MICROBIOTA, NECROTIZING ENTEROCOLITIS AND GENETIC VARIATIONS OF ZINC TRANSPORTER 2 IN PRETERM INFANTS

A Thesis in
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by
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ABSTRACT

Necrotizing Enterocolitis (NEC) is a surgical emergency in Neonatal Intensive Care Units (NICUs) affecting 2-20% of preterm infants. In 2013, the incidence of NEC in the NICU at Penn State Hershey Medical Center was 18%. NEC is characterized by inflammatory damage to the intestinal tissues. The mortality of NEC is estimated to be between 15-20% of infants diagnosed with the disease. Additionally, the majority of NEC survivors suffer from long-term complications, such as short bowel syndrome and neurodevelopmental delay. Combinations of different diagnostic tests are used to identify NEC; however, these tests are ordered when NEC is already clinically significant. Nearly 2/3 of infants diagnosed with NEC will need surgical interventions including laparatomy, bowel resection and ostomy establishment. These interventions have their own specific complications such as short bowel syndrome and long-term use of total parenteral nutrition. This represents a huge financial burden on the health care system. Given the acute onset and rapid progression of NEC, a non-invasive predictive method to identify risk factors that predispose premature infants to NEC will facilitate the development of interventions prior to the presentation of disease. Such a predictive method will be critical in reducing morbidity and mortality.

Recently, Paneth cell (PC) dysfunction has been implicated in the pathogenesis of NEC. PCs are highly specialized and granulated secretory cells, which reside at the base of the small intestinal crypts. PC granules contain antimicrobial agents, which are critical for microbial defense, and provide a host-friendly environment in the small intestine. PC granules also contain a large amount of zinc (Zn). Granular Zn prevents degradation of antimicrobial proteins such as α-defensins and stabilizes lysozyme in its monomeric, active conformation promoting appropriate microbial defense. More than 30 years ago Santulli et al. discovered the relationship between intestinal bacterial communities and NEC. Pneumatosis intestinalis, a
radiographic finding in many cases of NEC, is the result of intestinal microbial dysbiosis, illustrating the role of the microbial community in NEC pathogenesis. Additionally, the presence of specific bacteria such as Klebsiella seems to be elevated in the bowel before NEC development.

Our unpublished data indicate that the Zn transporter ZnT2 (SLC30A2), may be critical to Zn accumulation in PC granules and important for optimal PC function, as ZnT2 knock-out mice have defects in PC granule Zn accumulation, secretion and antimicrobial activity. We recently identified genetic variants in ZnT2, resulting in loss of function [2] and thus, these individuals may be at increased risk for enteric infections and inflammatory diseases that require coordinated secretion of PC granules (Podany et al, Submitted manuscript). Many of these variants occur in exons 2, 3 and 7, and affect ZnT2 function and Zn secretion. In addition, numerous variants (A28D, K66N, Q71H, D103E, A105P, Q137H, T288S and T312K) display changes in the management sub-cellular Zn pools and Zn secretion causing “loss of function” and “gain of function” in cultured cells. Interestingly, 2 variants (D103E and T288S) were found in ~12% of the study population and had a strong impact on mammary cell function suggesting that these defects are profound enough to influence the function of a secretory cell.

The objectives of this study were to determine if 1) variants in ZnT2 in premature infants are associated with alterations in gut microflora; and 2) if there is an association between variants of ZnT2 and the risk for NEC in premature infants. Our central hypothesis is that variants which result in the loss of ZnT2 function are associated with altered gut microbiota and NEC pathogenesis. In order to test this hypothesis, 51 preterm infants between the gestational ages of 26-36 weeks were recruited from the NICU at Penn State Hershey Medical Center within 48 hours after birth. DNA was collected by sterile buccal swab and the coding exons of SLC30A2 were sequenced. In addition, stool was collected with a sterile
applicator two weeks after feeding was initiated. Fecal microflora was analyzed by 16S DNA sequencing. Data on feeding, birth weight, growth, race, ethnicity, gestational age, use of antibiotics, length of stay, mode of delivery, NEC diagnosis and gender were collected.

