 days), 150 (± 7 days) and 180 (± 7 days) after the baseline visit (figure 2). There will be a 7 day grace period before and after the desired clinic visit dates.

Approximately, two weeks prior to the all of the visits (except baseline), the site coordinator will mail a detailed 3-days food record to the family. This form will be returned for analysis of oral diet and fluid intake, including formula and tube feeding intake (see below). During each visit, the following procedures will be performed with data entered without subject identifiers into the case report form:

1. Complete medical history (including information on BSI): Gestational age, age at diagnosis of NEC, omphalocele, gastroschisisor atresia. age at surgery, type of surgery, dates/age for BSI events, types of organisms cultured (genus and species) and susceptibility to antibacterials;
2. Anthropometric measurements of weight, length, mid arm circumference and head circumference in duplicate, when possible (see Aim 2);
3. Enteral nutrition assessment: The subject's usual nutrient intake (PN, solid and liquid oral diet provided by food sources, supplements and/or tube feedings) will be assessed by the site staff and investigators. All of the primary caregivers will receive a 3-days' food record to complete about 1-2 weeks prior to their clinic visit. The nutritionist at each site

will evaluate this form and the data will be entered into the ESHA Nutrient Analysis Program to calculate the enteral total caloric and protein intake. The mean daily intake of PN and enteral nutrition calories and grams of protein and fat during the week prior to each evaluation visit will be determined and change (Δ) in these variables from baseline will be calculated. **Enteral diet optimization will occur throughout the study as clinically indicated using our comprehensive feeding advancement principles and algorithm for SBS children, as previously outlined[18].** The percent of total energy and protein needs that are met enterally will be calculated. PN will also be assessed for total calories and proportion of calories provided by dextrose, protein and lipids.

4. The family will be instructed on how to administer GLN and placebo. During the baseline visit, the initial teaching will be done and they will be provided with the premeasured powder. The dose (0.6g/kg/d (0.3g/kg/dose) of GLN or ALA) will be measured out in the research pharmacy based on the weight of the child at the visit into individual packets. Children hospitalized during the study will receive GLN or ALA from the research pharmacy delivered to the patient's bedside to be administered by nursing staff. At subsequent visits (except visit on day 180), this teaching will be repeated as the dose to be administered will change based on the weight of the child. The family will also be given a weekly drug diary to complete in between visits. They will return the drug diary to the study staff during subsequent visits.
5. During the initial visit, stool will be collected into sterile containers and stored at -80ºF until analysis for sIgA and bacterial composition evaluation. It is anticipated that the majority of stool will be collected in clinic during the study visit. If stool is collected in clinic, the stool will be stored immediately in the -80ºF freezer without any need for DNA stabilizer.
   If stool is not collected during this visit, families will be instructed on how to collect stool for evaluation into a sterile container. They will put this in a storage bag and kept cold until picked up by the home health team or delivered to the research team.
   Patients already enrolled will be provided with a sterile container with instructions to collect stool and bring in to the clinic visit for storage and analysis. The family will be asked to collect stool 24-48 hours before their clinic visit and put the stool in a sterile container which will be supplied to them by the study team. They will put this in a storage bag and keep cold. They will bring the sample to the clinic visit. RNAlater may be used with samples when samples are unable to be obtained within 24 hours of the clinic visit.

Stool sIgA analysis: sIgA will be determined by ELISA as described [27,64].

During baseline visit and visit on day 180 the following additional procedures will be done: Extra 2 ml of blood will be collected for plasma glutamine, proinflammatory cytokines analysis, flagellin and LPS specific IgG levels.

During a BSI the following procedure will be done: stool collection for bacterial analysis and sIgA measurement. Extra 2 ml of blood will be collected for flagellin and LPS specific IgG levels. The blood will be obtained as soon as possible when the patient is being investigated for a suspected CA-BSI. In the event that the episode does not meet the criteria for a CA-BSI the samples will not be evaluated.

