Microbiome directed prevention of parenteral nutrition associated liver injury in the NICU

1. SPECIFIC AIMS AND HYPOTHESES

Parenteral nutrition (PN) is a lifesaving nutritional intervention in premature infants with delayed tolerance of oral or enteral feeds. PN-associated liver disease or cholestasis (PNAC) is a frequent complication of PN, with high morbidity and associated healthcare costs. Lipid sparing strategies and alternative lipid formulations mitigate the risk and severity of PNAC in the NICU, however these approaches are often used as reactive countermeasures–once liver injury has already occurred. The central theme of this proposal is to leverage the microbiome to predict–versus simply react to–PNAC, thereby setting the stage to prevent PNAC in high risk children. To that end, we propose pilot studies to elucidate the role of the gut microbiome in PNAC and identify microbiome signatures predictive of PNAC. Through an established iTHRIV UVA-Inova collaboration, we have for the first time identified blooms of gram-negative bacteria (Klebsiella, Veillonella, Enterobacter and Enterococcus) in the stool of 4 sets of premature twins discordant for PNAC. Importantly, clinical NICU infections with gram-negative bacteria are known risk factors for PNAC; however, the ability to predict PNAC based on a subclinical bloom of these bacteria in neonatal stools has not yet been fully explored. Further, no definitive chain of causality between these blooms and PNAC has been established. These knowledge gaps warrant further exploration in both neonates and preclinical models. Hence, our goals are two-fold: 1) Establish a microbiome-based prediction algorithm for PNAC to facilitate early intervention, 2) Determine the degree to which transplantation of PNAC-associated microbiota from infants to germ-free mice confers susceptibility to PNAC. If successful, these pilot studies will provide compelling preliminary data for comprehensive collaborative project proposals between the Departments of Pediatrics at UVa, Inova, Carillion, and VCU and UVA Biomedical Engineering.

We will leverage our group’s interdisciplinary expertise in the microbiome, pediatrics, mathematical modeling, and preclinical models of PNAC to address the following Specific Aims:

**Aim 1.** Develop a microbiome-based prediction tool for PNAC in neonates. *We hypothesize blooms of gram-negative taxa in the stool are a robust predictor of PNAC in NICU patients when combined with other clinical predictors.*

**Aim 2.** Determine the extent to which the stool microbiota of infants with PNAC confers greater susceptibility to PNAC in gnotobiotic mice. *We hypothesize a higher abundance of gut gram-negative taxa is a driver of PNAC.*
2. IMPACT

One in twelve babies in the United States is born too soon. Premature infants unable to tolerate feeds by mouth or directly to the gut require parenteral nutrition (PN) to grow until digestive function matures. Through mechanisms poorly understood, a subset of neonates on PN develop an associated cholestasis (PNAC) and liver injury, with an incidence exceeding 50% of infants with birth weights less than 1000 g and 85% of infants requiring PN for longer than 14 weeks (Christensen et al. 2007). Liver injury persists even after PN cessation, representing a vast health and economic burden (Mangalat et al. 2014). Recently, alternative fat or lipid sources in PN such as fish oil based lipids (Omegaven®) and mixed sources of lipids (Smoflipid®) have been shown to limit the progression and injury from PNAC (Park et al. 2015; Kasirer et al. 2019). However, due to cost and insurance limitations, these protective lipid formulations are currently reserved only for infants who have already developed PNAC. Figure 1 shows a rise conjugated bilirubin levels from a set of twins who received TPN simultaneously in the UVA NICU. Why did one develop PNAC and not the other? We and others have shown an intriguing association between the development of PNAC and the microbiome, (Figure 2; Cahova et al. 2017; Li et al. 2017; Lee et al. 2015). Further, PN itself is known to cause alterations to the gut microbiome. Thus the gut microbiome provides a compelling target for understanding and preventing PNAC. First, gut microbiome composition and function can be measured non-invasively. Second, the microbiome is modifiable with prebiotics, probiotics, antibiotics, and diet.

![Figure 1](image_url)

**Figure 1.** Conjugated bilirubin (CB) levels from 1 of 4 premature twin pairs identified in the UVA and INOVA NICUs who were discordant for parenteral nutrition associated cholestasis (PNAC).