Results indicated that there is an association between variants of ZnT2 and intestinal bacterial colonization in preterm infants. The relative abundance of *Enterobactericeae* is increased in infants with C-terminal and/or multiple variations of ZnT2. However, only one case of NEC was identified in our study. Overall, our study suggests that variants in ZnT2 create a less diverse microbial environment which may lead to short- or long-term health consequences.
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LIST OF ABBREVIATIONS

AMP – Antimicrobial Peptides
GA – Gestational Age
GWAS – Genome Wide Association Studies
HMC – Hershey Medical Center
KO – knockout
LPS – Lipopolysaccharide
NEC – Necrotizing Enterocolitis
NFkB1 – Nuclear Factor of Kappa
NICU – Neonatal Intensive Care Unit
PC – Paneth Cell
TLR – Toll –like receptor
TNF – Tumor necrosis Factor
Zn – Zinc
ZIP – Zinc Importer
ZnT – Zinc Transporter
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Chapter 1

Introduction

1.1 Necrotizing Enterocolitis

Necrotizing Enterocolitis (NEC) is an inflammatory disease resulting in intestinal tissue death, first described by Mizrahi et al in 1965 [3]. More than 85% of all NEC cases occur in premature infants with very low birth weights (infants who are less than 3lbs 3oz and less than 32 weeks gestational age) [4]. NEC has an incidence rate of 2-20% in preterm infants in neonatal intensive care units (NICUs) in the United States. The mortality rate for NEC is between 15-20%, making it the highest ranking gastrointestinal disease among preterm infants [3]. The progression of NEC requires 3 essential pre-conditions. These include a bowel which is already colonized but does not have a strong defense mechanism, a considerable amount of food in the bowel, and a stimulus which endangers the cohesion of the bowel [5].

As NEC develops in the premature gut, systemic symptoms include lethargy, hypotension, poor perfusion, pallor, increased episodes of apnea and bradycardia, temperature instability, and hyper- or hypoglycemia [6]. In severe cases the small intestine becomes inflamed, congested and discolored (gangrenous) [Figure 1]. Pneumatosis, a common sign in many cases of NEC, occurs following the fermentation and gas generation by bacterial population. Intestinal perforation occurs in the final stage of NEC [7]. Currently, there are no predictive methods to identify infants at risk for NEC and because its symptoms are associated with many other inflammatory reactions (e.g., sepsis), specific radiographic and laboratory tests such as abdominal ultra sound are required to diagnose NEC [4].
1.2 Pathogenesis of NEC

Recent studies have focused on the role of gut epithelium in pathogenesis of NEC [8]. NEC is caused by multiple factors. Some of the risk factors include hypoxia, intestinal ischemia, formula feeding, gut microbial dysbiosis, Paneth cell (PC) dysfunction, genetic predisposition, and prematurity (with prematurity being the most important factor in NEC pathogenesis) [9]. The most universally accepted hypothesis of NEC pathogenesis is that enteral feeding, accompanied by microbial colonization in the intestine, trigger a considerable amount of inflammatory reaction in the bowel of preterm infants [10].

1.2.1 Genetic predisposition

Epidemiological studies have shown that several genes may impact the severity and/or outcome of NEC. A novel study by Sampath et al. indicated that defects in genes that regulate Toll-Like
receptor signaling can enhance the pathogenesis of NEC [11]. Also in an animal model of early phase NEC, it was discovered that genes regulating tight junctions and cell adhesion molecules are altered in early NEC. Alterations of these cell adhesion molecules decreased tight junctions between intestinal epithelial cells leading to increased permeability [12]. Furthermore, it has been shown that several genetic polymorphisms in NOD2, a receptor greatly expressed in PCs and responsible for host response to pathogenic bacteria, are connected to the pathogenesis of two major categories of Inflammatory Bowel Disease: Crohn’s disease (CD) and ulcerative colitis [13]. Several genome wide association studies (GWAS) have been indicated the association between genetic susceptibility and complex diseases especially the gastrointestinal diseases such as CD and inflammatory bowel disease. However these studies have not considered variation in a specific candidate gene, as this is a GWAS. In association studies there are 2 cohorts, one with the specific disease (cases) and one without (controls). The DNA is genotyped from both groups and if specific genetic variants are found to be considerably more frequent in the disease group, we can say that there is an association between those variants and the disease. However it is possible that the related variants are not directly causing the disease alone, but are in linkage disequilibrium with the causal variant. The two major types of association studies are GWAS, and candidate gene studies. The advantage of GWAS is that there is no presumption about the importance of the functionality of the selected genes before study initiation, but it is a costly study and requires more resources. Also, due to the high throughput approach, some gene families are not well-covered. For this reason we may need a candidate gene study in order to identify the association between a specific gene and the disease so we can comprehensively test every variant in the gene [14]. A candidate gene study is a powerful and effective study in identifying the association between the specific variants and the disease. However it is not always a very practical study as it is restricted to a priori data about the specific gene functionality, but with a well-supported hypothesis a candidate gene study is often the strongest approach [15]. Our
study is a candidate gene study; we selected this study due to the strong evidence that this is strong candidate gene, and so we can be completely comprehensive and test all the variants in the gene. We have recently found that ZnT2 transports Zn into the PCs` granules. Our data showed that PCs knockout (KO) mice have defect in PCs resulting in degranulation. PCs dysfunction has been associated with NEC pathogenesis. Additionally we found novel genetic variants in ZnT2 that change the ZnT2 function leading to loss and/or gain of function in the study population, which affects the subcellular Zn. Our novel data are derived from approximately 15 years of studying the biology of ZnT2, so we are confident that our choice of this gene provides advantages over investing resources to study the entire genome.