All bacterial isolates obtained during the study from patients will be saved at their respective institutions (CCHMC and University of Michigan) until the end of the study in the event further work with them is required.

Plasma glutamine analysis: Plasma GLN will be performed using quantitative ion exchange chromatography[65].

Proinflammatory cytokine evaluation: Analysis will be performed utilizing Luminex xMAP technology (Austin TX) as previously described[66, 67].

Measurement of Flagellin and Lipopolysaccharide- specific Immunoglobulin G level: This will be done using enzyme-linked immunosorbent assay (ELISA) as previously described [18, 87-88].

Safety evaluation: The study coordinator will contact the family by phone to assess for the development of any serious adverse events on days 15(±7 days), 45 (±7 days), 75 (±7 days), 105 (±7 days), 135 (±7 days) and 165 (±7 days). There will be a 7 day grace period before and after the desired date of safety evaluation phone calls. The phone interview will assess safety and evaluate for any symptoms suggestive of adverse side effects related to L-GLN. If hospitalized, the safety evaluation will occur with the primary caregiver (bedside nurse or parent).

Additional safety precautions: If while enrolled in the study the patient develops multiorgan failure or requires vasopressors, the study drug will not be administered.

Clinical GI endoscopy
Patients with short bowel syndrome/Intestinal failure may have clinical indications for gastrointestinal endoscopies. These indications include:

- Gastrointestinal bleeding
- Persistent feeding intolerance leading to failure to advance enteral feeds

When the patient is scheduled for a clinically indicated gastrointestinal endoscopy, duodenal fluid (approximately 1 ml) will be aspirated into a sterile container. An additional tissue biopsy will also be obtained.
Patients will not have GI endoscopies only as part of the study procedures.

The samples of duodenal fluid and additional tissue collected will be stored in - 80º C freezer. They will subsequently be sent for bacterial composition evaluation at the University of Colorado using pyrosequencing techniques described below.

The following table summarizes Study Procedures:

Study Procedures Table for GLN/Placebo group

	Screening and Consent	Baseline (Day 0)	Day 15	30 day visit	Day 45	60 day visit	Day 75	90 day visit	Day 105	120 day visit	Day 135	150 day visit	Day 165	180 day visit (final visit)	When clinically indicated
Signed informed consent	X														
Medical History		X		X		X		X		X		X		X	
Physical Examination		X		X		X		X		X		X		X	
Blood collection for glutamine, proinflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8) and Antiflagellin and Anti-LPS IgG (2 ml))		X												X	X (During suspected or confirmed BSI)
Weight, length mid arm and head circumference velocities		X		X		X		X		X		X		X	
Nutritional intake evaluation		X		X		X		X		X		X		X	
stool collection for bacterial and sIgA analysis		X		X		X		X		X		X		X	X (During suspected or confirmed BSI)
Instruction on how to measure & administer GLN/placebo		X		X		X		X		X		X			
3-day food record completed by family				X		X		X		X		X		X	
Phone interview			X		X		X		X		X		X		
Aspiration of duodenal fluid (~1 ml) and 1 additional biopsy from the duodenum															X

Study procedures of control group:
Healthy age-matched infants (n=12; 6 at each site) will have serial stools collected on 4 occasions, each separated by 60 days [baseline, days 60(±15), 120(±15) and 180(±15)]. This is abbreviated compared to the study subjects.

A week prior to the scheduled date of stool collection, families will receive a brief medical questionnaire and stool container. They will be instructed to complete the questionnaire and send in with the stool specimen.

They will be provided with a sterile container with instructions to collect stool and bring in to the clinic for storage and analysis. They will be asked to collect stool on the day of the next anticipated clinic visit, put the stool in a sterile container (which may contain RNAlater) which will be supplied to them by the study team. They will put this in a storage bag, keep cool and bring the sample to the clinic visit.

Parents/Primary caregivers drop off stool specimen to the research team at the study site or will arrange with the team for the specimen to be collected from their homes.