Importantly, results from this study will allow us to determine the extent to which we can identify infants at risk for PNAC before the onset of liver injury. This will set the stage for larger, rigorously designed collaborative clinical and translational studies between iTHRIV institutions and future translation of these findings into a bedside clinical tool for early identification and intervention of infants at risk for PNAC, e.g., preemptive lipid sparing strategies, early transition from soy-based lipid formulations to next generation lipids, or the development of novel biomarkers. Furthermore, our findings will help generate mechanistic hypotheses for microbiome modification using prebiotic, probiotics, or targeted antibiotics as a viable strategy to prevent PNAC in the NICU.

3. PROJECT TEAM

Our proposal builds on a successful, established iTHRIV collaboration between Dr. Suchi Hourigan (INOVA Pediatric Gastroenterology), Dr. Sean Moore (UVA Pediatric Gastroenterology), and Dr. Jason Papin (UVA Biomedical Engineering). To support our new directions of predictive microbiome signatures in NICU patients and etiologic studies in gnotobiotic mice, we have added two essential collaborators: 1) Dr. Glynis Kolling (UVA Infectious Diseases) and 2) Dr. Ajay Kumar (UVA Pediatric Gastroenterology), who bring expertise in germ free mouse studies and mouse models of parenteral nutrition, respectively. We will leverage existing IRB approvals and collaborative agreements between our institutions as well as take advantage of an ongoing expansion of the gnotobiotic mouse facilities at the University of Virginia (see letter of support from Dr. Sandy Feldman) under UVA’s Equipment Trust Fund.

4. RESEARCH PLAN

**Specific Aim 1:** Develop a microbiome-based prediction tool for PNAC in neonates.

**Hypothesis:** We hypothesize blooms of gram-negative taxa in the stool are a robust predictor of PNAC in NICU patients when combined with clinical predictors.

**Rationale and Approach:** A number of liver disorders are associated with intestinal microbial dysbiosis, or imbalance in gut microbial communities (Fickert and Marschall 2019). Recently, through our iTHRIV UVA-Inova collaboration, we have shown that the stool microbiome of 4 premature twin sets discordant for PNAC differs in relative abundance of
specific genera, predominantly (Figure 2). We have presented this data as a platform presentation at Digestive Disease Week 2018 and the manuscript is currently in review at the Journal of Pediatric Gastroenterology & Nutrition. Our results add to evidence for a clear role of select gram-negative bacteria in the development of cholestasis (Bajaj et al. 2014; Tang et al. 2018). More specifically, our preliminary data showed a significant increase in the relative abundance of Klebsiella, Veillonella, Enterobacter and Enterococcus, with a decreased relative abundance of Escherichia/Shigella (p<0.05) in infants with PNAC compared to their twins who did not develop PNAC (Figures 1 and 2).

For the current proposal, we will extend and validate findings descriptive findings from our twin analysis to a larger cohort of UVa and Inova NICU patients to establish the temporal sequence of microbiome shifts in PNAC and infer causal relationships. 16s rRNA gene sequencing of the microbiome will be performed on serial stool samples from 30 infants who developed PNAC and 30 matched control infants who received PN but did not develop PNAC. Infants will be matched for gestational age, birth weight, antibiotic exposure and PN exposure, which are all risk factors for PNAC (Kim et al. 2016). Importantly, serial stool samples for these infants have already been collected and stored at both Inova and UVA through established IRB approved protocols (Inova IRB #15-1945, UVA IRB #18244). The use of this extended cohort will allow us to identify species of bacteria (as opposed to genera) associated with PNAC and assess which species bloom prior to the development of PNAC (Ong et al. 2013).

Comprehensive microbial and host metabolite identification and quantification will be performed on select stool samples in infants with PNAC prior to PNAC development and time matched stool samples from their control infants via mass spectrometry (Gould et al. 2018). This will allow us to identify microbial metabolites involved in PNAC development and clarify mechanisms by which the microbiome drives PNAC development. This finding would, in turn, set the stage for developing point-of-care tools for identification of metabolites predicted to be involved in PNAC (e.g., phytosterols, Zaloga et al. 2015).

**End Point Measurements:** Taxa at the species level involved in the development and progression of PNAC. Identification of stool metabolite shifts prior to PNAC development.

**Statistical Analysis:** We will use a machine learning technique, Area Under the ROC Curve Random Forest, to identify the minimal set of microbial species that are correlated with the development of PNAC. This method will be similar to and informed by our recently submitted publication. Additionally, we will use the same Random Forest method to identify metabolites that are correlated with the development of PNAC. Based on the accuracy found, the models generated using this method can be used to also predict if new samples are from patients who have or may soon develop PNAC. Cross-validation will be used to determine the accuracy of the optimal Random Forest models. In addition to machine learning, we will be applying standard statistical methods, such as corrected Wilcoxon rank sum tests, to confirm the significance of our findings. All methods will be implemented in R.

**Potential Problems and Alternatives:** There is an expected ~5% failure rate when performing 16S rRNA sequencing of fecal samples and occasionally repeat sequencing is required. We have accounted for this in the budget. In addition, environmental contaminants may affect results of both microbiome sequencing and metabolite analysis, therefore negative
control samples will also be run. In studies of the human microbiome, high interindividual variation makes inference challenging with small sample sizes. Our previous NICU microbiome studies have shown a more restricted repertoire of bacteria, which will facilitate comparisons between babies with and without PNAC. Forest models are designed specifically to determine which features, in our case species and metabolites, drive the major difference between classified groups. However, these models can not differentiate between features that are contributing mechanistically to the disease state versus simply biomarkers. We plan to address this limitation in Aim 2 mouse studies.

**Specific Aim 2: Determine the extent to which the microbiomes of infants who developed PNAC confers a higher risk of PNAC to gnotobiotic mice, thereby establishing a chain of causation.**

**Hypothesis:** Transplant of fecal microbiota from twin infants discordant for PNAC will confer differential sensitivity to PNAC in gnotobiotic mice.

**Experimental Approach:** Stool samples available from infants discordant for PNAC were collected from infants after PNAC development. Figures 3 and 4 summarize our design. Briefly, 6-week-old C57BL germ-free, female mice will be ordered and acclimatized for one week. Animals will be randomized and divided into two groups of five mice each. We will gavage 200 ul of fecal suspension for 3 consecutive days (Wong et al. 2017). To prevent contamination with after removal from the gnotobiotic facility, mice will be maintained in BSL3 cages and racks. We anticipate full colonization within 4 weeks after transplantation to the mouse (Wrzosek et al. 2018).

Animals will be cannulated in the right jugular vein and placed on TPN by Dr. Kumar, a postdoctoral fellow in the Moore Lab, who gained expertise in murine models of TPN during his training at the Department of Surgery, University of Wisconsin, Madison, under the guidance of Allen Kudsk, MD. Animals will be fed ad libitum chow diet and water access 48 hours post-cannulation for recovery purposes. Animals will receive intravenous constant infusion of total parenteral nutrition (TPN) in a graded fashion for the first three days (Pierre et al. 2015). Thereafter the animal will receive TPN at the highest rate (Sitren et al. 1983). In this model, mice on TPN develop liver complications after 2 weeks (Tazuke et al. 2009). Mice will also be administered intravenous soy oil which has been shown to induce steatohepatitis (Nandivada et al. 2017). At the end of study, animals will be euthanized and tissues will be harvested.

**End Point Measurements:** Liver Histology, Serum Biomarkers of Liver Inflammation- AST, ALT, Bile acids, Luminal Biomarkers of inflammation in feces, mouse fecal microbiome.

**Statistical Analysis:** Previous studies have shown that to achieve an experimental power of 80% in TPN induced steatosis, 15 animals per group will be sufficient (Xu Z. et al. 2019). The survival rate of animals after surgery is 70-80% (Tazuke et al. 2009), therefore we intend to repeat this study 4 times and pool the data together. The data will be analyzed in Graph-Pad Prism 7 by using two-tailed T test a 95% confidence level.

**Potential Problems and Alternatives:** Our team has been at the vanguard of germ free mice and associated experiments at UVA. To our knowledge, no one has attempted TPN administration in germ free mice colonized with human microbiota, hence we don't know whether mice will tolerate these conditions. If not, alternatives include antibiotic...
depletion of the microbiota or a polyethylene glycol cleanse prior to fecal microbiota transplant (Wrzosek et al. 2018). Together, these complementary approaches using previously acquired samples from NICU patients and novel studies in germ-free mice will allow us to elucidate a causal relationship between the gut microbiome and PNAC.

5. DESCRIPTION OF TEAM SCIENCE

Members of this team spearheaded one of the very first iTHRIV collaborations between INOVA and UVA, working together to generate novel data from pooled twin samples, deliver a platform presentation at Digestive Disease Week 2018, and generate a manuscript that is currently in review. Now, with the addition of Drs. Ajay Kumar, whose background is in nutritional sciences, immunology, and microbiology, we bring rare expertise in murine TPN models. Further, Dr. Kolling has worked closely with Comparative Medicine at UVA to expand our up and running gnotobiotic facilities. Dr. Hourigan at INOVA is a pediatric gastroenterologist and emerging leader in microbiome studies in neonates. Dr. Papin and student Tom Moutinho bring quantitative excellence to microbiome modeling studies. Finally, Sean Moore is a pediatric gastroenterologist and mentor to Dr. Kumar.

6. TIMELINE

<table>
<thead>
<tr>
<th>Aim 1</th>
<th>Quarter 1</th>
<th>Quarter 2</th>
<th>Quarter 3</th>
<th>Quarter 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbiome 16S rRNA sequencing</td>
<td>Prioritization of patient samples for retrieval from stool biobank; update DUA/MTA.</td>
<td>16S rRNA sequencing, identification</td>
<td>Microbiome bioinformatics</td>
<td>Abstract submission, manuscript preparation, grant submission</td>
</tr>
<tr>
<td>Mass spec metabolomics</td>
<td>Identification of infants for study and retrieval stool samples from biobank; update DUA/MTA</td>
<td>Metabolite identification</td>
<td>Metabolomic bioinformatics</td>
<td>Predictive modelling using both microbiome and metabolomic data</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Aim 2</th>
<th>Quarter 1</th>
<th>Quarter 2</th>
<th>Quarter 3</th>
<th>Quarter 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine TPN model</td>
<td>ACUC approval. TPN model refinement. Fecal microbiota transfer (FMT)</td>
<td>TPN studies with FMT</td>
<td>Sample analysis, liver histology, biomarkers, serum bilirubin and liver enzymes</td>
<td>Manuscript preparation, grant submission</td>
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7. PLANS FOR FOLLOW-ON STUDIES AND FUNDING PROPOSALS

This pilot and feasibility study will provide critical preliminary data for Dr. Hourigan’s K23 proposal, a murine model-focused K99/R00 award from Dr. Kumar, and a collaborative R01 or program project grant with NICUs across the iTHRIV network, including Inova, UVA, Carillion (Dr. Kimberly Dunsmore) and VCU (Dr. Flora Szabo). In addition, we anticipate strong commercialization potential findings relevant to bedside interventions and diagnostic tools to prevent PNAC in the NICU and other clinical settings.

8. REFERENCES

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This project will model intestinal effects of weanling undernutrition, deficiency of methyl donor nutrients, and intestinal stem cell specific deletion of DNA methyltransferase 1 in mice as a platform for understanding and reversing environmental enteropathy and its link to the reduced efficacy of live oral vaccines in low- and middle-income countries.

**Mechanisms of alanyl-glutamine oral rehydration and nutrition therapy**  
*Independent Scientist in Global Health Award*  
**K02 TW008767** (NIH/Fogarty International Center)  
September 2011 to August 2016  
Role: Principal Investigator  
This project will seek to define the mechanisms of alanyl-glutamine in a mouse model of malnutrition enteropathy and in a dose-response study among 140 undernourished children in Northeastern Brazil.

**Network dynamics of rhythmic biological processes**  
*Biochronicity Program*  
Defense Advanced Research Projects Agency – Department of Defense  
January 2012 to December 2016  
Role: Co-PI  
The major goals of this project are to: (1) Investigate temporal information of three distinct yet interconnected cellular processes (circadian rhythms, cell cycle, and DNA damage response using the filamentous (thread-like) fungi *Neurospora crassa*, and (2) to establish general principles of coupled network dynamics from *Neurospora* and apply those principles to mouse intestinal organoids.

**Global and hepatobiliary-specific knockout of PIGR to generate enteropathy in mice**  
The Bill & Melinda Gates Foundation  
August 2013 to August 2016  
Role: Principal Investigator  
This project will study the role of PIGR in protecting the small intestinal mucosa against damaging effects of endotoxin and undernutrition.