1.2.2 Hypoxia

Hypoxia is a condition where tissues are deprived of oxygen; it is very common in preterm infants who are suffering from underdeveloped lungs [16]. Hypoxia reduces cardiac output leading to decreased intestinal perfusion which can cause mucosal injuries [17]. Bacteria are then able to invade and translocate to lymph nodes and systemic blood circulation (bacterial translocation), which leads to sepsis, a life-threatening situation with infection in bloodstream.

1.2.3 Formula feeding

Formula feeding causes a delay in the physiological development of intestinal mucosa. This is due to lack of bioactive and immune-related elements which are present in breast milk. The absence of these bioactive elements affects intestinal development and microbial colonization which may increase intestinal permeability and lead to NEC [18, 19]. In one study, analysis of stool samples from 1032 infants indicated that gut microbiota population in exclusively formula-fed infants is dominated by \textit{E.coli}, \textit{C. difficile}, \textit{B. fragilis} while the gut of breast-fed infants is colonized by \textit{Lactobacilli} and \textit{Bifidobacteria}. These bacteria are important in
elaborating the gut maturity and improving gut immunity by downregulating the toll-like receptor-4 (TLR-4) expression, which is important to the innate immune system [20].

1.2.4 Prematurity

The immune system in preterm infants is immature. Prior to the establishment of feeding, their intestinal immune responses rely on the innate system rather than adaptive immune responses. The innate immune system is a non-specific response to invading pathogens. It includes phagocytic cells, physical barriers (skin) and TLRs. Many studies have shown that the undeveloped signaling cascade (due to prematurity) through TLRs is one of the most prominent stimulators of NEC [5]. In the bowel, TLR-4 gradually increases with gestational age until term birth, and have an important role in the gut defense system. These receptors are responsible for detecting intraluminal pathogens like lipopolysaccharide, a large molecule consisting of lipid and saccharides which are found in the outer part of most gram negative bacteria. There is a direct correlation between the severity of NEC and TLR-4 expression and apoptosis. TLR-4 stimulation triggers enterocyte apoptosis and decreases intestinal cell production and maturation, which is found in all experimental models of NEC [5]. The mechanism of apoptosis has not been well identified yet [21].

1.2.5 Microbial Dysbiosis

Previously, one of the risk factors of NEC was attributed to an imbalance in intraluminal bacteria, which activates enterocyte receptors. The presence of bacteria such as E. coli, Enterobacter and Klebsiella species from blood, peritoneal and stool cultures in severe cases of NEC have shown that an imbalance in bacterial colonization is a prerequisite for NEC development [22]. The estimated number of microbes in our gut is 10-100 times more than the
number of cells in the human body. Consequently, the frequency of microbial genes is 100-1,000 times more common than the genes in human genome and the large intestine hosts the largest microbial population of the human microbiome [23]. Microbial colonization has a broad impact on host physiology and immunity. Microbes have an important role in nutrient metabolism.

Symbiotically, the bacteria profit from a protective and nutrient-rich environment [24]. Multiple studies utilizing in vivo animal models have shown that a sterile (germ-free) gut has an impaired mucosal epithelial barrier, disorganized lymphoid structures, immature immune cells (T-cells and B-cells), and low level of immunoglobulins (Ig) [18]. These dynamic interactions between bacteria and immune cells lead to intestinal IgA synthesis [18]. There is an evolutionary symbiosis between all vertebrates and their microbiota. When an agent disturbs the microbial composition, it leads to “dysbiosis” and the host becomes susceptible to disease (Figure 2).

Dysbiosis not only permits infectious diseases but also has a prominent role in chronic intestinal inflammatory disorders[25].

Figure 2: Factors which enhance microbial dysbiosis (a condition in which the microbial population becomes unfavorably skewed leading to many disease). Taken from [26].
Many factors affect the compositional development of the gut microbiota. Some of them are mode of delivery (vaginal delivery vs. C-section), gestational age at birth, mother/infant diet, antibiotic administration, and illness, the need for hospitalization, stress or exposure to heavy metals. Among these, gestational age is known to have the greatest effect on intestinal microbial community.