Study Procedures Table for Control group

	Screening and Consent	Baseline (Day 0)	60-day visit	120-day visit	180-day visit (final visit)
Signed informed consent	X	X			
Medical History/questionnaire		X	X	X	X
stool collection for bacterial and sIgA analysis		X	X	X	X

I. Data Collection

Demographic and clinical information will be collected whenever clinical data are available via uniform baseline and follow up clinical data extraction forms to ensure accuracy and consistency. The essential demographic information includes age at diagnosis, gender, race and ethnicity, birth history and past medical history. The clinical information includes bowel length, dates and type of surgeries, nutrition data (formula type, amount, weaning foods, parenteral nutrition type and amount), infection type (genus, species and susceptibility to antibacterials) date infection is diagnosed) hospital admissions and hospital length of stay.

J. Compensation

Participants will receive $10 for every stool collected for analysis during study participation. Subjects will receive study medication, clinical supplies, and laboratory evaluations related to the study free of charge.

X. Assessment of Efficacy

Hypothesis 1: Enteral GLN supplementation in infants with SBS after massive bowel resection for NEC will decrease BSI, and thereby decrease systemic proinflammatory cytokine concentrations.

Specific Aim 1: To perform a double-blind, randomized, placebo controlled, 6-months pilot study of enteral GLN (0.6g/kg) vs. isocaloric, isonitrogenous alanine (placebo) treatment in PN-dependent SBS children due to NEC to evaluate the efficacy of GLN to decrease: a) BSI (primary endpoint) and b) serial serum concentrations of TNF-α, IL1- β, IL-6 and IL-8 (secondary endpoint). **The primary outcome is the incidence of BSI during the 6 months on GLN supplementation.** BSI is defined as laboratory-confirmed, culture-proven bloodstream infections that meet the Centers for Disease Control and Prevention (CDC) definition[68, 91]. This allows for the distinction between catheter related-BSI and other causes of BSI.

Secondary outcomes: 1) Plasma proinflammatory cytokine levels (TNF-α, IL-1β, IL-6, IL-8); 2) readmissions and hospital length of stay; 3) proportion of nutrition delivered enterally versus parenterally; 4) stool sIgA levels; 5) Plasma glutamine.

Expected results: We expect that that the SBS children on enteral GLN supplementation will have significantly fewer BSI and greater decline in blood cytokine levels during the period of therapy.

Hypothesis 2: Enteral GLN supplementation will improve somatic growth parameters in Aim 1 patients.

Specific Aim 2: To assess the efficacy of 6 months of therapy with enteral GLN on somatic growth parameters [length velocity (primary endpoint), weight velocity, mid arm circumference and head circumference] in Aim 1 subjects and the impact of BSI on these growth parameters.

The primary outcome for specific aim #2 is length velocity. Secondary outcomes are growth parameters of weight mid arm circumference and head circumference velocities. These are clinically relevant growth parameters identified by our preliminary data in which SBS children had growth delay with short lengths and smaller head circumferences than infants without NEC or SBS[8].

Data collection: During CNC/clinic visits (baseline, days 30, 60, 90, 120, 150 and 180) as described above, each child will have weight, length mid arm circumference and head circumference measured in duplicate. These measurements will be done by a trained member of the study staff at each center. The individual at each site will be trained to perform these

measurements according to standard methods previously described[69, 70]. Institutional scales, length boards and measuring tapes will be used at each center.

Expected results: We expect that SBS children on enteral GLN supplementation will have more rapid growth as measured by the parameters above. The group with no BSI or decreased BSI (2 episodes) during the study period is expected to have a greater increase in growth parameters compared to the group with > 2 BSI.

Hypothesis 3: The gut lumenal microbiome is altered in pediatric SBS versus non-SBS controls, correlates with BSI and is altered by enteral GLN supplementation.