There is a common misconception that the intrauterine environment of newborn infants is sterile until delivery [27]. In fact, microbial DNA and cell structures from gut microbes have been detected in placental amniotic fluid and fetal membranes in term pregnancies without any signs of inflammation, rupture of membranes or onset of labor [23]. The presence of bacteria in the intrauterine environment could affect the prenatal colonization of the meconium (infant’s first stool) [27]. Vaginal and fecal microbiota of the mother may be the origin of the infant’s gut bacterial colony [28]. Additional sources include mother’s skin, oral microbes, mammary gland during breast feeding and other environmental agents.

1.2.6 Paneth cell dysfunction

Gustav Schwalbe and Joseph Paneth were the first to describe PCs in the small intestine as columnar epithelial cells (Figure 3). These cells inhabit the base of the crypts of Lieberkuhn and are rich in secretory granules containing lysozyme, microbial defensins peptides (cryptidins) and large amounts of zinc (Zn) [29, 30]. Different stimuli, including acetylcholinergic agonists, bacterial toxins, and other TLR agonists trigger PCs to release their granules into the lumen of the crypt by exocytosis. The antimicrobial peptides (AMPs) of PCs play an important role in protecting the host from enteric pathogens, thereby preventing microbial dysbiosis [29].
Figure 3: Transmission electron microscopy image of PCs in the intestinal crypt. Cells are columnar with many secretory granules in the cytoplasm. Modified from [29].

Recently PC dysfunction has been implicated in NEC. There are two existing scenarios regarding PC dysfunction and NEC pathogenesis. The first scenario is known as “Top to Bottom” while the most recent scenario is referred to as “Bottom to Top”.

The “Top to bottom” describes that intraluminal bacteria activate enterocyte TLRs leading to apoptosis and bacterial dislocation (Figure 4A) [1]. In brief, bacterial imbalance is the primary insult which leads to the development of NEC. In contrast, the “Bottom to Top” scenario postures that dysfunctional PCs are not able to mediate an effective response to microbial toxins, causing inflammation via cytokine release, immune cell invasion, platelet-activating factor (a biological element with lipid structure produced by different cells in response to specific stimulation) and coagulation necrosis (a common and definitive sign of NEC characterized by thick, necrotic intestinal tissues) which leads to the development of NEC (Figure 4B) [1, 31].
Figure 4: Current models of NEC pathogenesis. A) Classic “Top to Bottom” Scenario: Bacterial dysbiosis causes activation of epithelial cell receptors which leads to apoptosis and bacterial dislocation B) “Bottom to TOP” scenario, which is more recent, postures that PC disruption by microbial toxins causes intravascular inflammation (cytokine release, neutrophil invasion, PAF activation, decreased NO production) which leads to coagulation necrosis. Taken from [1].
One of several clinical findings, which support the role of PC dysfunction in NEC, is that corticosteroids, which are given to preterm infants to decrease the incidence of respiratory distress syndrome, also reduce the incidence of NEC. This finding suggests that hydrocortisone accelerates the maturation of PCs [1]. Additionally, a novel experiment implicated PC disruption in the pathogenesis of NEC. In this experiment, 14-16 day old mice were treated with dithizone (a Zn chelator) and soon after were exposed to Klebsiella pneumoniae. This novel two-hit treatment significantly and consistently induced small intestinal injury that is consistent with human NEC (Figure 5). However, neither dithizone nor internal infection alone was capable of inducing NEC in immature mice—the presence of both was required [32]. A study by Zhang et al. indicated a lack of PC granules in the surgical samples of infants with NEC in comparison with age–matched infants with Spontaneous Intestinal Perforation.

Figure 5: Paneth cell death by Zn chelation and exposure to Klebsiella. This causes a NEC-like injury in 14-to 16 day old mice. There are 4 groups: Control=31, dithizone only=42, Klebsiella only n=13 and Dith/Kleb n=30. Mice were given an injection of dithizone and 6 hours later a gavage of Klebsiella. They were under observation 10 hours after klebsiella gavage and mice which were treated with dithizone/Klebsiella had more damage to their intestinal tissues which is important for NEC-like disease. Taken from [32]
1.3 PCs and Zinc (Zn)

PC granules contain large amounts of Zn. This Zn is important for α-defensin activation which is responsible for defending the host against bacteria and fungi. In addition, Zn has antimicrobial properties and has been used to treat diarrhea in developing countries [30]. Some experimental studies have shown that when Zn is chelated, PC granules and numbers are depleted [30]. Zn has a unique and essential role in many biological processes, such as immune function, growth, and reproduction [33]. Many enzymes and other proteins require Zn for catalytic or structural stability. However, Zn is also cytotoxic in high amounts; therefore, living organisms must maintain adequate Zn levels for survival. An imbalance of Zn, caused by low intake, physiological and pathological stimuli, or genetic defects in Zn transporters, can compromise immune and neuronal function.

Intracellular Zn in humans is controlled by two families of Zn transporters (SLC30A [ZnT] and SLC39A [ZIP]). ZnTs lower cytoplasmic Zn by transporting Zn into subcellular compartments or across the plasma membrane to extracellular spaces, while ZIP proteins raise cytoplasmic Zn by transporting from subcellular compartments or across the plasma membrane [34]. At this time only ten members of the ZnT family have been identified [35]. Among the ZnT family, ZnT2, ZnT3, ZnT4 and ZnT8 are associated with secretory granules [35]. Our unpublished data indicate that ZnT2 imports Zn into PC granules, and that ZnT2 KO mice have defects in PC granule secretion. Five non-synonymous mutations have been identified in ZnT2 which result in low Zn secretion into milk and lead to transient neonatal zinc deficiency [36]. A study by Lopez et al showed that there are two different ZnT2 isoforms (~42 and 35 kDa) that are localized to distinct subcellular compartment and functionally transport Zn [37]. Seo et al identified two single nucleotide polymorphism (SNPs) (Leu 23 Pro and Arg 340 Cys) in SLC30A2 in mammary epithelial cells that affect Zn metabolism and may compromise the
mammary cell function [38]. We were the first to identify a loss-of-function mutation in ZnT2 (H54R) that resulted in decreased Zn secretion into milk [39]. In addition, Lasry et al. identified a second mutation (G87R) which was found in two nursing Ashkenazi Jewish mothers whose infants were suffering from transient neonatal Zn deficiency [36]. In addition, our recent study showed that 36% of women have variants in ZnT2 [2]. We postulate these individuals may be at increased risk for enteric infections and inflammatory diseases that require coordinated secretion of PC granules. Based on this information we propose that variants in ZnT2 are a critical determinant of PC function. We will test the hypothesis that variants of ZnT2 lead to gut microbial dysbiosis and may predispose preterm infants to NEC. To test this hypothesis, we have conducted an observational study from July 2014-July 2015 in the NICU at PSHMC.

Figure 6: ZIPs and ZnTs activities in balancing the intra and extracellular Zn. Taken from [40].
Chapter 2

Materials and Methods

We recruited 51 preterm infants from the PSHMC NICU following IRB approval (IRB# 00000138). The inclusion criteria are all preterm infants born between 26-36 weeks of gestational age who are admitted in the NICU within 72 hours after birth. This includes the infants who were born in other center but were transferred to the NICU at PSHMC within 72 hours after birth. Additionally the mothers of all eligible infants were eligible to participate the study.

Exclusion Criteria:

The presence of some congenital anomalies such as heart, gastrointestinal and renal in preterm infants. Infants were born from the mother who had a history of drug abuse, alcoholism, long-term depression disorders.

2.1 Sequence analysis of ZnT2

Genomic DNA (100 ng) isolated from buccal swab samples was amplified by PCR using primers specific for each of the 8 individual ZnT2 exons. Primer sequences used in PCR have been previously reported [41]. The Expand High Fidelity PCR System (Roche) was used to amplify ZnT2 exons using conditions as follows: initial denaturation 94°C for 4 min; 94°C for 30 s; 60°C (55°C for exon 2), for 30 s followed by 72°C for 1 min, for 40 cycles; final extension at 72°C for 10 min. PCR products were purified using QIAquick PCR Purification Kit (Qiagen) per manufacturers' instructions. Polymorphisms were identified by direct sequencing of PCR products using the 5’ primers used to amplify the respective exons. The sequencing chromatograms were base-called with Applied Biosystems Sequencing Analysis 5.2 Patch 2 software. Additionally, chromatograms were visually analyzed for changes in DNA sequence and
compared with the predicted protein sequence deposited in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/protein/NP_001004434.1). To exclude potential artifacts, PCR amplifications from original genomic DNA samples were replicated at least three times, and were considered to be confirmed when nucleotide changes were reproduced in all independent PCR amplifications.

2.2 Isolation of DNA from stool samples

Nucleic acid extractions were performed on approximately 0.25 g of human feces ($n = 32$) using a MoBio power fecal DNA isolation kit following standard procedure (MoBio Carlsbad, CA). The beadbeating step was performed in the Disruptor Genie cell disruptor (Scientific Industries, Bohemia, NY). Genomic DNA was eluted in 50 ul of 10 mM Tris.