Specific Aim 3: To comprehensively assess the gut lumenal microbiome (using state-of-the art molecular methods) in Aim 1 SBS patients versus non-SBS, age-matched control subjects (primary endpoint), to correlate major bacterial dynamics present in the lumen with BSI due to specific microbes and to explore whether the gut lumenal microbiome is altered by enteral GLN treatment.
Primary outcome: Increased *K. pneumonia* and *E. faecalis* in the gut microbiome of SBS infants with BSI.
Secondary outcome: Alteration in other commensals in the gut lumenal microbiome of SBS infants.

Subjects: Infants recruited in specific aim 1 (n=48 – subjects- 36; controls- 12)
Healthy age matched infants (n=12) will be recruited from the Gastroenterology and surgery clinics.

Sample Procurement: Stool samples (~500 mcg), will be obtained from infants' diapers or ostomy bags. Stool specimens will be collected prospectively during visits at baseline, days 30, 60, 90, 120, 150 and 180. **Stool will also be collected whenever the patient has a BSI.**

The controls (12 healthy infants) will have serial stools collected on 4 occasions, each separated by 60 days (baseline, days 60, 120 and 180).

It is anticipated that stool samples will be collected during the clinic visit. All stool samples will be scooped into a stool container and stored immediately in a -80°C freezer. For samples collected at home, subjects will be instructed to collect in a container containing RNAlater tissue reagent. This will be stored in a cold at home and when delivered to the study team will be stored in -80°C freezer until processed. RNAlater provides immediate stabilization of DNA, RNA, and protein in samples at room temperature. This preserves the in vivo profile of DNA, RNA, and proteins, allowing reliable downstream analysis. Stabilized samples can be transported at 15–25°C for up to 7 days, or stored at 2–8°C for up to 12 months. For longer storage, stabilized samples can be archived at –80°C.

Small bowel fluid and tissue procurement: If the patient is scheduled for a clinically indicated gastrointestinal endoscopic procedure; duodenal fluid (~1 ml) will be aspirated and an additional duodenal biopsies will be collected. Specimens will then be stored at -80°C within 10 minutes of obtaining until processing. They will subsequently be sent for bacterial composition evaluation at the University of Colorado using pyrosequencing techniques described below.

Genomic DNA Preparation: Total genomic DNA will be prepared from specimens using a robust protocol that entails chemical and mechanical disruption of cells, which is performed in a HEPA-filtered, UV-sterilized PCR hood[71-74]. Sham extractions are performed in parallel to detect contaminated reagents. **Broad-Range PCR:** DNA lysates are subjected to PCR with pan-bacterial 16S rDNA primers (27FYM+3 and 338R), yielding libraries of PCR amplicons representative of all bacteria in a specimen[74, 75]. Triplicate PCR reactions will be performed and amplicons pooled for each sample. To alleviate representational bias in PCR amplicon libraries, we will determine empirically the minimum number of PCR cycles required to generate the quantities of DNA required for direct sequencing by broad-range rDNA Q-PCR. **High throughput DNA Pyrosequencing**: Subject PCR amplicon libraries will be sequenced using the high-throughput Genome Sequencer™ FLX pyrosequencing platform of Roche/454 Life Sciences, Inc., available through the University of Colorado's Consortium for Comparative Genomics. Barcoded PCR primers will be used to multiplex ~200 libraries per pyrosequencer run[76-79]. Approximately 5000 rRNA gene sequences will be determined for each sample library, which based on our preliminary studies will comprise a comprehensive census of the resident microbes. Additional sequences will be screened if coverage is less than 95%[79-81].

XI. Assessment of Safety

A DSMB will monitor the study for the occurrence of adverse events (both serious and otherwise). A significant increase in the rate of adverse events would be cause for concern for the safety of participants in the study. Information on adverse events will be presented in several ways: (1) listings of serious adverse events with accompanying narrative summary by the PI: (2) summaries of adverse events by body system and type of event. This information will be presented to the DSMB.