2.3 PCR amplification

Illumina iTag Polymerase Chain Reactions (PCR) were run at a total volume of 25 µL for each sample and contained final concentrations of 1X PCR buffer, 0.8 mM dNTP's, .625 U Taq, 0.2 µM 515F forward primer, 0.2 µM illumina 806R reverse barcoded primer and ~20 ng of template DNA per reaction. PCR was carried out on a MJ Research PTC-200 thermocycler (Bio-Rad, Hercules, CA) using the following cycling conditions: 98°C for 3 min; 35 cycles of 98°C for 1 min, 55°C for 40 s, and 72°C for 1 min; 72°C for 10 min; and kept at 4°C. PCR products were visualized on a 1% CYBRsafe E-gel (Life Technologies, Carlsbad, CA)
2.4 Library purification, verification and sequencing

Pooled PCR products were gel purified using the Qiagen Gel Purification Kit (Qiagen, Frederick, MD). Clean PCR products were quantified using the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA), and samples were pooled in equimolar amounts. Prior to submission for sequencing, libraries were quality checked using the 2100 Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA). Pooled libraries were stored at -20°C until they were shipped on dry ice to the California State University (North Ridge, CA) for sequencing.

Library pools were size verified using the Fragment Analyzer CE (Advanced Analytical Technologies Inc., Ames IA) and quantified using the Qubit high sensitivity dsDNA kit (Life Technologies, Carlsbad, CA). After dilution to a final concentration of 1nM and a 10% spike of PhiX V3 library (Illumina, San Diego CA), pools were denatured for 5 minutes in an equal volume of 0.1N NaOH then further diluted to 12 pM in Illumina’s HT1 buffer. The denatured and PhiX-spiked 12 pM pool was loaded on an Illumina MiSeq V2 300 cycle kit cassette with 16S rRNA library sequencing primers and set for 150 base, paired-end reads.

2.5 Statistical Analysis

Principle Coordinate Analysis was performed using Qiime1.9 software while relative abundance of OTUs was analyzed using USEARCH. Correlation between variants in ZnT2 and variables which are considered to be associated with NEC pathogenesis (gestational age, modes of delivery, birth weight, antibiotic administration and duration and feeding cessation) after stratification for race were analyzed with SAS® 9.4 (SAS Institute, Inc). Fisher’s exact test was used to analyze data with categorical variables and Student’s T-tests were used to analyze continuous variables. Statistical significance was determined by a p value < 0.05.
Chapter 3

Results

3.1 Population Characteristics

The data in this project is based on 50 premature infants (26 males and 24 females). Of the 51 infants, there were 6 African Americans, 8 Hispanic and 37 Caucasians, with a mean gestational age of 32 weeks. The mean birth weight was 1800gm. The study population contained 5 sets of twins (4 sets non-identical and 1 set is unknown) and one set of triplets. There was only 1 official diagnosis of NEC; 6 infants were suspected to have NEC and therefore feeding was withheld. There were 15 vaginal deliveries and 35 deliveries by cesarean. The SLC30A2 gene sequencing indicated that 19 were true wild type (no variations) and the rest had at least 1 variation in SLC30A2. (See Figure 8: racial frequency of SLC30A2 variations

3.2 The relationship between genetic variation in ZnT2 and development of NEC

The objectives of this study were: 1) to identify the association between variants of ZnT2 and NEC; and 2) to determine whether variants in ZnT2 affect the intestinal microbial community. Variants in ZnT2 were common in the study population (Table 1A and 1B); however, the association between variants in ZnT2 and NEC could not be completed due to only one case of NEC. Additionally we used the electronic medical records to determine the association between variants in ZnT2 and variables that are considered to be important in pathogenesis of NEC. These variables were: gestational age, modes of delivery, birth weight,
antibiotic administration and duration of feeding cessation. We did not find a strong correlation between variants in ZnT2 and variables of interest after stratification for race.

Table 1. The frequencies of Variation of ZnT2. A) Frequency of infants with variants in ZnT2. B) Racial groups frequency of ZnT2

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3.3 The effect of genetic variations of ZnT2 in the alteration of intestinal microbiota

In order to determine the relationship between variants in ZnT2 and intestinal microbiota, we sequenced microbial DNA in the stool samples of infants by 16sRNA. Principal coordinates analysis (PCoA) plots were generated from a Weighted Unifrac distance matrix within Qiime-1.9.0 to compare beta diversity (phylogenetic diversity between samples). A Unifrac distance matrix is a qualitative measure that exclusively uses a phylogenetic tree and accounts for the history of shared common ancestors between microbial communities. Differences in microbial communities are measured in terms of the total unique branch lengths between samples versus the total number of shared branch lengths. A weighted Unifrac distance matrix additionally accounts for the abundance of each bacterial taxa. Our data suggest that phylogenetically their microbial communities share a high number of common ancestors, and thus they are not (as a whole community) significantly phylogenetically different.