Interim monitoring reports for the DSMB will include, but will not be limited to, such tabulations as:

1. Frequency of BSI and hospitalization
2. coagulation studies
3. liver profiles
4. renal profiles
5. Frequency of adverse events

The DSMB will meet at the start of the study, then after 5 patients have received treatment. Subsequent meetings will be at the discretion of the chair given the expected speed of recruitment and study medication administration.

The DSMB chair (or designee) will be asked to review SAE reports within 48 hours after initial receipt of the information by the investigator(s), to review the PIs assignment of SAEs as related or unrelated to treatment, to confirm the grading of any toxicities. This will also be reported to the site IRB and the study sponsor.

A safety analysis will be performed after approximately 5 patients have received treatment or, if enrollment begins slowly, 12 months after the first patient is enrolled. Safety data will be shared with the Data and Safety Monitoring Board (DSMB). An investigator in this study will not be a member of the DSMB. Members of the DSMB will receive safety data approximately every 6 months for review or more frequently if requested by the chair. Safety assessments will consist of vital signs, AEs, and laboratory evaluations. A cumulative listing of patient withdrawals, dose adjustments, and serious adverse events (SAEs) will also be reviewed. DSMB members will be notified of all SAEs reported expeditiously to regulatory authorities.

Dr. James E. Heubi, Division of Gastroenterology, will act as the chair of the DSMB. There will be 2 other members appointed to the DSMB: a statistician (Dr. Christopher Lindsell) and a neonatologist (Dr. Kurt Schibler), who are not involved in the conduct of the study. Dr. Sean Moore will serve as the safety officer for this study. The safety officer is not a member of the DSMB and he is an expert on glutamine metabolism and use in children with chronic and persistent diarrhea.

Study Monitoring

An investigational new drug (IND) application was submitted by the PI for the use of L-GLN in the management of infants with SBS. An independent monitor from the CCHMC Clinical Trials Office will conduct routine independent monitoring visits that shall occur one or more times during the period after study initiation but before study closeout. Guidelines for scheduling monitoring visits shall be determined according to the stage of development, complexity of the study, the rate of subject accrual and other factors. Monitoring visits are conducted for routine monitoring only and are intended to ensure that the protocol and applicable regulatory requirements are being followed, that subjects' rights and safety are protected, and to confirm data integrity and quality.

XII. Adverse Events

An adverse event (AE) is any untoward medical occurrence in a patient participating in this trial. An adverse event can be an unfavorable and/or unintended sign (including an abnormal laboratory finding), symptom, syndrome or disease associated with or occurring during the use of an investigational product whether or not considered related to the investigational product.

A serious adverse event (SAE) is one that:

1. results in death
2. is life threatening
3. requires an inpatient hospitalization or prolonged existing hospitalization
4. is a persistent or significant incapacity or substantial disruption of ability to conduct normal life functions
5. is a congenital anomaly/birth defect
6. is an event that does not meet the above criteria but may jeopardize the patient or may require medical or surgical intervention to prevent one of the outcomes listed above.

IRB Notification of SAEs

Per Institutional Review Board (IRB) Requirements, the Investigator will report serious adverse events to the IRB Office within the time period required at each institution (at CCHMC this is outlined in research policy R-18). All other adverse events that are not serious will be reported to the IRB at the time of continuing review, per IRB requirements.

AE's require prompt (within 7 days after knowledge of the problem) reporting to the IRB when meeting the following criteria:

- Any unanticipated problem involving risk to subjects
- Deviations from or changes to, the protocol to eliminate immediate hazards to the trial participants
- Changes increasing the risk to subjects and/or significantly affecting the conduct of the trial.
- All adverse drug reactions that are both serious and unexpected
- New information that may adversely affect the safety of the subjects or conduct of the trial.

These events will be reviewed by the safety officer and are required to be reported to the FDA within 7 calendar days of the sponsor's initial receipt of the information if characterized as fatal or life-threatening. Non-fatal or non-life threatening events must be reported in 15 calendar days or at the time for annual review (reporting will be based on the FDA criteria for reporting AEs). In addition, these events are to be reported to the IRB based on IRB reporting guidelines. .