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<th>BLACK% (n=6)</th>
<th>PACIFIC ISLANDER % (n=1)</th>
<th>HISPANIC% (n=8)</th>
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Figure 8. Principal Coordinates Analysis (PCoA) of bacterial community in infant guts. Plots revealed differences in bacterial community structure between infants with and without variants in ZnT2. A) A comparison of infant fecal samples with and without C-terminal genetic variants (variants) in ZnT2. Significant clustering was not observed between the sample groups (Y, blue, indicates presence of variants n = 9, No, red, no variants in this region n = 22, ANOSIM=0.941). B) A comparison of infant fecal samples with and without multiple variants. Significant clustering was not observed between sample groups (Yes, blue, indicates presence of variants n = 10, No, red, single or no variants n = 21, ANOSIM=0.863).

The Kruskal-Wallis test was rerun to present taxa enriched in multiple SNP +/- samples and C-terminal SNP +/- samples stratified by race. This analysis was only conducted on Caucasian subjects as the number of non-Caucasians was too small to analyze. At \( \alpha = 0.025 \), Caucasian infants with multiple and or C-terminal variation inZnT2 have a more enriched taxa. A total of 998 unique OTUs were analyzed, and tests were run with 1000 permutations. (Table 2A and B)
Table 2. Kruskal-Wallis nonparametric ANOVA test P-values reveal taxa enriched with (A) Multiple SNPs and (B) C-Terminus SNPs. This may indicate dysbiotic intestinal microbial community Caucasian.

A.

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<tr>
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B.

<table>
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The data derived from stool analysis revealed that the fecal microbial community was dominated by Enterobacteriaceae (44%). Three unknown species in the Enterobacteriaceae family strongly correlated (p<0.05) with a region of dense variants in the C-terminus, after accounting for
multiple testing via FDR correction. These species were each approximately 5-fold more abundant in infants with variants than in those without. Recent studies highlight the importance of establishing normal microbiota after birth, especially in preterm infants. Because several members of Enterobacteriaceae are known pro-inflammatory opportunistic pathogens, our study suggests that variants in SLC30A2 affects the establishment of normal microbiota, which could potentially increase risk for enteric infections (Figure 9A and B) (39).

Figure 9 A and B. Relative abundance of OTUs at the family taxonomic rank comparing effects of (A) C-Terminus variants; and (B) Multiple variants sample categories. Relative abundance outputs were generated from an unrarefied OTU table picked using the USEARCH sequence analysis tool. The x-axis displays percent family abundance. The 9 families with the greatest abundance across all samples are shown, while all additional families of lower abundance are grouped together into the ‘other’ category.
Chapter 4

Discussion

We are interested in PC dysfunction because it has been implicated in NEC pathogenesis. We recently determined that ZnT2 regulates Zn in the PCs of mice. Localization of ZnT2 in the PCs of the small intestine was confirmed by immunofluorescence in ZnT2 KO and wild-type mice; further characterization indicated that ZnT2 KO mice have degranulated or depleted PCs in comparison to wild-type mice. PCs are highly granulated and specialized cells in the crypt of the small intestine. They have an important role in host defense and regulating host microbiota.

Currently, few GWAS or candidate gene studies regarding the pathogenesis of NEC have been conducted. Previously we determined that variants in ZnT2 are common and lead to changes in the management of sub-cellular Zn pools in cultured cells. Interestingly, we found that the variants in ZnT2 are common in our study population, which is dominated by Caucasian infants. Further diversity in our study population is required in order to investigate the frequency of these variants in other races, as it may be drastically different in each racial group.

Our central hypothesis was that variants of ZnT2 cause PC dysfunction, leads to microbial dysbiosis and predisposes preterm infants for NEC. Due to only one case of NEC in our study population, we could not determine if there is an association between variants in ZnT2 and NEC. However, here we found that infants with variants (non-synonymous amino acid substitutions) in exons 2, 3, 7 and 8 of SLC30A2 have a more skewed intestinal microbial community. Enterobactericeae, Cyanobacteria, Rhodospirillaceae and Oscillatoriophycidae were abundant in the stool of preterm infants with variants of ZnT2, whereas infants without variants had Bacteriodaceae, Costridiceae and Porphyromonadaceae in their stool. Matamoros et al. notes that it is very difficult to identify a “normal” human intestinal microbiota [27]. Most of our knowledge about infant microbiome comes from research on term infants. There are few studies,
which focus on the gut microbiota in preterm and low birth-weight infants. However the presence of bacterial translocation in utero, microbial communities in preterm infants’ meconium and lipopolysaccharide (LPS) in the cord blood of preterm infants indicates preterm infants are not born sterile [42]. Preterm and term infants have different microbial communities in their gut.