Recording of Adverse Events

Adverse event recording will begin after administration of study medication.

Diagnostic and therapeutic non-invasive and invasive (i.e. surgical) procedures will not be reported as adverse events. However, the medical condition for which the procedure was performed must be reported if it meets the definition of an adverse event, unless it is pre-existing (prior to initiating GLN).

Medical conditions/diseases present before starting drug are only considered adverse events if they worsen after starting treatment.

Symptoms of the original or targeted disease are not to be considered adverse events in this treatment. The following symptoms are indicative of underlying disease and will not be reported as adverse events: Malabsorption, growth failure, liver disease and BSI.

Abnormal laboratory values or test results constitute adverse events if they induce clinical signs or symptoms or require therapy. They are to be recorded on the Adverse Events CRF under the signs, symptoms or diagnoses associated with them. CA-BSI will only be reported as an adverse effect if the rate is higher than 20% of what is standard for the center.

As far as possible, each adverse event will be assessed by the Investigator with respect to its duration (start and end dates), the severity grade (mild, moderate, severe, life-threatening, fatal), its relationship to the drug (unrelated, unlikely to be related, possibly, probably and definitely related), the action(s) taken, the outcome (i.e. resolved, resolved with sequelae, continuing), gravity (SERIOUS or NOT SERIOUS), and expected/unexpected on the basis of drug insert.

Grading (Severity) of Adverse Events

Each adverse event will be graded to describe its intensity according to the Severity scale listed for each AE found on the
NCI's Common Terminology Criteria for Adverse Events Version 4.0.

Relationship to Drug

For all adverse events that occur while the patient is on drug, the relationship to the drug must be assessed by the Investigator using the following scale:

1 = not related
2 = unlikely
3 = possibly related
4 = probably related
5 = definitely related

For Grade > 3 adverse reactions which are possibly (3), probably (4) or definitely (5) related to the drug, study medication should be discontinued as described above.

Please note: For safety data analysis, not related and unlikely are considered "not attributable;" possible, probable and definite are considered "attributable." Assessment of relationship to drug must be carried out by the Investigator according to the 5-point scale above.

Gravity (Seriousness) of Adverse Events

Each adverse event is to be classified by the Investigator as SERIOUS or NOT SERIOUS.

Follow-Up after Adverse Events

All adverse events will be followed until they are resolved or the patient's participation in the treatment ends (i.e., until a final report is completed for that patient).

In addition, all SAEs and those non serious events assessed by the Investigator as attributable (i.e. possibly, probably, definitely related to the drug) will continue to be followed even after the patient's participation in the treatment is over. Such events will be followed for 30 days post treatment. Resolution of such events is to be documented in the CRF.

Expedited Reporting

SAE's require expedited reporting when meeting the following criteria:

- Serious
- Unexpected
- At least possibly related to the study agent or other protocol specific activity

Serious Adverse Events meeting the above criteria are required to be reported to the FDA as follows:

- If characterized as fatal or life-threatening, within 7 calendar days of the sponsor's initial receipt of the information
- If non-fatal or non-life threatening, within 15 calendar days.

In addition, the sponsor is required to submit serious adverse events to the DSMB for review, and serious adverse events requiring expedited reporting will be reported to the IRB concurrently.

Significant unplanned deviations from the protocol will be reviewed and will be reported as stipulated above.

XIII. Statistical Analysis Plan

To assess the comparability of the two treatment groups, demographic and baseline data will be summarized for each treatment group. For continuous data, mean, standard deviation, median, min, max, first quartile and third quartile will be presented. Comparisons will be made using the two-independent sample t-test or the Wilcoxon Rank sum test, depending on the distribution of the variable. For categorical data, frequencies and percentages in each group will be presented. Comparisons will be made using Fisher's exact test. These tests will be conducted at the two-sided, 5% level of significance. In addition, demographic and baseline data for the control group will be summarized and presented as described above. The statistical analysis plan for each aim is presented below.

Aim 1 Hypothesis: Enteral GLN supplementation in infants with SBS after massive bowel resection for NEC will decrease BSI, and thereby decrease systemic proinflammatory cytokine concentrations.

The primary outcome variable is the incidence of BSI during the 6 months on GLN supplementation. BSI is defined as laboratory-confirmed, culture-proven bloodstream infections that meet the Centers for Disease Control and Prevention (CDC) definition[69]. To compare the incidence of BSI during the 6 month time period, the percent of patients with at least one BSI will be calculated in each group. Since this is a pilot study, the desire is to detect a trend in improvement of BSI with GLN supplementation. Therefore the 2 groups will be compared using the Fisher's Exact test at the one-sided 10% level of significance which will indicate a significant trend. As an additional analysis, time to first BSI will be displayed using Kaplan-Meier (K-M) curves. The log-rank test will be used to test for differences in curves between treatment groups. Patients will be censored if they exit the study prior to having their first BSI. As a further exploratory analysis, time to multiple-event analyses techniques will be used to compare groups where the multiple events represent multiple episodes of BSI within patients.

Secondary outcomes include the following and will be tested at the two-sided 5% level of significance:

1) Plasma proinflammatory cytokine levels: TNF-α, IL1-β, IL-6, and IL-8. Change from baseline values at 180 days will be computed for cytokine levels. Change from baseline to 180 days values will also be calculated for the flagellin and LPS IgG levels. Appropriate transformations of the data (e.g. log) will be done prior to calculating change from baseline in order to meet the assumptions of the analyses (i.e. normality, equal variance). Analysis of covariance techniques will be used to compare the change from baseline scores of the treatment groups. Covariates included in the analysis will be baseline value, age, gender, ostomy status, feeding type (formula, breast milk).
2) Length of hospital stay and number of readmissions: For length of hospital stay, treatment groups will be compared using the Wilcoxon Rank Sum test. For number of readmissions, either a Wilcoxon Rank Sum test or a Chi-Square test for homogeneity will be used to compare groups, depending on the variability of the number of readmissions.
3) Stool sIgA levels: Treatment groups will be compared using the random effects repeated measures analysis of covariance. The response variables for each of the parameters will be change from baseline. The time points included in the analyses will be 30, 60, 90, 120, 150, 180 days post-baseline. If required to meet the assumptions of the analyses (normality, equal variances), appropriate transformations will be used. Treatment groups will be compared at each individual time point using appropriate contrasts and the error term from the repeated measures model. Covariates will include baseline value, age, gender, ostomy status, feeding type (formula, breast milk).
4) Proportion of nutrition delivered enterally versus parenterally: The statistically analysis approach for this endpoint will be the same as for the Stool sIgA levels discussed above. Transformations appropriate for proportions may be used, if necessary, to meet assumptions of the analyses.
5) Plasma glutamine: The statistical analysis approach for this endpoint will be the same as for the proinflammatory cytokine levels discussed above.
6) Impact of level of compliance on outcome will be evaluated for a dose effect analysis.

Aim 2 Hypothesis: Enteral GLN supplementation will improve somatic growth parameters in Aim 1 patients.

7) The two treatment groups will be compared using the random effects repeated measures analysis of covariance for the following parameters: length velocity, weight velocity, mid arm circumference and head circumference. Length velocity and weight velocity will be analyzed as the post baseline scores. Mid arm circumference and head circumference will be analyzed as change from baseline. The time points included in the analyses will be 30, 60, 90, 120, 150, 180 days post-baseline. If required to meet the assumptions of the analyses (normality, equal variances), appropriate transformations will be used (e.g. log). Treatment groups will be compared at each individual time point using appropriate contrasts and the error term from the repeated measures model. Covariates will include baseline value, age, gender, ostomy status, feeding type (formula, breast milk). For length velocity and weight velocity, the baseline value will be the baseline length and weight values.

   To evaluate the impact of BSI on the growth parameters listed above, point biserial correlation coefficients will be constructed overall and within each treatment group and tested to determine significance.

Aim 3 Hypothesis: The gut luminal microbiome is altered in pediatric SBS versus non-SBS controls, correlates with BSI and is altered by *enteral* GLN supplementation.

8) Intestinal microbial groups (e.g., species, genera) will be correlated with the treatment or control arms of the GLN supplementation trial and with bacteria identified during any diagnosed BSI. The frequency of rRNA sequences will be interpreted as the relative abundances of organisms in samples of stool and standardized by gram of stool weight. Bacteria present in specimens will be preliminarily identified by use of the Naïve Bayesian Classifier Tool of the Ribosomal Database Project[82.83]. The strength of association of individual microbial groups with treatment arms and/or main outcome measures will be measured by comparing the abundances of these groups using the non-parametric Kruskal-Wallis test. Experimental reproducibility and inter- and intra-individual similarities in microbial populations will be assessed by calculating and comparing the sequence-abundance-based Morisita-Horn index[81,]. In all statistical analyses 2-sided *p*-values less than 0.05 will be considered statistically significant. Results will be corroborated through the phylogeny-based UniFrac metric, which assesses population-wide differences in microbial lineages between different groups of subjects (SBS vs. control) [82]. Stool-associated bacteria collected at BSI incidence will be compared to the species identified on blood culture to determine whether intestinal bacteria are potential sources for BSI. Alternatively, absence of particular species from the stool at the time of BSI diagnosis will indicate that lack of specific bacterial species from the stool predisposes to BSI. Tissue samples collected during endoscopy or surgery and intestinal fluid aspirated during endoscopy will be analyzed in parallel with stool samples in order to assess the use of stool samples as proxies for intestinal samples. Patterns will also be reviewed to assess for relationships to enteral feeding rate.

These methods have been used and demonstrated in 2 published studies by one of the collaborators/co-investigators [89, 90].

Sample size

This is a pilot study and will provide estimates of effect sizes and variability to be used to power a future definitive study. The power of this pilot study was investigated based on the primary endpoint of BSI infection rate and the study was powered to detect a trend toward improvement in BSI rates. For this study, we defined "trend" as a significant improvement tested at the one-sided, 10% level of significance. A total of 18 patients per treatment group will be enrolled in the study. Assuming a 10% dropout rate, it is expected that 16 patients per treatment group will complete the study. A sample size of 16 patients per treatment group will provide at least 85% power to detect a trend if the true BSI rates are 75% versus 30% BSI rate at the one-sided 10% level of significance. This calculation is based on the Fisher's exact test for two proportions.

A sample size of 16 patients per treatment group will also provide at least 80% power to detect a geometric mean ratio of 2.0 for the two treatment groups for the proinflammatory cytokines assuming a coefficient of variation of 0.7 using a two-sided, 5% level of significance test.

We anticipate applying for a follow up R01 /U01 to attract other centers that will allow a larger more definitive study regarding the use of GLN in pediatric SBS.

IX. Data Management

Each subject will be given a unique identifier. Only members of the study team will be able to link this identifier to the subject. No patient identifiers will be published. The specific data items to be captured will be stored in a secure area that will be a locked filing cabinet or in a password protected electronic file.

REDCap (*Research Electronic Data Capture*) is a secure, web-based application designed exclusively to support data capture for research studies. This application will be used for data entry during the study. REDCap provides: 1) an intuitive interface for data entry (with data validation); 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages (*SPSS, SAS, Stata, R*); and 4) procedures for importing data from external sources

Deidentified samples and data will be retained until all the final data analysis and publication. Data/specimens/identifier will be destroyed at the earliest opportunity consistent with the research plan.

Unused plasma, stool and mucosal tissue will be deidentified and stored for future analysis if the participant provides consent for this purpose. These will be used in the future for further analysis of bacterial DNA analysis as newer methods are developed in the field of pyrosequencing, nutrition metabolism and microbial DNA analysis.

X. Financing

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