The presence of potentially pathogenic bacteria, such as *Enterobacteriaceae*, *Clostridium difficile*, or *Klebsiella pneumoniae*, are greater in pre-term infants; whereas in full-term infants fecal microbiota are more diverse, with *Bifidobacterium*, *Lactobacillus*, and *Streptococcus* dominating [27]. As a result, preterm infants are very vulnerable to dysbiosis in comparison to term infants as their immune system is not fully developed and their gastrointestinal tract is immature [43]. The first days of life are very important in shaping the microbial population in the gut. These microbial communities play a critical role in generating the gut barrier as an immune-regulator. However, a key question that remains is whether we can call the skewedness of intestinal flora “dysbiosis”. The term dysbiosis represents a pathological condition, which is associated with various diseases such as NEC, autism, childhood obesity and autoimmune diseases. Because we only had one documented case of NEC in our study, we cannot make this conclusion. Therefore, we need to follow these infants to determine long-term effects of this skewedness on health status. The outcomes we would be most interested in are weight gain, autoimmune disease susceptibility, asthma, childhood allergy, etc.

**Study limitations**

Our small sample size with only one case of NEC limits our ability to draw conclusions. This may result from the fact that most of our participants were late-preterm infants, and as it was previously mentioned, the higher gestational age has an inverse effect on NEC incidence. Also, concurrent with our study in the NICU at PSHMC, there were interventions initiated which may have decreased the incidence of NEC. Some of these interventions were the initiation of a
Prolacta (a human-based milk fortifier), feeding preterm infants less than 34 weeks whose mothers do not have a great supply of breast milk with donor breast milk (as it is preferred to be used in place of formula and is believed to have more protective effects than formula in NEC prevention), limitation of antibiotics (antibiotics are well-known to damage the natural microbial communities and create dysbiosis) and limited H2 blocker administration (as they promote the pathological organisms growth by decreasing the pH level in the stomach) [44] and more meticulous policies regarding withholding the feeding before during and after blood transfusion (as feeding during transfusion may perturb the intestinal perfusion and predispose the infants for intestinal ischemia and increase the risk for infusion-associated NEC) [45]. However as NEC is a multifactorial disease and up to now there has not been a specific risk factor for pathogenesis of this disease, we cannot be certain that these interventions are 100% effective in NEC prevention.

**Future Plans**

This study will continue in the NICU at PSHMC and our aim is to recruit more infants (200 preterm infants). Also we are planning to follow up these infants to gather information about the effect of dysbiosis on physical and mental status as they grow. Also, we collected infant feeding data. Our plan is to analyze the feeding type for micro/macro nutrients such as protein and calories in order to investigate the correlation between feeding type and infant intestinal communities. Breast milk changes dynamically during each time of breast-feeding. Many factors such as gestational age, chronological age of the infant and the mother’s health status affect breast milk composition [46]. Furthermore the milk composition in mothers who deliver preterm infants is vastly different from milk of mothers who deliver term infants [47]. Premature milk is well known to be high in protein. In a study by Dutta et al. showed significant changes in the milk composition in preterm infants. They discovered an accelerating change in triglycerides and sodium, and a continuous decline in protein [47]. We also want to explore Donor Breast Milk
(DBM) consumption in preterm infants, as there are several obstacles in providing mother breast milk (MBM) for preterm infants. Preterm labor and delivery have tremendous stress on parents. The mother has to begin pumping milk soon after giving birth and has to pump 8-12 times per day in order to increase the infant’s chance to be fed with her own milk. The use of DBM in preterm infants has been increased as a beneficial intervention in decreasing in-hospital morbidity in addition to decreasing NEC incidence [48]. According to Underwood nutrition, safety, supply, and immune protection are noticeable issues in feeding the preterm infants with DBM [49]. However, most DBM from milk banks is from later periods of lactation in breastfeeding mothers who had term deliveries. Their milk components are low in protein, fat and many bioactive elements [49]. Also DBM pasteurization, to decrease risk of infection, results in decreased antimicrobial components [48]. The preterm infant’s naïve and altered intestinal microbial community may not be able to react properly in response to DBM components and may predispose the infants for many metabolic diseases. The overestimation of the protective characteristics of DBM, the ethical issues regarding for-profit bargaining of human breast milk and possible long-term health outcomes of DBM consumption in preterm infants, are encouraging the idea that we need to increase the number of women who breastfeed their preterm infants [49].

In conclusion, our study suggests that Zn mismanagement in PC granules due to variants in ZnT2, may increase susceptibility for dysbiosis, which may predispose the preterm infants not only to NEC but several childhood diseases. This is supported by many studies, which have indicated that there is an association between dysbiosis and childhood disease such as celiac disease, childhood obesity, autism and cancer. It is apparent that these microbial communities play a critical role in generating the gut barrier as an immune-regulator. Further studies must be conducted to monitor preterm infants with variants in ZnT2 and altered gut microbiota for long-term health outcomes.
BIBLIOGRAPHY:


