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Moderate Acute Malnutrition: Inflammatory Response, Microbiota, and Potential Treatments

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Moderate Acute Malnutrition: Inflammatory Response, Microbiota, and Potential Treatments

by

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Acute malnutrition is directly or indirectly responsible for nearly half of all deaths in children under five. Even when treatment is provided in a timely manner, some children fail to recover and others relapse within months. Recovery has traditionally been measured by anthropometric scores, but metabolic and immunological recovery takes longer than weight gain. Thus, markers are needed that signify recovery not just of weight, but of metabolism and immunity. An understanding of the underlying pathology of malnutrition is required to identify markers that indicate full recovery. While the pathology of SAM and resultant attenuated inflammatory response have been characterized, there is minimal and often conflicting data available about the effects of MAM on inflammation and response to inflammatory stimuli. In this work, we observed increased inflammatory cytokine production at baseline and in response to LPS in whole blood of children with MAM. These children also exhibited signs of impaired fatty acid metabolism, intestinal damage, and liver dysfunction. Markers of these dysfunctions (iFABP and LBP) may have potential as markers of recovery from MAM. Studies in a mouse model of MAM corroborated the chronic inflammatory state and metabolic dysfunction observed in children. MN mice also exhibited intestinal dysbiosis and increased bacterial translocation. Use of the antibiotic

vancomycin further exacerbated intestinal dysbiosis in MN mice by increasing the proportion of Gammaproteobacteria; these mice also had increased inflammatory cytokine response to LPS challenge. Conversely, MN mice fed anti-inflammatory omega-3 PUFAs exhibited heightened intestinal immune function, decreased proportion of intestinal Gammaproteobacteria, and reduced inflammatory response during intradermal LPS challenge. Malnutrition-induced expansion of Gammaproteobacteria may therefore drive systemic inflammation and inflammatory response via heightened exposure to LPS. Mice fed omega-3 PUFAs were also able to better maintain weight during systemic LPS challenge, suggesting that omega-3 supplementation may provide protection from malnutrition-related morbidity even during the recovery process. These results provide new insight into the pathology of MAM and identify targets for intervention and monitoring during the course of treatment.

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List of Abbreviations

AC	acylcarnitine
ALA	alpha-linoleic acid
APR	acute phase response
cDNA	complementary deoxyribonucleic acid
BHI	brain heart infusion
CFU	colony forming unit
CMAM	Community Management of Acute Malnutrition
COL	colistin
CSB	corn-soy blend
DALY	disability adjusted life year
DHA	docosahexaenoic acid
CRP	C-reactive protein
DC	dendritic cell
DNA	deoxyribonucleic Acid
EE	environmental enteropathy
EED	environmental enteric dysfunction
EndoCAb	endotoxin core antibodies
EPA	eicosapentaenoic acid
FBF	fortified blended food
GH	growth hormone
HAZ	height-for-age Z score

HFIAS	household food insecurity access scale
HMO	human milk oligosaccharides
FITC	fluorescein isothiocyanate
FO	fish oil
iFABP	intestinal fatty acid binding protein
IL	interleukin
IP	intraperitoneal
IQ	intelligence quotient
IUGR	intra-uterine growth restriction
IGF-1	insulin like growth factor 1
LBP	lipopolysaccharide binding protein
LCFA	long chain fatty acid
LNS	lipid-based nutrient supplement
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAM	moderate acute malnutrition
MDCF	microbiota directed complementary foods
MLN	mesenteric lymph node
MN	malnourished
MNP	micronutrient powder
mRNA	messenger ribonucleic acid
MUAC	mid-upper arm circumference
MUFA	mono-unsaturated fatty acid

NF-KB	nuclear factor kappa-light-chain enhancer of activated B cells
NK	natural killer cell
ORS	oral rehydration solution
OTU	operational taxonomic unit
P/S	Penicillin/Streptomycin
PBMC	peripheral blood mononuclear cell
PC	phosphatidylcholine
PCA	principal components analysis
PEM	protein energy malnutrition
PLA2	phospholipase A2
PLSDA	partial least squares discriminant analysis
PLT	platelet
PP	Peyer's patch
PPARα	peroxisome proliferator-activated receptor alpha
PUFA	poly-unsaturated fatty acid
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RBC	red blood cell
ROUT	robust regression and outlier removal
RUSF	ready-to-use supplemental food
RUTF	ready-to-use therapeutic food
SAM	severe acute malnutrition
sCD14	soluble cluster of differentiation 14
SCFA	short chain fatty acid

SES	socioeconomic status
SFA	saturated fatty acid
SGA	small for gestational age
SM	sphingomyelin
TLR	toll-like receptor
UC	ulcerative colitis
UNICEF	United Nations Children's Fund
USD	United States Dollar
VANC	vancomycin
WASH	water, sanitation, and hygiene
WAZ	weight-for-age Z score
WBC	white blood cell
WHO	World Health Organization
WHZ	weight-for-height Z score
WN	well-nourished

INTRODUCTION

Chapter 1. An Introduction to Moderate Acute Malnutrition[1]

INTRODUCTION TO UNDERNUTRITION

Definitions

Malnutrition is defined as a physiological process that occurs when nutrient intake or utilization is not appropriate to maintain normal health and encompasses a spectrum of conditions from obesity to acute wasting. More narrowly, undernutrition describes micronutrient deficiencies such as anemia as well as underweight, wasting, and stunting. Acute malnutrition, or wasting, is driven by an intense period of energy and or nutrient deprivation, leading to dangerously reduced weight. Chronic malnutrition, or stunting, is a result of less severe nutrient deprivation over a long period of time, leading to growth faltering and other developmental shortfalls. These forms of undernutrition are driven primarily by insufficient food intake and repeated bouts of infection, yet the treatments, and mechanisms underlying pathology of various forms of undernutrition differ.

Diagnostic Measures

The WHO defines severe acute malnutrition (SAM) in children between the ages of 6-59 months as either a mid-upper arm circumference (MUAC) <115 mm or a weight-for-height Z score (WHZ) >-3 [1]. Any children with bilateral pitting oedema should also be treated for malnutrition. Moderate acute malnutrition (MAM) is defined for the same age group as a MUAC > 115 mm but < 125 mm and WHZ ≤ -2 and > -3. Z scores describe

1 Some of the content of this chapter has previously been published: Thaxton GE, Melby PC, Manary MJ, Preidis GA. New Insights into the Pathogenesis and Treatment of Malnutrition. Gastroenterol Clin N Am, 47 (2018) 813-827. https://doi.org/10.1016/j.gtc.2018.07.007. Reproduced with permission.

the number of standard deviations a child's measurement differs from the mean of a healthy reference population of the same age group and sex (Figure 1.1). The WHO released new standards for anthropometric measurements in 2006, and subsequent studies have compared the accuracy of using either MUAC or WHZ alone to diagnose acutely malnourished children [2]. WHZ is difficult to accurately and rapidly calculate in the community and can also misidentify children with fluid retention as non-malnourished [3]. MUAC can be used to easily screen large numbers of children in a community setting and is an adequate proxy for weight change [4], but often identifies different groups of malnourished children than WHZ [5,6]. Use of MUAC alone for diagnosis is also limited by the fact that MUAC correlation with WHZ differs with sex and age [7,8]. Using the current anthropometric standards, MUAC alone is not sufficient to identify the majority of

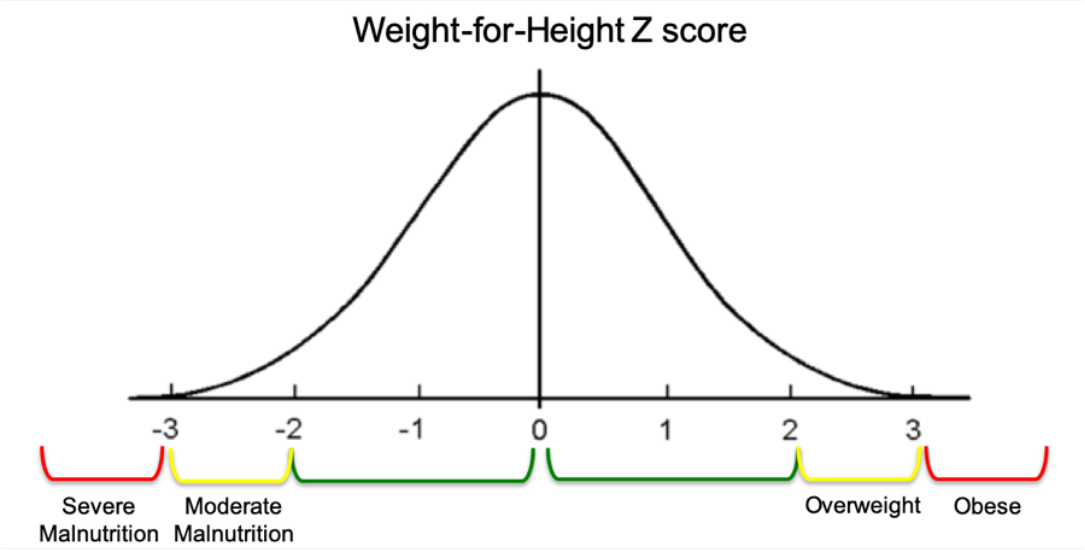


Figure 1.1: Weight-for-height Z scores for diagnosis of malnutrition.

Z scores used to diagnose malnutrition are calculated using the mean and standard deviation weigh-for-height score for a reference population of the same age and sex. In a healthy population, only 5% of children should fall outside 2 standard deviations from the mean. Children are considered malnourished if their Z score is more than two standard deviations from the mean.

cases of SAM in the community. However, either increasing the cutoff MUAC [6,9] or developing a set of MUAC cutoffs stratified by age and gender [8] could render MUAC satisfactory as stand-alone admissions criteria.

The WHO guidelines no longer support using percent weight gain as discharge criterion from treatment programs for malnutrition [1]. Rather, children should be discharged when they meet nutritional recovery based on the anthropometric marker used to diagnose them. Recovery is defined as WHZ ≥ -2 or MUAC ≥ 125 mm, coupled with no oedema in the last two weeks. While use of either anthropometric marker is better than percent weight gain for protecting vulnerable groups from early discharge [10,11], MUAC alone has proven effective a stand-alone discharge criterion, regardless of intake criterion [12,13].

Global Impact of Wasting

Globally, 49.5 million children under the age of five suffer from either MAM or SAM [14]. Wasting predominantly affects developing countries, with 75% of all wasted children living in low-middle income nations. This burden is divided largely between two regions: 33.8 million in Asia and 14 million in Africa [14]. The distribution of these cases should be considered to develop effective treatment programs and reduce global rates of wasting. While the burden of malnutrition is highest in Southern Asia, the majority of research on malnutrition is conducted in Africa. Cultural and geographical factors may impact efficacy of treatments developed in one location and adopted in another, thus studies of malnutrition should be repeated in multiple locations when possible.

Acute malnutrition contributes heavily to childhood mortality. The burden of severe wasting in 2016 was 16.9 million [15]. Severely wasted children have a 9.4-fold increased risk of dying compared to healthy-weight children [16] and wasting is directly responsible for 4.7% of all deaths in children under the age of five, totaling 2 million deaths per year [17]. All forms of malnutrition together are indirectly involved in up to 45% of all

child deaths [16], due primarily to increased susceptibility to infectious disease. Malnutrition increases morbidity and mortality from a wide variety of infectious diseases, particularly respiratory, gastrointestinal, and systemic infections [18]. Infection perpetuates wasting and can hinder recovery, driving a vicious cycle of growth faltering and illness.

Wasting perpetuates cycles of poverty on an individual and national level. Children who experience a bout of acute malnutrition during infancy are more likely to have lower household income [19] and impaired IQ [20] as adults. High burdens of childhood malnutrition drain economic resources and human capital in developing countries [21]. It is estimated that malnutrition will reduce gross domestic product in Asia and Africa by up to 11% [14] and wasting is directly responsible for 14.6% of disability adjusted life years (DALYs) in children under five [16]. Treatment of SAM is expensive, at 200 USD per episode [22]. Use of complimentary foods to prevent and treat MAM is less expensive, costing 40-80 USD per child [22]. Treatment of MAM using common interventions is cost-effective across multiple regions [23-25] and reduces costs associated with SAM and infectious disease treatment. However, public health spending has been on the decline in low-income countries [14]. Government investment in prevention and treatment of malnutrition will be required to manage the growing burden of malnutrition and limit related economic damage.

ORIGINS OF ACUTE MALNUTRITION

Socioeconomic Factors

Socioeconomic Status

Chronic and acute malnutrition are associated with correlates of low socioeconomic status (SES) such as parental education and income. Parental education level has been correlated with underweight and stunting [26-28]. Education for mothers on breastfeeding,

child nutrition, and childcare is among available interventions with the highest potential for reducing multiple forms of malnutrition [29-31]. Household income is often tested for association with child malnutrition, but a more meaningful measure is wealth. Wealth provides protection from personal economic disasters and is inversely associated with chronic malnutrition and underweight [26-28,32]. Increased wealth often lead to increases in dietary diversity [33,34] and cash transfers support recovery from SAM in poor families [35]. The effects of SES on child malnutrition are mediated through more proximal determinants, such as environment exposures, access to clean water, and access to food. Thus, step-wise improvements in SES may not provide much benefit in reduction of malnutrition rates until a relatively high threshold of standard of living is achieved. A survey of a community in the Gambia only observed the benefit of higher SES on child anthropometric measures in the highest tier of SES [36]. Children in all other groups had similar levels of stunting and wasting.

Food Insecurity and Dietary Diversity

Food insecurity is unreliable access to affordable, nutritious food. A modest but significant negative relationship between food insecurity and stunting (but not wasting) can be observed when controlling for sociodemographic factors [37]. The evidence supporting this relationship is correlational and of variable strength [26,38,39]. Studies have also observed decreased weight gain and growth [40], maternal thinness (but not child malnutrition) [41], and low weight-for-age (on a regional scale [42] and individually [43]) in food insecure children. While mild food insecurity appears to have little direct effect on childhood malnutrition, severe food insecurity that arises seasonally or due to political or natural crises can cause outbreaks of malnutrition and child mortality [44,45]. Social safety nets such as cash transfers have been proposed to limit seasonal food insecurity and wasting, but a trial program was not successful in reducing incidence or duration of wasting in this context [46].

Dietary diversity is another measure of nutritional adequacy that is related to food insecurity [47] and is inversely associated with malnutrition. Low dietary diversity is typically a strong predictor for stunting in children under the age of 5 [48,49], even when controlled for socioeconomic factors [34]. Children who consume a greater proportion of nutritionally deficient staple foods are more likely to be stunted or wasted [50], while children with higher dietary diversity are more likely to consume adequate micronutrients [51]. While dietary diversity is only weakly linked to wasting, studies have demonstrated that low dietary diversity is correlated with development of kwashiorkor over marasmus [52]. Just as important as macronutrient quantity are protein quality and digestibility [53]. Protein inadequacy, which correlates with stunting [54], is highest in Africa and southern Asia [55]. Up to 70% of protein consumption in these regions is in the form of cereals and roots, which lack many of the essential amino acids found in animal meat and dairy proteins.

Environment, Infection, and Microbiota

Water, Sanitation, and Hygiene

The physical environment has a major impact on the development of malnutrition. Stunting and wasting are often correlated with unimproved water sources and sanitation facilities [37]. Water, sanitation, and hygiene (WASH) interventions have achieved some success in reducing rates of wasting and stunting [28,56-58] but not nearly to the level predicted based on the link between diarrheal diseases and malnutrition [59]. A model based on reviews of hygiene intervention studies predicted that 99% implementation of hygiene interventions in countries with 90% stunting would only reduce rates of stunting by 2.4% among ages 0-36 months [29]. There are likely several factors influencing the modest effect of WASH interventions on malnutrition rates. It is difficult for any one person to improve their physical environment, as it is predominantly a result of socio-economic and political factors. Coverage of interventions must be high to have significant

effects on child health [60]. Exposure to fecal pathogens in the soil and water is nearly ubiquitous in certain regions, even if interventions to improve WASH are implemented [58,61]. Children in low-income countries begin continuously acquiring and clearing intestinal pathogens as early as one month old, even while breastfeeding [62,63]. Both well-nourished and malnourished children acquire and clear enteric infections at the same rate, suggesting that the high enteric pathogen load in malnourished children is a result of heavy exposure rather than an inability to clear infection [62]. Further, WASH intervention studies may not measure appropriate endpoints to assess their impact on more proximal determinants of malnutrition. Handwashing interventions are known to reduce risk of diarrhea by 30% [29], but studies of these interventions do not always measure pathogen load or inflammation. Infection with enteric pathogens such as entero-aggregative E. coli, even in absence of episodes of diarrhea, are known to cause growth faltering [54,64-67]. Studies that only assess days of diarrhea lack critical information regarding effects of any subclinical infections on intestinal and systemic inflammation.

Environmental Enteropathy

The impact of subclinical infection and chronic inflammation on malnutrition is described by the condition of environmental enteropathy (EE). EE has not yet been directly and causatively linked to malnutrition but is nearly ubiquitous in regions with high prevalence of malnutrition and is correlated with lower anthropometric indices [68]. EE is a subclinical condition characterized by physiological, anatomical, and functional changes to the intestine. These changes include villous blunting, crypt hyperplasia, immune cell infiltration, impaired absorption, and increased intestinal permeability [69]. EE is a result of frequent (and often subclinical) infection with enteropathogens, generally by continued exposure to an unhygienic environment [70]. The condition can be resolved simply by removing the affected individual to a more hygienic setting. EE is not easily diagnosed in children, though discovery of fecal biomarkers such as calprotectin and myeloperoxidase

show promise as diagnostic criterion [68,70-72]. EE is thought to influence development of malnutrition in several ways. Firstly, chronic intestinal damage and exposure to enteric pathogens can result in metabolically costly chronic intestinal and systemic inflammation [69]. Children with EE are less able to absorb macro and micronutrients from the diet, further compounding the energy deficit [73,74]. Also, children with EE have increased intestinal permeability that could lead to increased microbial translocation and systemic inflammation, though evidence linking this pathway to stunting is lacking [69]. A multi-site study of the determinants and effects of EE observed a high enteropathogen load and markers of intestinal inflammation in non-diarrheal stool samples [54,70]. Enteropathogen load and systemic inflammation mediated linear growth defects, while intestinal inflammation mediated acute malnutrition.

Enteropathogens and Microbiota

Enteropathogens induce malnutrition, promoting growth impairment by reducing nutrient absorption and increasing nutrient and energy needs [67]. Most malnourished children in low-income countries harbor multiple pathogens [75], including *Giardia lamblia* [76-79], Cryptosporodium *[62,75,76,80]*, enterotoxigenic *Escherichia coli* [62,75,81], *Campylobacter jejunum* [76,77,82], Shigella [75,77], rotavirus [62,75,77], and norovirus [83]. As the number of pathogens isolated from stool increases, WAZ and HAZ decrease [76]. A single episode of diarrhea can impact mortality and linear growth for 2-3 months after infection [75,77]. Commonly used drugs for treatment of intestinal parasites such as nitazoxanide, albendazole, and metronidazole are ineffective or used suboptimally in malnourished children, and resistance to such drugs is growing [80].

However, even in the absence of diarrhea, the malnourished gut microbiota is abnormal [84-86]. Decades ago, culture-dependent studies revealed bacterial overgrowth in the proximal gastrointestinal tract, and microbial DNA sequencing technologies have facilitated a more detailed characterization of this malnutrition-associated "dysbiosis"[86].

Numerous factors may drive these microbiome alterations. For example, a monotonous diet containing specific non-digestible dietary carbohydrates [87,88] provides a selective advantage to microbes that metabolize these substrates. Likewise, inflammation can alter the microbiome by triggering an immune response in which subsets of commensal microbes may be eliminated by host-secreted antimicrobial peptides [89], by disruption of the oxygen gradient at the mucosal surface [90-92], and by generation of reactive oxygen and nitrogen species [93,94].

Recent preclinical studies demonstrate a causal link between the malnourished microbiome and growth impairment. Fecal microbes isolated from malnourished children can induce weight loss in gnotobiotic mice under specific conditions [95-97]. Similarly, mice iteratively challenged with a combination of non-pathogenic commensal microbes demonstrate impaired growth [98]. Intriguingly, the microbiota's effect on growth in each of these mouse models is dependent on administration of a low-protein, low-fat diet – if the animals consume standard chow, growth impairment is not observed. Similarly, the presence of a malnourished microbiota can exacerbate weight loss due to pathogenic infection [99]. On the other hand, specific beneficial microbes have been positively linked to growth. For example, *Lactobacillus plantarum* increases insulin-like growth factor-1 (IGF-1) expression and linear growth in a model of chronic malnutrition [100].

Maternal Factors

Maternal underweight and macronutrient malnutrition is correlated with infant and child malnutrition. Mothers who are moderately malnourished during pregnancy frequently give birth to stunted infants [101] and their children are more likely to develop acute malnutrition [102]. Balanced protein-energy supplementation during pregnancy can reduce risk of stillbirth and neonatal mortality [103], and mothers given lipid-based nutrient supplements (LNS) had infants with increased birth weight and reduced stunting [104,105]. Prenatal health is also impacted by the age and height of the mother, which may reflect her

nutritional history and development. Teen mothers with malnutrition were found to have infants with smaller length-for-age than older women, despite the fact that the teen mothers had a better nutritional status [106,107]. Shorter maternal height has also been repeatedly identified as a strong predictor of child stunting [54,102,106,107].

Intrauterine growth restriction (IUGR), small for gestational age (SGA), and preterm birth all contribute to child mortality [108] and malnutrition [109]. Maternal micronutrient status is one determinant of low birth weight and IUGR. Iron supplementation during pregnancy reduces the risk of low birthweight and child mortality within the first five years of life [110], and multiple micronutrient supplementation during pregnancy increases birthweight and decreases infant mortality [111]. Low vitamin D receptor expression has been observed in placentas of IUGR pregnancies [112], and single nucleotide polymorphisms in placental genes governing vitamin D metabolism are associated with low birthweight [113]. IUGR can be driven by many other factors, including low IGF-1 [114], highlighting the complex, systemic nature of metabolic derangements in malnutrition.

Low birthweight could promote malnutrition via fetal epigenetic alterations [115]. Differential DNA methylation in infants with and without IUGR was observed in genes involved in lipid metabolism, transcriptional regulation, metabolic disease, and T cell development [116]. Epigenetic changes caused by episodes of prenatal or childhood malnutrition can persist for generations [117]; however, some changes can be rescued by early nutrient supplementation in preclinical models [118].

Maternal genotype also influences the risk of child malnutrition. For example, vitamin D status and fetal growth are impacted by maternal variants of vitamin D metabolizing genes [119]. IUGR might be avoided in certain cases by individualizing prenatal supplementation regimens. Similarly, mothers lacking a functional *FUT2* gene secrete lower concentrations of fucosylated human milk oligosaccharides (HMOs) in breastmilk, and are more likely to have stunted children [88]. When nutritional quality of

breastmilk is inadequate, complementary feeding might be required to reduce the infant's risk of malnutrition. In low-income countries, children who are exclusively breastfed beyond the recommended 6 months of age are at increased risk of dietary protein inadequacy [120] and can greatly reduce the risk of stunting and underweight with complementary feeding [121].

CONSEQUENCES OF MALNUTRITION

Immediate Biological Consequences

Metabolic Derangement

Although stunted children have lower circulating levels of all essential amino acids, it is uncertain whether this results from inadequate intake, increased catabolism, or both. Malnourished children are particularly deficient in arginine, glycine, glutamine, asparagine, glutamate, and serine [122]. These amino acids serve in a variety of biological roles including protein synthesis, enterocyte growth, bile acid conjugation, intestinal barrier function, and neurotransmitter biosynthesis. Serum amino acids are sensed by, and influence the activity of, the mTORC1 pathway, a master regulator of growth [123]. Protein synthesis, proteolysis, and bone growth are inhibited during SAM, as lipolysis and fatty acid oxidation meet a greater proportion of energy needs [124-126]. Decreased circulating polyunsaturated fatty acid levels further suggest compensatory fat catabolism in SAM [127], while elevated cortisol and growth hormone and decreased leptin and insulin may reflect hormonal regulation of these processes [124,128]. Indeed, decreased leptin is a strong independent predictor of mortality in children with SAM [124,126]. Finally, while the metabolic profile of a child with SAM changes upon stabilization, it still differs profoundly from stunted and non-stunted controls [129]. Control metabolomes exhibit higher concentrations of sphingomyelins and most phosphotidylcholines, which may indicate continued liver dysfunction and lipid malabsorption. This suggests that metabolic

dysfunction persists even after nutritional recovery from SAM and may contribute to continued growth faltering.

Systemic Immune Deficiencies

Malnutrition causes deficits in both adaptive and innate immune function, leading to increased childhood mortality from infectious disease [130]. These deficits are multifactorial, driven in part by impaired immune cell production and function. Animal models of protein malnutrition demonstrate bone marrow atrophy and decreased numbers of hematopoietic stem cells and hematopoietic progenitor cells. Cell cycle arrest occurs in the latter due to reduced expression of cell cycle proteins and increased expression of inhibitory proteins [131]. Bone marrow mesenchymal stem cells are more likely to differentiate into adipocytes in protein-malnourished mice, further limiting their ability to produce cytokines [132]. Bone marrow polymorphonuclear cells from protein-malnourished mice also exhibit reduced migration and IL-1B production in response to lipopolysaccharide challenge [133]. Lymphoid organs, including thymus, spleen, and lymph nodes, also show atrophy, reduced cellularity, arrested cell cycle, and impaired cellular function in acute malnutrition [18].

In addition to the reduced cellularity of hematopoietic and lymphoid tissues, a shortened lifespan from increased apoptosis also contributes to reduced numbers of circulating monocytes, macrophages, dendritic cell, and natural killer cells [18]. Circulating innate immune cells from malnourished mice also exhibit impaired cytokine expression in response to LPS as a result of NF-KB dysregulation [18]. In addition to protein deficiency, multiple micronutrient deficiencies can contribute to immune dysfunction [18]. In preclinical models, NK cell and neutrophil function are restored with reversal of vitamin A and C deficiency, respectively [134,135].

Reduced numbers and function of innate immune cells contribute to deficits in adaptive immunity. Dendritic cells from severely malnourished children have reduced

HLA-DR expression and consequently are unable to stimulate T cells [136]. Peripheral blood mononuclear cells from malnourished children under-express Th1 differentiation cytokines and overexpress Th2 cytokines, contributing to their inability to clear certain infections [137,138]. CD3+ T cells from cord blood of children with IUGR revealed hypermethylation of genes that participate in T cell regulation and activation and metabolic diseases [139]. T cells from malnourished children also over-express the apoptotic marker CD95 [137]. CD8+ T cells from malnourished mice recovered their functional deficits when transferred to a healthy mouse [140], suggesting that environmental cues contribute to impaired function. T cells require glucose uptake and metabolism – both leptin-dependent processes. Low leptin levels in malnutrition inhibit T cell activation and skew differentiation of T cells from Th1 to Th2 [141,142]. Leptin also protects against thymic atrophy, prevents apoptosis of innate immune cells, and improves cytokine production in macrophages and T cells in models of malnutrition [142,143].

Liver Dysfunction

Protein deficiency results in liver dysfunction, which has both metabolic and immunological consequences. The most dramatic manifestation is steatosis [144], although mechanisms by which this occurs are poorly understood. A murine model of protein deficiency linked mitochondrial dysfunction and loss of peroxisomes to impaired fatty acid oxidation and steatosis. By stimulating the nutrient-sensing nuclear receptor peroxisome proliferator-activated receptor-alpha (PPARα), peroxisome numbers and fatty acid oxidation and steatosis were normalized. Interestingly, peroxisome loss was associated with decreased markers of bile acid synthesis [145], suggesting that peroxisomal dysfunction may contribute to the altered bile acid profiles observed in SAM. Specifically, children with SAM have increased total bile acids in serum, while their intestine contains decreased conjugated and increased secondary bile acids [146]. Secondary bile acids deoxycholic acid and lithocholic acid, products of metabolism by gut microbes, can be

toxic to intestinal epithelial cells, increasing permeability and apoptosis [147]. Thus, while liver dysfunction and microbiome alterations influence bile acid metabolism, the resulting bile acids may in turn cause liver and intestinal dysfunction. In a neonatal mouse model of protein deficiency, primary and secondary bile acid content within liver was decreased >80%; mice exhibited evidence of oxidative stress, inflammation, autophagy, and liver dysfunction [148]. Decreased intestinal conjugated bile acids likely also contribute to the impaired fat digestion, fat-soluble vitamin deficiencies, and small bowel bacterial overgrowth that contribute to the clinical picture of SAM. Malnutrition also impairs hepatic synthesis of complement proteins, especially in children with edematous malnutrition, among whom low circulating C3 correlates with low serum albumin [130]. However, increased consumption of complement, measured via elevated circulating levels of the C3 degradation product C3d, might also contribute to the low levels reported in numerous studies of malnourished children [146].

Intestinal Damage and Immune Function

Malnutrition affects all organ systems, including the intestinal mucosa (Figure 1.2). Hallmark histological changes include mucosal and villous atrophy, crypt branching, and narrowing of the brush border [149]. Malnourished children also have inflammatory cells infiltration in the lamina propria, increased numbers and activity of CD3+ cells, increased macrophage number and activity, and reduced IL-10 production [150]. Changes to the gut microbiota drive differentiation of Th17 cells over Treg cells, as transcription factors for each cell type are sensitive to different commensal species [151]. Animal models of protein malnutrition reveal an inverse relationship between dietary protein quantity and the severity of intestinal histopathology [152,153]. This intestinal damage impairs digestion and absorption of macro- and micronutrients, increasing nutritional requirements [154]. Proteins from breast milk and animal sources are more bioavailable than those derived from plants, which could explain why dairy proteins improve growth in children with SAM

[155,156]. Additionally, lactose is more easily digested by children with SAM than other carbohydrates [157].

Animal models of malnutrition exhibit minimal intestinal histopathology unless an infectious insult is provided. Nonetheless, human studies and animal models suggest that malnutrition (with or without infection) impairs intestinal barrier function [66,153] by altering the expression of antimicrobial peptides [153] and tight junction proteins [158,159]. Historically, intestinal absorption and permeability has been assessed with the lactulose:mannitol test, which has high variability in children due to inaccurate carbohydrate dosing, incorrect urine collection, variable rates of gastric emptying or renal excretion, and concurrent diarrhea [160]. Recent studies have sought to identify biomarkers that correlate with intestinal damage, inflammation and barrier function. Promising candidates include serum endotoxin core antibody, circulating bacterial products such as

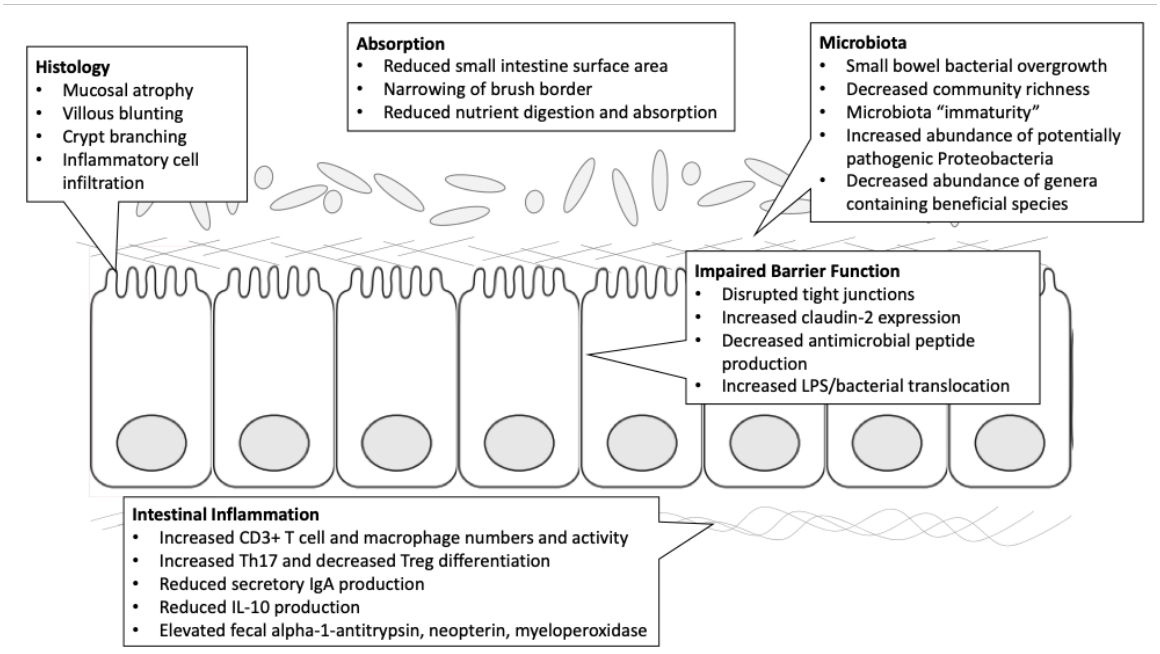


Figure 1.2: Effects of malnutrition on intestinal function.

Observations predominantly from clinical studies, although some mechanistic data are from preclinical models of malnutrition.

lipopolysaccharide and flagellin, and fecal markers including alpha-1-antitrypsin, myeloperoxidase, and neopterin [69].

Finally, deficits in mucosal immunity in malnourished children can result in poor response to mucosal vaccines [161]. In a mouse model of malnutrition, poor secretory IgA production mediated decreased response to *Salmonella* and cholera vaccines [162]. Serum LPS and bacterial 16S DNA are elevated in children with SAM, and correlate with decreased expression of mucosal repair peptides and IGF-1, which suggests growth hormone (GH) resistance [158]. Thus, decreased barrier function and bacterial translocation may contribute to chronic inflammation and growth failure by modulating the GH/IGF-1 axis [125,128]. Not surprisingly, intestinal and systemic inflammatory markers and elevated GH predict mortality in malnourished children [77,128].

Microbiota

Malnutrition drastically alters the intestinal microbiota, subsequently influencing intestinal and systemic processes including inflammation, immune function, metabolism, and growth [85,163]. Incomplete microbiota recovery may mediate treatment failure and relapse in children released from SAM treatment programs, as the microbiota of children who have recovered from SAM resembles that of a child with persistent MAM [164]. Different microbiota compositions exhibit different responses to the same nutritional interventions, which may also contribute to treatment failure [165].

The malnourished microbiota has several distinguishing features. Malnourished children experience microbiota "immaturity" [166,167]. A child's gut microbiota matures during the first two years of life to accommodate the dietary transition from breastmilk to solid food [164,166]. This transition is halted or slowed in malnourished children and is only transiently remedied by nutritional interventions [95,166]. Key age-discriminatory taxa that can predict development stage include *Bifidobacterium longum, Faecalibacterium prausnitzii, Prevotella copri, Streptococcus thermophilus,* and

Lactobacillus ruminis [164,168]. This group is correlated with markers of growth and reduced inflammation [169]. The malnourished microbiota is also less diverse than the healthy microbiota [170] and exhibits a loss of bacterial genes associated with growth and metabolism [171]. The malnourished microbiota typically exhibits increased proportions of Proteobacteria, including potential enteropathogens such as *Shigella* and *E. coli* [170,172] and increased expression of bacterial virulence and pathogenesis genes [171]. While it is critical to understand the ecology and interactions of the entire microbiota, single species can have outsized impacts on growth and development in a dysbiotic malnourished microbiota. Infection with pathogenic strains of *Bacteroides fragilis* exacerbated weight loss in an undernourished microbiota, but not in a healthy microbiota [99]. Conversely, *Lactobacillus plantarum* increases IGF1 expression and linear growth, even during chronic undernutrition [100].

Early life exposure to antibiotics may also irreversibly alter the intestinal microbiome in a way that impacts metabolism and immunity [173,174]. Mice exposed to low dose penicillin prenatally and during weaning developed a distinctive intestinal microbiota that mediated increased fat mass and bone growth, hampered intestinal immune development, and disrupted liver function and insulin response [173]. These changes persisted even after microbiota recovery. These effects on weight gain have been observed in humans as well. Children in low-income settings exposed to antibiotics in the first six months had higher WAZ than unexposed children. However, it remains to be seen if the metabolic and immune changes observed in mice also take place in children [175]. The positive benefits of subtherapeutic doses of antibiotics must be considered in light of the known and potential negative effects, such as impaired immunity, increased antibiotic resistance, and altered host physiology and metabolism [176].

Susceptibility to Infection

Children with acute malnutrition are at an increased risk of contracting and dying from a wide variety of infectious diseases [16,177]. Over half of all child deaths from diarrheal disease, pneumonia, and malaria can be attributed in part to malnutrition [178]. Malnutrition has been identified as a risk factor for respiratory syncytial virus infection in multiple studies and is associated with colonization by *Streptococcus pneumoniae* [18]. Malnutrition also increases susceptibility to gastrointestinal infection with bacterial or parasitic pathogens. There is little research on the impact of malnutrition on viral enteric pathogens and no clear consensus on how malnutrition affects rotavirus vaccination [18]. However, traits of the malnourished intestine such as dysbiosis, intestinal damage, reduced barrier function, and dampened immune function all contribute to increased susceptibility to most enteric pathogens. Preclinical models of PEM exhibited reduced oral cholera and *Salmonella enterica* serovar Typhimurium vaccine efficacy [162]. Children with malnutrition vaccinated orally (but not intramuscularly) against polio developed lower titers to the vaccine than healthy children [179], suggesting that damage to the intestine impairs mucosal immune response [180]. Lastly, malnutrition also increases susceptibility to systemic infections. Approximately 17% of children hospitalized with SAM also have bacteremia at time of admission and recorded case-fatality rates range from 10-28.9% [18]. Gram-negative bacteria are detected in just over half of these cases. Common isolates include *Salmonella enterica* serovar Typhimurium, *E. coli, Klebsiella pneumoniae, Staphylococcus aureus,* and *S. pneumoniae* [18]. In severely malnourished children, bacteremia can develop into life-threatening septic shock. Children with both SAM and septic shock require careful monitoring due to their increased risk of renal and cardiac failure [18]. Bacteremia may be more common in children with malnutrition due to combined effects of respiratory and intestinal immune dysfunction and increased exposure to pathogens in the environment as discussed previously. Malnutrition also contributes to increased mortality from other systemic infectious diseases, including Tuberculosis (TB), malaria, and visceral leishmaniasis. It is difficult to definitively assert malnutrition as a risk

factor for TB, but children with malnutrition exhibit poorer response to the BCG vaccine [181] and low BMI adults with TB have reduced protective cytokine response [182]. Relative risk of malaria mortality is increased 9.49 in children with SAM and 4.48 in children with MAM [183]. Children with SAM also clear anti-malarial drugs faster and become reinfected faster than healthy children [184]. Finally, malnutrition increases severity of visceral leishmaniasis. Studies in preclinical models attribute this increased severity to impaired immune cell trafficking and increased parasite dissemination [18].

Long Term Consequences

Both acute and chronic malnutrition can cause long-term cognitive deficits, especially if suffered during early childhood development. Low birth weight, stunting, and low body weight have been correlated with lower scores on intelligence tests and developmental delays [185,186]. The first year of life is critical to neural development and deficits in this time period are often irreversible [187]. Although children who suffer an episode of acute malnutrition in the first year of life can achieve catch-up growth physically, cognitive and behavioral deficits persist into adulthood [20]. Adults who have experienced a childhood episode of MAM or SAM are 9 times more likely to have an IQ in the intellectually disabled range [20]. This low IQ translates to decreased lifetime earnings and makes it more difficult to break the cycle of poverty and malnutrition [188]. However, the effects of poor nutrition on child development can be mitigated to some extent by wealth status, level of psychosocial stimulation, and level of physical activity [189].

Episodes of prenatal or childhood malnutrition can also cause epigenetic changes that persist for generations [190]. Muscle biopsies from adults who survived childhood marasmus or kwashiorkor (non-oedematous or oedematous SAM) exhibited different epigenetic signatures in genes related to metabolism, growth, immunity, and neuronal development [191]. It has been proposed that these epigenetic changes occur in low birth

weight infants to allow for a more stable metabolism and higher odds of SAM survival. While a "thrifty phenotype" may be protective during early-life nutrient deprivation, its persistence into adulthood can have detrimental effects, such as poor glucose tolerance, blood pressure control, and cardiac function [192]. Adult survivors of infant or prenatal famines (and their offspring) experience higher rates of obesity, hypertension, and diabetes [193-197], contributing to the growing global double burden of undernutrition and obesity [110].

TREATMENT OF MALNUTRITION

The WHO has released guidelines and updates on the treatment of severe acute malnutrition six times between 1981 and 2013 [1,2,198-201]. Early guidelines were based on clinical experience and physician anecdotes and the quality of evidence for today's guidelines remain poor, ranging from low to very low, to unclassifiable [1,202]. The lack of empirical evidence for the current treatment guidelines may explain why SAM mortality rates are still as high as 10-40% in some regions [202]. Innovations that improve treatment coverage and caregiver education show promise in reducing the burden of malnutrition. Current research on novel interventions is guided by new insights into the pathology of malnutrition and target the intestinal microbiota and inflammation.

Current Guidelines for SAM and MAM

According to the most recent WHO recommendations [1], all children diagnosed with SAM should be admitted to a treatment program. Children with complicated SAM require inpatient treatment. Complications include oedema, lack of appetite, or signs of other medical complications. If the child is dehydrated upon admission, low-osmolarity oral rehydration solution (ORS) should be administered. Once admitted, the child should be stabilized using therapeutic milks F-100 (and later F-75) and begin treatment with an antibiotic, such as amoxicillin. Current WHO guidelines recommend treating all cases of

complicated or uncomplicated SAM with broad-spectrum antibiotics because their use results in decreased mortality, and one study associated early-life antibiotic exposure with an increase in ponderal growth among children in low-income settings [175]. These guidelines are warranted, but vigilance must be kept for adverse events that may emerge as well [173,203]. Upon stabilization, the child can be transitioned to outpatient care, and treated with ready to use therapeutic foods (RUTFs). Children who pass the appetite test (eat when food is offered) and have uncomplicated SAM can progress directly to outpatient therapy and RUTF, though they too should be given a course of oral antibiotics. Children should be monitored until they reach recovery, defined as WHZ ≥ -2 or MUAC ≥ 125, and two weeks without oedema.

The WHO guidelines for treatment of MAM have remained relatively the same for the past 30 years. There are currently no evidence-based recommendations for treatment of MAM, but the WHO suggests children with MAM should be treated with nutrient dense, locally available food [204]. However, when that is not feasible, children with MAM can be treated with supplemental foods. The type and amount given should depend on the nutritional needs of the child, the likelihood of the food being shared with other children in the family, and if the child will be consuming it as a main food source or a supplement. The WHO also supports upstream interventions such as education on breastfeeding and nutrition, stabilization of infrastructure, and crop diversification. Systematic reviews of the use of supplemental foods to treat MAM found that any specially formulated food (and particularly lipid-based nutrient supplements (LNS)) in comparison to standard, local care improved recovery rates and WHZ [205,206].

Therapeutic and Supplemental Foods

A wide variety of supplemental foods are used in the prevention and treatment of malnutrition. Composition varies according to use, but can be broadly organized into therapeutic milks, LNS, and fortified blended foods (FBFs)[207]. Therapeutic milks such

as low-protein F-75 and higher protein F-100 are given during the stabilization and transition phases of recovery from complicated SAM. LNS are a category of ready to use foods that do not require cooking. They are typically shelf stable and composed of a lipid, peanut-paste, dry milk, and micronutrients and vitamins. They encompass RUTF, which provide all daily macro and micronutrients during recovery from SAM, and ready to use supplemental foods (RUSFs), which supplement breastfeeding or other foods in the treatment and prevention of MAM. Emergence of RUTF and RUSF has allowed for home treatment of acute malnutrition, but these foods are often expensive and at risk of shortage as they are not typically produced locally. However, the WHO recommends the use of RUTF for both complicated and uncomplicated SAM [1]. Finally, FBFs are blends of milled and partially cooked fortified cereals, soya, and legumes that can be used in the treatment and prevention of MAM [207]. The most common FBF is a corn-soy blend (CSB). These are inexpensive and can be produced locally but are generally inferior to RUTF in supporting recovery due to their low energy density and micronutrient content [208-210]. In an attempt to improve the nutritional composition and energy density of CSB, versions have been made with oil (CSB+) and oil and milk powder (CSB++) [1,211].

Research suggests that RUTFs and RUSFs are generally superior at producing recovery from SAM and MAM as compared to FBF or local nutrient dense foods [208-210,212-214], though this evidence has been deemed low quality [1,206]. Some groups have attempted to improve the nutrient composition of CSB in hopes of providing a cheaper alternative to RUTF/RUSF, and have generally found CSB+ and CSB++ noninferior to RUTF/RUSF [215,216]. However, the ease of use of RUTF/RUSF compared with CSB has resulted in higher rates of feeding compliance and acceptability [217-219], suggesting that nutrient composition is not the only roadblock to CSB+ and CSB++ use.

A major issue with RUTF/RUSF usage is high cost compared to FBF. This high cost is mainly driven by the use of dry skimmed milk powder. Studies to find a non-dairy RUTF have had mixed success. RUTF with reduced milk was found to be inferior in

producing recovery from SAM [220,221], but versions of milk-free RUTFs with added whey protein or amino acids are in many cases noninferior [222-224]. In fact, amino acid supplementation was found to accelerate catch up growth and protein synthesis during recovery from SAM [225]. Another important factor in RUTF/RUSF manufacturing and utilization is acceptability to patients. Several studies have found that locally produced RUTFs/RUSFs are equivalent to centrally produced RUTF/RUSF in terms of acceptability and recovery rates [213,226-228], and are usually less expensive and easier to keep in stock.

Finally, multi-nutrient powders (MNP) and vitamin supplementations have been examined to treat malnutrition. MNP or vitamin supplements were not found to improve rates of recovery from MAM or SAM, but did reduce rates of anemia and iron deficiency [229-232]. However, one intervention of iron and zinc was able to increase WAZ over placebo in malnourished children [232]. The 2013 guidelines for treatment of SAM do not address MNP use beyond recommending specific micronutrient content of RUTF and RUSF, but do suggest vitamin A supplementation for all children not already receiving vitamin A through therapeutic and supplemental foods [1]. Vitamin A has utility in reducing morbidity and mortality in malnourished children, though opinion varies on the appropriate dosage [233,234].

Outpatient and Community Management of Acute Malnutrition

Historically, the WHO recommended inpatient treatment for all forms of SAM for a minimum of four weeks [198,200,201]. In 2007, WHO endorsed the use of community-based management of acute malnutrition (CMAM) for cases of uncomplicated cases or complicated but stabilized SAM [199]. By 2013, over 70 countries had implemented CMAM [207]. CMAM uses community volunteers to screen the community for cases of acute malnutrition and then triage them based on the level of malnutrition. CMAM programs are comprised of treatment sites close to the community and an inpatient facility for occasional complicated cases of SAM. Uncomplicated cases of SAM are treated at

home with RUTF. CMAM may or may not include protocols for the treatment of MAM, though this is suggested to reduce development of SAM cases. At home, the malnourished child receives regular visits from the community health workers to offer guidance and counseling and referrals to other health programs if necessary. Monitoring stops once the child reaches the discharge criteria. CMAM avoids the majority of drawbacks associated with inpatient treatment programs, such as lengthy and expensive hospital stays, cross-infections, premature program discharge, and low program coverage [207].

Though CMAM is a relatively new phenomenon, data have been collected from multiple continents on the implementation, cost, efficacy, and challenges of CMAM. Early analysis of CMAM focused predominantly on humanitarian aid settings, and found cost and health benefits in developing nations that implemented CMAM over inpatient care [235-237]. While most CMAM programs have had similar or better rates of treatment than inpatient protocols [238,239], there are situations in which such programs fail to make an impact in the community. This often depends on the level of government support, occurrence of RUTF shortages, and accessibility [240]. RUTF shortages can be combatted through local production of RUTFs and nutritional education. Studies in Vietnam, Ethiopia, Ghana, Pakistan, and India [226,241] assessed cost-benefits and cultural acceptability of locally produced RUTFs. Locally produced RUTFs were on average 60% cheaper than standard RUTF, due to differences in materials and reduced costs of manufacturing and shipping from a distant location. Locally produced RUTFs were comprised of foods that are familiar and accessible to the local population, were well tolerated, and were liked just as much as standard RUTF in nearly all cases. However, poor accessibility is the main factor driving the existing enormous coverage gap, with <15% [242] of affected children globally, including <2% in East Asia and the Pacific [243], having access to malnutrition treatment. Barriers to access include caretaker awareness of malnutrition and local treatment programs, high opportunity costs of seeking treatment, and proximity [242]. Interventions that may reduce the coverage gap include educating and

engaging mothers, integrating community-based management of SAM with existing health programs, improving staff competency [244-246], and strengthening government involvement to increase coverage and data collection [243].

Microbiota Directed Therapies

Greater awareness of the intestinal microbiota's role in malnutrition has driven interest in the effects of prebiotics and/or probiotics as supplements to the standard nutritional and antibiotic treatment for SAM. Probiotics and prebiotics provide health benefits in non-malnourished children and adults [247-249] but little data is available on their effects in malnourished children. The PRONUT study in Malawi provided a mixed probiotic/prebiotic (Synbiotic) to children with SAM after the initial stabilization phase [250]. There was no difference in nutritional recovery rate but a trend towards decreased outpatient deaths, especially in HIV seronegative children. However, children treated with Synbiotic also had longer coughing, vomiting, and severe diarrhea as inpatients. The greatest benefits were observed in children not given antibiotics, which are recommended by WHO for all cases of SAM. A study in Uganda gave probiotics to children upon admission with SAM [251]. Probiotics reduced days with diarrhea during the outpatient phase of treatment by 26% but had no effect during inpatient treatment. These studies suggest that probiotics may be beneficial in long term recovery from SAM and could be integrated into outpatient rather than inpatient protocols. Prebiotic and antibiotic use in malnutrition treatment protocols is currently under investigation for treatment of MAM as well [252]. A three-arm study in four African nations will examine the effect of FBF, FBF + prebiotic, or FBF + azithromycin on child weight recovery and intestinal microbiota. The use of the antibiotic azithromycin in this study is notable, as it is a macrolide with a narrow spectrum of activity that is not commonly used as a first line treatment. Mass administered azithromycin has already proven to reduce all-cause mortality rates among children in sub-

Saharan Africa, potentially due to its anti-inflammatory and immunomodulatory effects [253].

Although trials of probiotics and prebiotics have not revealed growth benefits for malnourished children, it is important to note that these microbiome-targeting therapies were not tailored to microbial species or functional deficiencies within the target populations; thus, the full potential of microbiome-targeting therapies for child malnutrition has not yet been realized. However, a recent study based on individuals from Bangladesh identified a core "ecogroup" of 15 covarying bacterial taxa associated with healthy child development that can be used to assess microbiota recovery from SAM to MAM to full health [164]. A novel microbiota directed complementary food (MDCF) was developed with local foods that supported growth of this ecogroup [169]. Children with MAM treated with this MDCF experienced similar levels of growth as children treated with standard RUSF, but also had increased levels of biomarkers associated with growth, bone development, neurodevelopment, and immune function. Thus, microbiota-directed therapies can have far-reaching impact in supporting a child's return to a healthy development track. This model of MDCF development should be deployed in other regions with high burden of MAM and follow-up extended to see if any long-term benefits exist.

Impetus for this Project

Recent research has produced insights into the interconnectedness of nutrition, metabolism, microbiota, and immunity in the context of acute malnutrition. This information has already proven useful in guiding design of interventions that promote recovery beyond weight gain. However, the literature lacks evidence on the inflammatory response in children with malnutrition. Children with malnutrition have greater morbidity and mortality during infection, but the underlying mechanisms are not fully understood [16]. It is also not clear what baseline inflammatory status children with malnutrition have and what role different systems (metabolism, microbiota, intestinal permeability) play.

Much of the available research on these topics was conducted in the context of SAM or stunting, both of which produce different physiological effects than MAM [130]. However, MAM comprises two thirds of the burden of acute malnutrition [15]. Interventions targeted to the prevention and treatment of MAM also have the benefit of preventing SAM and minimizing the associated long-term developmental damage.

Within this dissertation we characterize MAM-related inflammation and inflammatory response in MAM in children and mouse models, identify potential underlying contributors to this inflammation, and explore interventions targeted to those mechanisms. Chapter 2 of this dissertation addresses baseline inflammation and inflammatory response in children with MAM and identifies associated socioeconomic and biological factors. Chapter 3 characterizes inflammation and inflammatory response in a mouse model of MAM and explores the role played by microbiota. Chapter 4 then examines incorporation of an anti-inflammatory lipid in the malnourished diet to reduce inflammation and potentially support recovery from MAM and appropriate response to inflammatory stimulus.

CHARACTERIZATION OF SYSTEMIC INFLAMMATION AND INFLAMMATORY RESPONSE IN CHILDREN WITH MAM

Chapter 2. Association of Socioeconomic, Environmental, and Biologic Factors in Children with Moderate Acute Malnutrition: An Exploratory Study

ABSTRACT

Acute malnutrition, or wasting, affects 50.5 million children worldwide. These children are at an increased risk of morbidity and mortality from infectious disease. However, the mechanisms underlying this increased risk are not well understood and may differ with severity of malnutrition, geography, and environment. Our objective was to identify differences in inflammatory responses between well-nourished and moderately malnourished children and search for physiological, environmental, or sociodemographic traits that may influence this response. Sixteen children with moderate acute malnutrition and 16 healthy controls under 5 years of age were studied in Nairobi, Kenya. Demographic and health data were provided by their primary caregivers. Blood samples were taken to measure markers of inflammation and intestinal damage, metabolites, and the leukocyte response to bacterial stimuli. Children with malnutrition more frequently came from households with contaminated water, crowding, and unstable income sources. They had evidence of metabolic changes, basal inflammation, intestinal damage, and an exaggerated whole blood inflammatory response to bacterial lipopolysaccharide. These exploratory findings suggest convergence of multiple factors to promote dysregulated inflammatory responses and prompt several mechanistic hypotheses that can be pursued to better

understand the increased morbidity and mortality from infectious diseases in children with malnutrition.

INTRODUCTION

Acute malnutrition (wasting) affects 50.5 million children under the age of five [254]. The World Health Organization (WHO) defines two categories of acute malnutrition, identified by the weight-for-height Z score (WHZ) or mid-upper arm circumference (MUAC) [1]. Moderate acute malnutrition (MAM) is defined as either WHZ ≤-2 and >-3 or MUAC <12.5 cm and ≥11.5 cm. Severe acute malnutrition (SAM) is defined as WHZ ≤-3.0 or MUAC <11.5 cm [2]. While WHZ is the gold standard for diagnosis of acute malnutrition, MUAC is more easily deployed in the community and can be used to screen large numbers of children quickly. However, children that meet the diagnostic criteria for malnutrition using MUAC may not meet the criteria using WHZ, and vice versa [6]. MUAC correlation with WHZ differs with sex and age [7,8], and malnourished children with fluid retention can be erroneously diagnosed as healthy when using WHZ alone [3]. Two-thirds of children with acute malnutrition are moderately wasted [15], but little is understood about the pathophysiology of MAM. Two other anthropometric measures are relevant to understanding acute malnutrition. Weight-for-age Z score (WAZ) is used to define underweight without discriminating between wasting or stunting or pace of evolution (acute vs. chronic). Height-for-age Z score (HAZ) is used to define stunting and does not measure wasting. It is commonly used as a measure of chronic undernutrition.

SAM and MAM increase a child's odds of dying approximately 9-fold and 3-fold, respectively, compared to healthy children [16]. The majority of the malnutrition-related increase in childhood mortality is due to an increased susceptibly to a broad array of pathogens [18]. Gastrointestinal infections in particular drive the vicious cycle of malnutrition, in which malnourished children are more susceptible to infection and infection makes children more likely to develop malnutrition. Animal models of

malnutrition have demonstrated increased susceptibility to enteroaggregative *E. coli* and subsequent growth impairment. This is likely due to increased nutrient loss, malabsorption, and diversion of energy to the immune response [255]. Intestinal damage during infection probably contributes to the relatively high rates of bacteremia observed in children with SAM. Two studies found that 17% of children admitted with SAM also had bacteremia and the majority of cases were due to gram-negative enteric species such as *Salmonella* and *E. coli*. The high prevalence of bacteremia in children with SAM is likely due to failure of the physical and immunological gut barriers, impaired removal of bacteria in the mesenteric lymph node and liver, and reduced function of anti-bacterial phagocytic cells. Systemic infection with enteric bacteria carries high morbidity and mortality in malnourished children [18].

While there are currently no globally standardized approaches for treatment of MAM[256,257], the WHO recommends that children with MAM should be given locally sourced energy-dense supplementary food for nutritional recovery and monitored for growth recovery [204]. However, with this approach approximately 20-30% of children fail to recover, or relapse within a year after recovery [210,214,258]. Persistent alteration of the intestinal microbiome appears to be a driver of relapse in children who have recovered from SAM [166]. Additionally, the metabolic profile of children with SAM is markedly different from healthy children and does not normalize after nutritional stabilization [129]. Furthermore, growth recovery from SAM does not appear to reduce the incidence and mortality of infectious disease [259], suggesting that recovery of immune function may not accompany growth recovery. Studies are needed to better characterize the deficits in immune function and host defense in SAM and MAM and to assess immunological recovery following nutritional intervention.

Studies in both children and mouse models of SAM have found evidence of intestinal damage and reduced barrier function, liver dysfunction, and impaired immune cell function [18,130,260]. However, there are few studies to describe the pathology of MAM

in children and no markers of host defense or inflammatory status have been consistently associated with MAM. Studies that attempt to identify such markers often produce contrasting results about levels of circulating inflammatory cytokines such as TNF-α and IL-6 and acute phase response proteins [18,130]. Differences in study populations and presence of overt or subclinical infections make comparison of results between studies difficult. Nevertheless, they are important to develop an understanding how variables such as infection status, geography, and social status impact the pathogenesis of malnutrition.

The intestinal damage observed in children with malnutrition [153] may create chronic, low level systemic exposure to lipopolysaccharide (LPS) and other bacterial products. In a study of children with SAM, the level of endotoxemia was inversely correlated with activation status of dendritic cells [136], akin to the dampened inflammatory response seen in endotoxin tolerance [261]. This is likely a protective mechanism to ensure malnourished children with chronic exposure to LPS are not constantly experiencing inflammatory cytokine storm. This anergic response may, however, contribute to increased susceptibility to some pathogens. On the other hand, exposure to even lower levels of LPS can lead to "endotoxin priming" [261], in which a second LPS exposure leads to a potentially detrimental exaggerated inflammatory response.

With this study, we seek to identify circulating biomarkers of immune function and inflammation in children with moderate acute malnutrition in urban Nairobi, Kenya. We also characterize cytokine responses to bacterial lipopolysaccharide in children with MAM compared to healthy controls. By evaluating measures of inflammation, markers of intestinal damage, and circulating metabolites, and correlating these measures with environmental and sociodemographic data, we provide an exploratory model from which multiple hypotheses can be tested in future studies to gain a better mechanistic understanding of the pathogenesis of MAM. These markers of intestinal, metabolic, or immune function that are associated with MAM can also be explored as potential correlates of recovery of immunological and metabolic function after treatment for MAM.

MATERIALS AND METHODS

Subject Enrollment and Evaluation

The present study was an exploratory observational study of cohorts of 16 children with moderate acute malnutrition (MAM) and 16 healthy controls in Nairobi, Kenya. The cohort of MAM subjects for the study presented here was obtained from a larger prospective observational study (60 subjects with MAM) that was designed to identify risk factors for infection and relapse in children with MAM. For the immune function sub-study presented here, children aged 18-36 months were classified as either MAM or healthy controls (n=16 per group), based on mid-upper arm circumference (MUAC). A MUAC equal to or less than 12.5 cm identified subjects in the MAM group according to WHO guidelines. Healthy controls had a MUAC >12.5 and a WHZ >-1. Children with congenital malformations, chronic disease (including HIV infection), and symptoms of an acute infectious disease in the previous 2 weeks were excluded from the sub-study. Upon enrollment, caregivers of children with MAM and healthy controls were administered a questionnaire on family sociodemographic traits and the child's health and development. Weight and height were measured and urine, stool, and blood samples were collected. Children with MAM were treated according to WHO guidelines [204] with supplemental foods and followed for a year post enrollment. During the longitudinal follow-up, time to recovery of growth metrics, incidence of relapse, and frequency of fever, cough, and diarrhea were collected.

Anthropometric Measurement

The child's length was measured using the UNICEF child measuring height board (model: S0114530) to a precision of 0.1 cm. The child's weight was measured using the electronic baby scale (model; 336Seca) to a precision of 0.01 kg. The middle upper arm circumference (MUAC) was measured at the midpoint of the left arm using a non-stretch

insertion tape to a precision of 0.1cm. The anthropometric measures were expressed as z scores based on WHO child growth standards [2].

Stool Microscopy

Fresh stool was collected for microscopy to determine presence of ova and cysts of intestinal parasites. Stool samples were collected in a plain collection vial (AlphaTec, USA) and a vial containing preservative (ProtofixTMCLR, AlphaTec, USA). Each stool sample was processed and evaluated using wet-preparation, Kato- Katz and formol-ether concentration techniques by an experienced laboratory technician. The presence of protozoa or helminth eggs was determined.

Complete Blood Count and Whole Blood Assay

We collected blood samples from all 16 healthy controls and the 16 subjects from the MAM group for analysis of plasma biomarkers and whole blood stimulation assay. We minimized confounding effects of an acute infection on immune/inflammatory status by excluding children who had symptoms of an acute infectious disease in the previous two weeks. 1.0 mL EDTA whole blood was drawn for full blood count. An additional 5.0 mL heparinized blood was collected for immune/inflammatory biomarker analysis. 3 mL of the heparinized whole blood was centrifuged and the naïve plasma collected and stored at -80°C.Two 1 mL aliquots of the heparinized whole blood were incubated in capped 3 mL polystyrene culture tubes (Grenier) for 20 hours at 37°C with either phosphate-buffered saline (PBS) as control or 0.2 μg/mL lipopolysaccharide (LPS) from *E. coli* O55:B5 (Sigma-Aldrich, Missouri, USA) in PBS as stimulation. After incubation, the whole blood was centrifuged and the plasma collected and stored at -80°C. Each of the three types of plasma samples (naïve, incubated-unstimulated, and incubated-LPS-stimulated) were then analyzed for expression of ten cytokines via Quantibody Array (Ray Biotech, Georgia,

USA). Concentrations were calculated from MFI using a log-log regression curve. Concentrations below the limit of detection were given the value of the LOD.

Biomarker Measurement and Analysis

ELISA kits were used to measure insulin-like growth factor 1 (IGF-1) (Sigma-Aldrich, Missouri, USA), endotoxin core antibody IgM (EndoCAb) (Hycult Biotech, Pennsylvania, USA), haptoglobin (Hycult Biotech, Pennsylvania, USA), LPS binding protein (LBP) (Abcam, Massachusetts, USA), and intestinal fatty acid-binding protein 2 (iFABP or FABP2) (Abcam, Massachusetts, USA) in naïve plasma samples. Magnetic Luminex Assays (R&D Systems, Minnesota, USA) were used to measure C-reactive protein (CRP), procalcitonin, growth hormone (GH), leptin, insulin, and adiponectin in naïve plasma samples. Data was acquired on a Bio-Plex 200 System and standard curve calculated via five-parameter logistic curve.

Plasma Metabolite Measurement and Analysis

180 plasma metabolites including amino acids, biogenic amines, phospholipids, sphingolipids, glycerophospholipids, and acylcarnitines were measured by Biocrates Life Sciences (Biocrates, Innsbruck, Austria) in naïve plasma samples. Amino acids and biogenic amines were measured by liquid chromatography-mass spectrometry, while other metabolites were measured by flow-injection mass spectrometry. Metabolites were reported as μM concentration. The dataset was cleaned, and metabolites that were measured below the limit of detection in greater than 20% of samples were removed from further analysis. Remaining missing values were imputed using a Logspline imputation method. The imputed dataset was then log2 transformed prior to univariate statistical analysis. Microsoft Excel and R were used to perform metabolite analysis.

Primary PBMC Fatty Acid Stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated from a healthy adult volunteer for stimulation with saturated long chain fatty acids and LPS. Blood was drawn into a heparinized vacuette tube and diluted 1:2 with Minimum Essential Media (MEM). Diluted blood was overlaid with Ficoll-paque Plus at a 4:3 ratio and centrifuged at 400g for 40 minutes at room temperature. Cells were transferred from the gradient ring to a new tube and washed in MEM twice and culture mledium (RPMI with L-glutamine and HEPES, 10% FBS, 1% penicillin/streptomycin) once. Wells in a 96-well plate were seeded with 200,000 cells in 100 μl culture medium and left to rest at 37°C and 5% CO_2 for 30 minutes.

Working solutions of albumin-bound fatty acids C14, C16, and C18 (M3128, P0500, S4751; Sigma-Aldrich, Missouri, USA) were prepared with fatty-acid free bovine serum albumin (AK8909; Akron Biotech, Florida, USA) as described previously [262]. These solutions (or vehicle control) were then added to cells to produce a final concentration of 200 μM. Some cells were also simultaneously exposed to LPS at a final concentration of 10 ng/mL. Cells were incubated at 37°C with 5% CO_2 for 24 hours. Supernatants were collected and stored at -80°C until analysis with Bio-Plex for IL-6 (171BK29MR2), IL-1β (171BK26MR2), and TNF-α (171BK55MR2) (Bio-Rad, California, USA). Data was acquired on a Bio-Plex 200 System and standard curve calculated via five-parameter logistic curve. Concentrations above the upper limit of quantification (ULOQ) were given the value of the ULOQ.

Data Management

Data from the questionnaire given at enrollment was de-identified, entered into RedCap, and exported into Excel. For this study, only data from the 32 participants (16 WN and 16 MN) from the immune function sub-study were analyzed. Data was cleaned in Excel and formatted for analysis in SAS. Graphics were generated in GraphPad Prism (version 8.1.2 for macOS, GraphPad Prism Software, La Jolla, California, USA) and in R 3.4.3.

Statistical Analysis

The final data file developed for analysis contained four broad groups of variables: sociodemographic variables, health variables, anthropometric variables, and protein expression variables. Descriptive statistics, such as mean, standard deviation (SD), median and interquartile (IQR) for continuous variables, and frequency and proportion for categorical variables, were calculated among all subjects and by groups as appropriate. Chi-Squared test was used to compare the distribution of the categorical variables (proportions) between two groups. Fisher's exact test was used in the condition with sparse cell. Wilcoxon Rank Sum test was used to compare continuous variables between groups. MUAC was used for post-hoc stratification of the 32 children in our study into WN and MN groups (using WHO-defined cut-off values) before comparing protein expression levels via Wilcoxon Rank Sum test. We used Spearman's correlation to identify any correlations between biomarker expression and demographic or anthropometric variables. All tests conducted in the above analysis were two-sided. A p-value of < 0.05 was deemed statistically significant and a p-value of 0.1 regarded as a statistical trend. All analyses were explorative and were performed using SAS software (SAS Institute, Inc., Cary, North Carolina, United States).

Ethical Review and Approval

Caregivers of all subjects gave their informed consent for inclusion before they participated in the study. Consent forms were available in Kiswahili and English and was read aloud for caregivers unable to read or write. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was reviewed and approved by the Scientific and Ethics Review Unit of the Kenya Medical Research Institute (SERU/SSC Protocol 2871).

RESULTS

Subject Characteristics

Subject characteristics are shown in Table 2.1. MUAC was used to classify subjects into WN (≥12.5 cm) and MN (<12.5 cm) groups. Children in the malnourished group were significantly younger (740 vs 851 days, $p = 0.026$), shorter (80.1 vs 85.7 cm, $p = 0.005$), and weighed less (9.1 vs 11.8 kg, $p < 0.001$) than the healthy control group. Malnourished children were significantly more wasted by WHZ (-1.6 vs -0.2, $p < 0.001$) and underweight (WAZ -2.2 vs -0.9, $p < 0.001$). However, only three of 16 children in the MN group met the criteria for MAM by WHZ. The median height-for-age Z Score (HAZ) was similar between groups (-1.9 vs -1.7, $p = 0.137$).

All children in this cohort presented at enrollment with normal temperature, heart rate, and pulse. None showed any sign of current infection, though nearly all had had some illness in the last month. Stool tests showed no evidence of current parasitic infestation, though malnourished children had significantly increased percentages of eosinophils in their blood (1.5% vs 0%, $p = 0.003$). Malnourished children also had decreased percentage of circulating monocytes (4% vs 7%, $p = 0.047$). The frequency of anemia (50% vs 31%, $p = 0.473$) and elevated platelet counts (81% vs 81%, $p = 1.000$) were high across the cohort but did not differ significantly between groups.

Several sociodemographic characteristics were significantly different between the healthy control and MAM groups. Children with MAM lived in more cramped households (5 people per room vs 4, $p = 0.045$) with less wealth, as demonstrated by the number of common appliances owned (1.6 vs 2.2, $p = 0.014$). Children with MAM were also less likely to have fathers with external employment who were not dependent on relatives, friends, or self-employment for income (27% vs 73%). Finally, children with MAM were more likely to consume water purchased from a street vendor rather than piped (44% vs 0%, $p = 0.007$), and less likely to have that water treated before consumption (53% vs 94%,

$p = 0.044$). The purity of the water and containers from street vendors is not known but is generally considered to have more contaminants than piped water.

Table 2.1. Demographics, Anthropometrics, and Health Metrics at Enrollment

	Healthy Controls (n = 16)	*MAM (n = 16)*	*P Value (WN-MN)*
DEMOGRAPHICS/SES			
Gender: Female (%)	8 (50%)	8 (50%)	1.000
Age (months)	27.9 (21.2-34.6)	24.2 (17.8-30.7)	**0.025**
Birth Position	2 (1-3)	2 (2-3.5)	0.256
Living Siblings	1 (1-2)	1 (1-3)	0.246
Crowding Score[1]	4 (1-7)	5 (2-8)	**0.045**
Caregiver Age	26 (22-28)	26 (22-31)	0.484
Caregiver's Source of Income			0.073
Husband	*11 (73%)*	*6 (38%)*	
Other[2]	*4 (27%)*	*10 (63%)*	
Caregiver Education			0.458
Some/All Primary	*9 (56%)*	*12 (75%)*	
Some/All Secondary	*7 (44%)*	*4 (25%)*	
Father's Source of Income			**0.024**
Wife/Relatives/Friends	*0 (0%)*	*4 (27%)*	
Employment	*11 (73%)*	*4 (27%)*	
Self-Employment/Business	*4 (27%)*	*7 (47%)*	
Father Education			0.450
Some or all primary	*8 (53.3%)*	*11 (73.3%)*	
Some or all secondary/College	*7 (46.7%)*	*4 (26.7%)*	
Monthly family income (KES)	10,000 (5,000-11,000)	5,400 (3,000-9,000)	0.111
Appliances Owned (TV/Radio/Cell Phone)			**0.014**
One	*4 (25%)*	*8 (50%)*	
Two	*5 (31%)*	*6 (38%)*	
Three	*7 (44%)*	*2 (13%)*	
Water Source			**0.007**
Piped	*16 (100%)*	*9 (56%)*	
Purchased	*0 (0%)*	*7 (44%)*	
Water Treatment			**0.044**
Chlorine	*6 (38%)*	*3 (20%)*	

Boiling	*9 (56%)*	*5 (33%)*	
No Treatment	*1 (6%)*	*7 (47%)*	
HFIAS Food Security Grade			0.315
Food Secure	2 (12.50%)	0 (0%)	
Mild Food Insecurity	1 (6.25%)	0 (0%)	
Moderate Food Insecurity	3 (18.75%)	2 (12.5%)	
Severe Food Insecurity	10 (62.5%)	14 (87.5%)	
ANTHROPOMETRICS			
Weight (kg)	11.8 (10.6-13.1)	9.1 (8.4-9.5)	**<0.001**
Height (cm)	85.7 (81.5-86.8)	80.1 (78.7-82.0)	**0.005**
MUAC	14.5 (14.0-15.4)	12.4 (12.1-12.5)	**<0.001**
WHZ	-0.2 (-0.6-1.0)	-1.6 (-1.3- -2.1)	**<0.001**
Healthy	*15 (47%)*	*12 (38%)*	*0.159*
Moderately Malnourished	*0 (0%)*	*3 (9%)*	
Severely Malnourished[3]	*0 (0%)*	*1 (3%)*	
HAZ	-1.7 (-1.9- -0.8)	-1.9 (-2.4 - -1.5)	0.137
Healthy	*14 (44%)*	*10 (31%)*	*0.356*
Moderately Stunted	*1 (3%)*	*4 (13%)*	
Severely Stunted	*1 (3%)*	*2 (6%)*	
WAZ	-0.9 (-1.5- 0.4)	-2.2 (-2.7 - -1.6)	**<0.001**
Healthy	*12 (38%)*	*6 (19%)*	***0.034***
Moderately Underweight	*3 (9%)*	*7 (22%)*	
Severely Underweight	*0 (0%)*	*3 (9%)*	
HEALTH METRICS			
Diarrhea within month before enrollment	3 (19%)	4 (25%)	1.000
Fever within month before enrollment	7 (44%)	8 (50%)	1.000
Cough within month before enrollment	11 (69%)	9 (56%)	0.716
Vomiting within month before enrollment	0 (0%)	1 (6%)	1.000
Hemoglobin g/dL (Elevation Adjusted)[4]	10.7 (9.6-11.6)	10.2 (9.5-10.9)	0.071
Anemia (Hb<11.0 g/dl)[5]	5 (31%)	8 (50%)	0.473
% Monocytes	7 (4.5-9.5)	4 (1-7)	**0.047**
WBC (10^9/L)	11.2 (8.9-13.4)	13.4 (11.2-15.6)	0.274
RBC (10^12/L)	4.8 (4.5-5.1)	4.6 (4.4-4.8)	0.235
PLT Count (10^9/L)	509 (399.3-618.8)	485 (363.8-606.3)	0.867
% Neutrophils (Segmented)	39 (32.5-45.5)	37.5 (31.5-43.5)	0.428
% Lymphocytes	52.5 (45.3-59.8)	54 (47.5-60.5)	0.766
% Eosinophils	0 (0)	1.5 (0-5.5)	**0.003**

Data expressed as Median (IQR) or Number (%). P value by Wilcoxon Rank Sum test for continuous variables, Fisher's Exact (two-sided Pr ≤P) for categorical variables.
[1] Crowding score is calculated by dividing the number of people residing in the home by the number of rooms in the home.
[2] "Other" sources of caregiver income reported include self-employment, business, employment, other relatives or friends, or other sources.
[3] One child in the study was found to be severely malnourished when diagnosed with WHZ, though they were considered moderately malnourished when diagnosed by MUAC.
[4]Due to Nairobi's high elevation, all hemoglobin levels were adjusted by subtracting 0.5 based on WHO recommendations [263]
[5]Anemia cutoffs taken from WHO [263]

Children with MAM have Evidence of Damage to Small Intestine

Since poor water quality can increase exposure to enteric pathogens, we measured markers of intestinal damage and bacterial translocation in naïve plasma samples (Figure 2.1A). We found that malnourished children had elevated concentrations of iFABP (2053 vs 65 pg/mL, $p < 0.001$), a marker of recent damage to the tips of villi in the small intestine. Children with MAM also had reduced levels of citrulline (13.88 vs 18.20 pg/mL, $p = 0.028$), which is produced by healthy enterocytes. Plasma EndoCab IgM and sCD14, which

are markers of bacterial translocation from the gastrointestinal tract, were not significantly different between subjects with MAM and healthy controls.

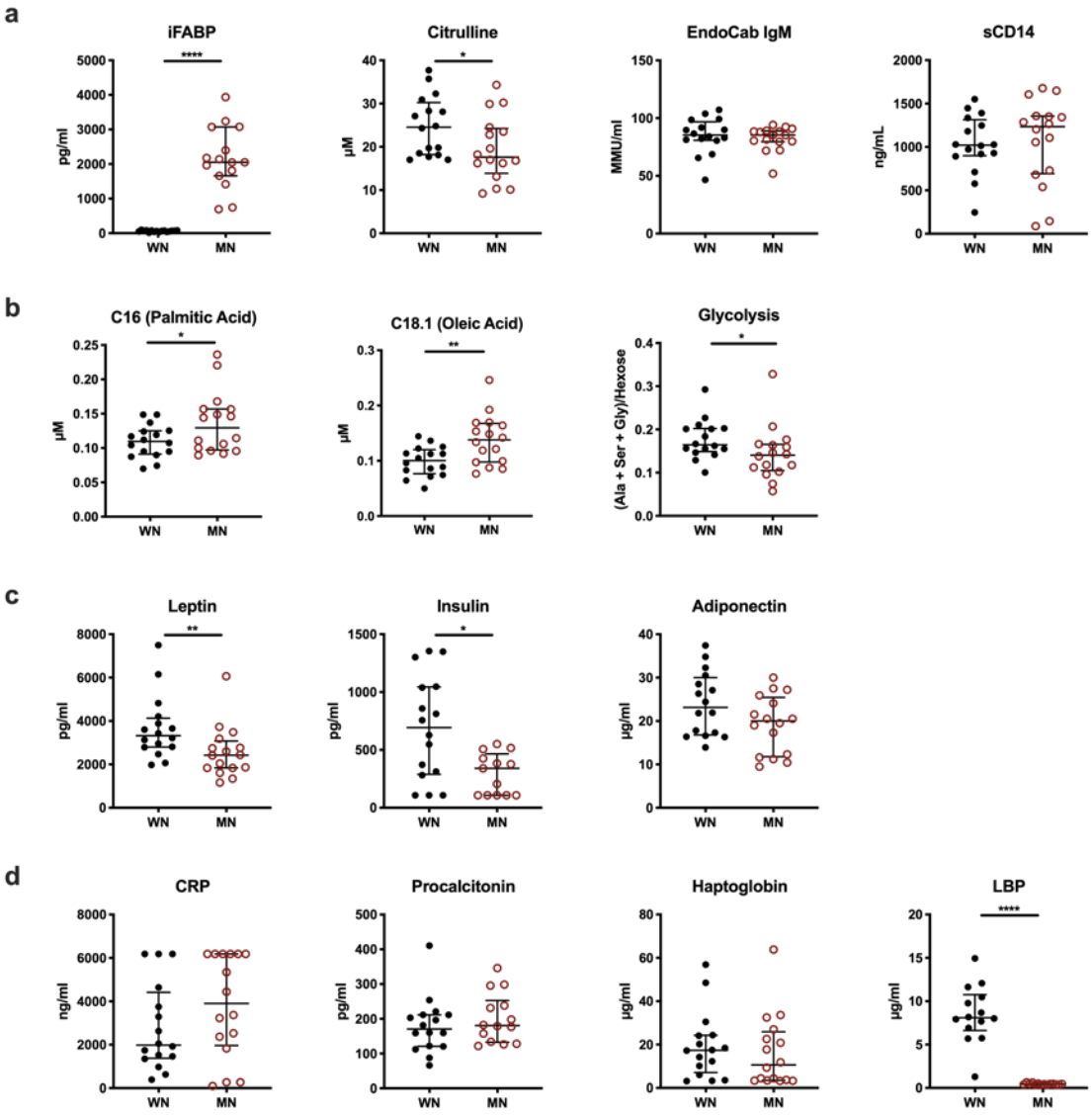


Figure 2.1. Concentrations of Markers of Intestinal Health, Metabolism, and Inflammation in Naïve Plasma.

Concentrations of markers related to intestinal health (a), metabolites (b), metabolic hormones (c), and acute phase response proteins (d) in naïve plasma from children with and without MAM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Children with MAM have Elevated Markers of Basal Systemic Inflammation and an Exaggerated Inflammatory Response to Bacterial Lipopolysaccharide

Intestinal damage and liver dysfunction can contribute to endotoxemia due to increased translocation and reduced clearance of bacteria and bacterial LPS [264]. To determine if children with MAM exhibit signs of systemic inflammation, we measured eight inflammatory cytokines in naïve plasma (Table 2.2). We found significantly elevated levels of IL-6 (40.3 vs 8.4 pg/mL, $p = 0.009$), IL-1β (7.8 vs 2.6 pg/mL, $p = 0.047$), and IFN-γ (69.2 vs 35.9 pg/mL, $p = 0.005$) in children with MAM (Figure 2.2).

Table 2.2: Median (IQR) Cytokine Concentration (pg/mL) in Naïve Plasma

Group	IL-1α	IL-1β	IL-6	IL-8	IL-10	IFN-γ	TNF-α	CCL2
Healthy	**9.4**	**2.6**	**8.4**	**3.4**	**2.8**	**35.9**	**2.8**	**100.2**
	(7.4-17.2)	(1.8-6.9)	(4.7-15.7)	(1.3-8.3)	(1.9-3.7)	(33.4-50.7)	(2.5-3.0)	(53.5-126.0)
MAM	**9.4**	**7.8**	**40.3**	**6.7**	**3.7**	**69.2**	**2.8**	**93.2**
	(5.3-13.8)	(2.1-13.5)	(12.3-88.2)	(2.1-9.3)	(3.2-5.6)	(47.5-104.5)	(2.5-2.8)	(66.8-145.3)
P value	0.703	*0.047*	*0.009*	0.221	0.099	*0.005*	0.647	0.926

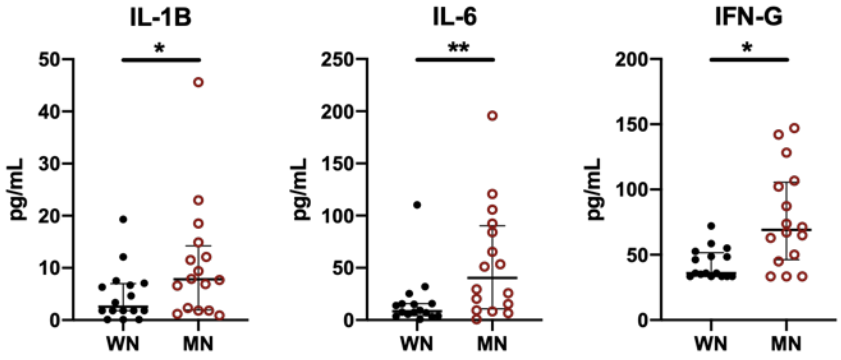


Figure 2.2. Cytokine Concentration in Naïve Plasma.

Plasma was isolated from heparinized whole blood samples and frozen at -80 C before quantification of cytokines. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

The concentrations of acute phase proteins C-Reactive Protein (CRP), procalcitonin, and haptoglobin were not significantly different in children with MAM and healthy controls (Figure 2.1D). LPS binding protein, an acute phase protein that has dual

functions of facilitating LPS-mediated activation of TLR4 signaling and detoxifying circulating LPS, was significantly lower in children with MAM (0.44 vs 8.09 pg/mL, $p < 0.0001$). Low concentrations of LBP lead to enhanced activation of mononuclear cells exposed to endotoxin.

To examine the response to a bacterial inflammatory stimulus, we incubated whole blood with LPS for 24 hours and measured plasma cytokine levels (Table 2.3). Blood from children with MAM exhibited increased IL-6 (4000 vs 3287 pg/mL, $p = 0.018$) (Figure 2.3) production and a trend towards increased IFN-γ (694 vs 470 pg/mL, $p = 0.051$).

Table 2.3: Median (IQR) Cytokine Concentration (pg/mL) in Plasma from LPS-Stimulated Whole Blood

Group	IL-1α	IL-1β	IL-6	IL-8	IL-10	IFN-γ	TNF-α	CCL2
Healthy	**214** (115.1-403.8)	**791** (739.7-886.8)	**3287** (2756.8-4000)	**223** (155.3-511.9)	**301** (232.3-356.4)	**470** (325.2-653.0)	**620** (350.3-1225.3)	**823** (470.5-1374.4)
MAM	**314** (160.5-509.0)	**820** (771.9-1036.7)	**4000** (3592.6-4000)	**205** (153.0-607.7)	**258** (177.2-442.6)	**694** (473.6-1184.1)	**612** (411.5-895.5)	**814** (651.0-1077.9)
P value	0.224	0.402	*0.018*	0.956	0.838	0.051	0.867	0.956

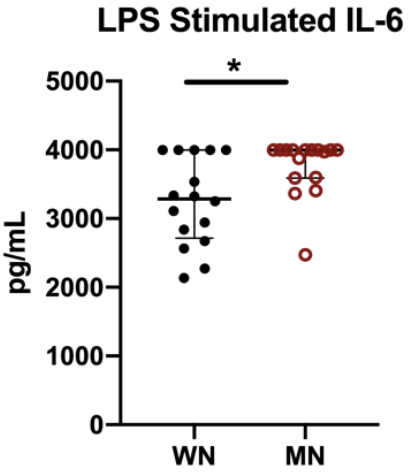


Figure 2.3. Cytokine Concentration in Plasma from LPS-Stimulated Whole Blood.

Heparinized whole blood samples were stimulated with LPS and incubated at 37°C for 24 hours prior to quantification of cytokines. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Children with MAM Exhibit Metabolic Changes

As metabolism is greatly affected by malnutrition and influences immunity, we measured concentrations of circulating amino acids and biogenic amines, acylcarnitines, and hexose. Levels of acylcarnitines C16 (0.14 vs 0.11 μM, $p = 0.049$) and C18:1 (0.14 vs 0.10 μM, $p = 0.007$) were significantly elevated, indicating impaired fatty acid oxidation in this group (Figure 2.1B). Levels of hexose and all amino acids and biogenic amines were similar between groups. The calculated glycolysis rate [(Alanine + Glycine +Serine)/Hexose] was significantly reduced in children with MAM (0.14 vs 0.16, $p = 0.039$) (Figure 2.1B). The metabolic hormones leptin (2423 vs 3325 pg/mL, $p = 0.005$) and insulin (341 vs 693 pg/mL, $p = 0.022$) were significantly lower in the MN group, while adiponectin levels were similar between groups (Figure 2.1C).

Associations Between Anthropometric Measures and Variables of Intestinal health, Immunity, and Metabolism

We conducted Spearman's correlation to examine the relationships between different measures of malnutrition and the variables that were different between children with MAM and healthy controls. Significant correlations are displayed in Figure 2.4 and variables are arranged by hierarchical clustering. MUAC, WHZ, and to a lesser degree WAZ had similar profiles of correlation. HAZ correlated with markers of underweight and wasting but had limited, weaker correlation with other variables. Variables most strongly associated with MUAC/WHZ include leptin, LBP, iFABP, C18:1, and naïve plasma IFN-γ. While both LBP and iFABP strongly correlate with measures of MAM, LBP alone is inversely correlated with both naïve and LPS-stimulated cytokine concentrations (Figure 2.4). Also, while leptin level is strongly correlated with measures of MAM, it is not correlated with iFABP or LBP.

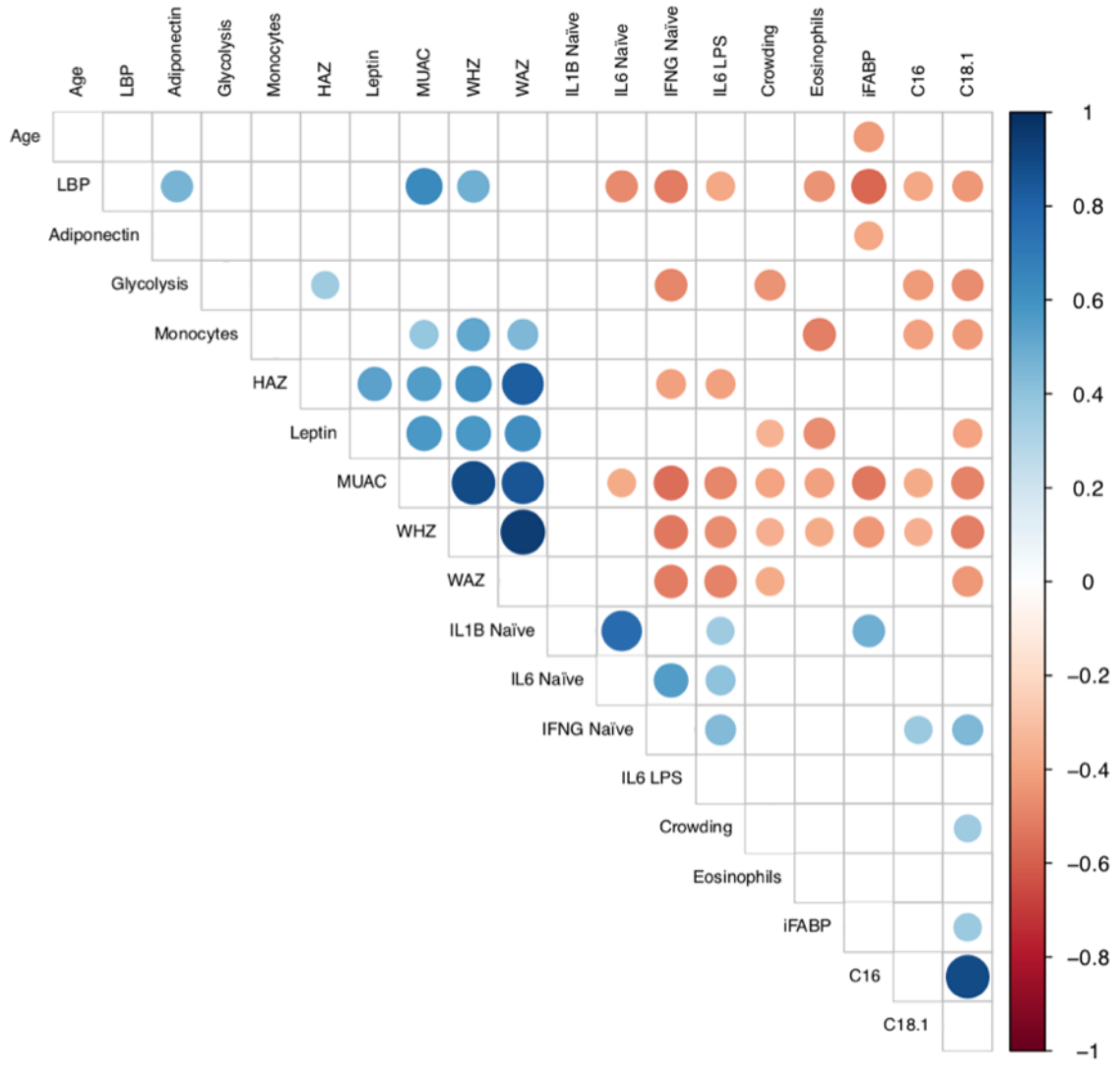


Figure 2.4. Correlations between variables associated with malnutrition.

Correlations that were not significant ($p > 0.05$) are shown as empty boxes. Size of dot correlates to level of significance; a larger dot indicates a smaller p value. Color indicates correlation coefficient as indicated by the range given on the side of the graphic. Variables are grouped by hierarchical clustering. Glycolysis is defined as the ratio of the concentrations of specific amino acids or the concentration of hexoses (Ala+ Gly +Ser)/Hexose.

Saturated Long Chain Fatty Acids Induce Inflammatory Cytokine Expression in PBMCs

Children with MAM have alterations in metabolites and metabolic processes that indicate impaired long chain fatty acid (LCFA) oxidation (Figure 2.1).

Because previous studies demonstrated that saturated medium and long chain fatty acids induced inflammatory cytokine production by human and mouse macrophage cell lines

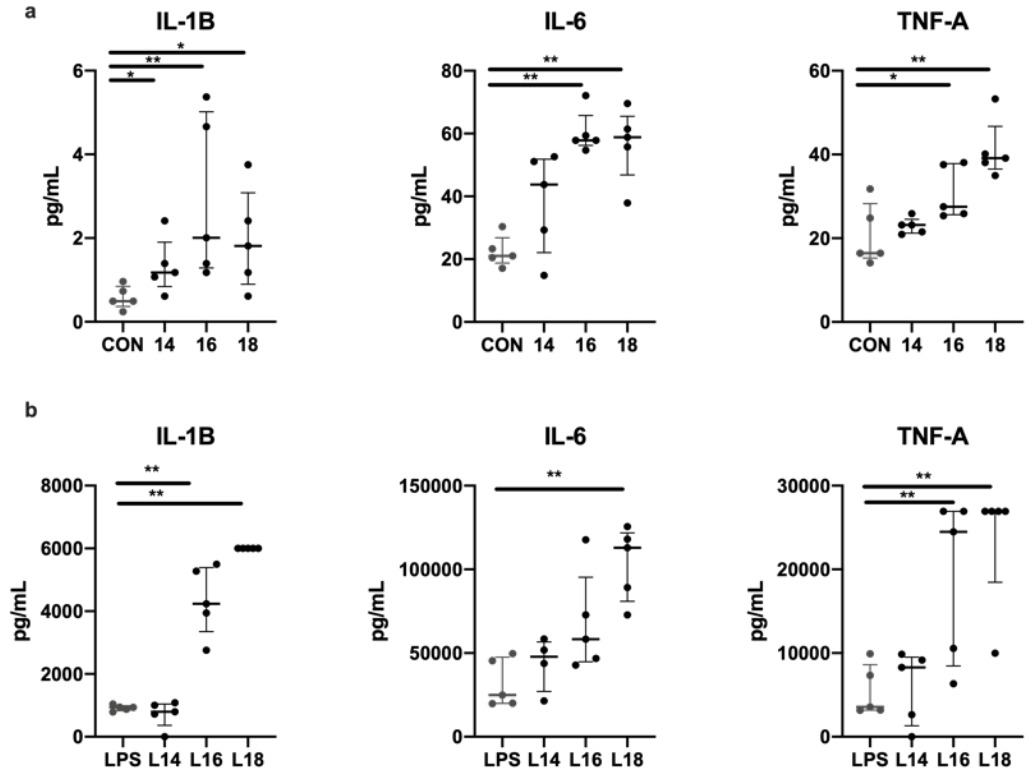


Figure 2.5 Cytokine production in human PBMCs upon stimulation with free fatty acids and/or LPS.

PBMCs were isolated from a healthy adult volunteer and stimulated with an albumin-bound saturated long chain fatty acid or a vehicle control (**a**). Some cells were also treated with 10 ng/mL LPS (b). After 24 hours, cytokines were measured in supernatant via Bio-Plex. Data representative of two experiments. 14 = myristic acid, 16 = palmitic acid, 18 = stearic acid, L = LPS. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

[265,266], we reasoned that the elevated plasma LCFAs in children with MAM could contribute to the increased basal and LPS-induced inflammatory cytokine production. To test this, we measured inflammatory cytokine production in response to myristic acid (C14), palmitic acid (C16), and stearic acid (C18), with and without simultaneous LPS exposure, in PBMCs from a healthy adult volunteer (Figure 2.5). We observed significant increases in TNF-α, IL-6, and IL-1β production in cells treated with C16 and C18 compared to controls and a trend in cells treated with C14. We also observed a synergistic effect in which addition of C16 or C18 greatly increased cytokine expression in LPS-stimulated cells. Thus, increased concentrations of plasma free fatty acids, especially in the presence of LPS, can promote inflammatory cytokine production by leukocytes.

DISCUSSION

In a cohort of children with MAM from an urban slum in Nairobi, Kenya, we evaluated environmental, sociodemographic, physiological and immunological variables with the intent to generate a preliminary model from which new hypotheses could be generated and tested to help understand the pathogenesis of MAM and identify potential correlates of disease and recovery. Our data suggest that low socioeconomic status and environmental exposure to non-purified (likely contaminated) water are associated with MAM. Children with MAM had markers of intestinal damage and metabolic changes that promote systemic inflammation and a heightened whole blood inflammatory response to bacterial LPS.

Comparison of children with MAM to healthy controls from the same urban slum environment demonstrated an association of MAM with measures of reduced wealth, including income instability, fewer appliances owned and increased household crowding. The association of these traits with MAM supports the idea that the psychological and physiological stresses of economic instability in a household have a detrimental impact on child health [28,267].

Water source and water treatment were the strongest determinants of MAM among environmental variables. Water quality has been linked to stunting, environmental enteric dysfunction (EED), and wasting in cohorts around the world [57,58,68]. While we did not observe ongoing intestinal infection in any of the children in the subset used for this study, repeated exposure to enteric pathogens over time (perhaps through contaminated water) may result in persistent intestinal damage and weight faltering [65]. There also may be non-pathogen contaminants in purchased water versus piped water that impact intestinal health and development of malnutrition. Regardless, these data indicate that improving water quality may reduce malnutrition rates in this population. In support of this, a previous study found that use of water storage containers with fitted lids was a strong predictor of sustained recovery after treatment of MAM [268].

While we did not observe a direct association between water source and signs of intestinal damage (iFABP and citrulline concentrations), we did observe strong associations between MUAC and iFABP. IFABP is a marker of recent intestinal damage as it enters the circulation when the tips of villi in the small intestine are damaged [269]. Citrulline is produced by healthy enterocytes in the small intestine, and low concentrations correlate with several acute and chronic intestinal pathologies [270]. Together, these markers suggest that children with MAM experience intestinal damage that could contribute to poor nutrient absorption and other metabolic changes. Plasma iFABP levels in this cohort were inversely correlated with the levels of adiponectin. Adiponectin is a protein that modulates glucose regulation and fatty acid oxidation [271], processes that appear to be impaired in this cohort.

Intestinal damage in children with MAM would be expected to lead to decreased intestinal barrier function and increased translocation of bacteria and bacterial products into circulation. However, we saw no differences in concentration of proteins commonly used as proxies for systemic endotoxin exposure (Endocab IgM or sCD14) between healthy children and children with MAM. We did not directly measure bacterial LPS, bacterial

DNA, or other bacterial products in the blood, so we cannot conclusively assert that there was no difference in endotoxin exposure between groups. Reduced protein production by the liver may cause reduced levels of these proteins, or the level of LPS leakage may be so low as to not trigger IgM or sCD14 production. However, the elevated levels of IFN-γ, IL-6, and IL-1β observed in the naïve plasma of children with MAM suggest that some stimulus is triggering chronic, low level inflammation.

LPS binding protein (LBP) was strikingly lower in the MAM group compared to controls. LBP is produced by hepatocytes and intestinal cells [272], so dysfunction of either may drive the low LBP concentrations observed in children with MAM. LBP regulates the cellular response to LPS in a concentration-dependent manner. At high concentrations, LBP either binds LPS to form large aggregates or transfers LPS to plasma lipoproteins, in both instances inhibiting inflammatory response [272,273]. However, at low concentrations LBP transfers LPS to CD14, amplifying the expression of inflammatory cytokines such as IL-1β, TNF-α, and IL-6 [272]. We observed correlations between levels of LBP and levels of IL6 in naïve and LPS-stimulated plasma in our cohort. Thus, low LBP may be a key factor in the increased baseline inflammation and heightened inflammatory response to LPS observed in children with MAM. Further, elevated baseline levels of IFN-γ (such as those observed in children with MAM in this cohort) can sensitize cells to stimuli such as endotoxin [264]. This may explain the correlation we see between naïve IFN-γ levels and LPS-induced IL-6 response and contribute to the overall pattern of systemic inflammation observed in children with MAM.

Other indicators of changes in liver function and metabolism have also been linked with both intestinal permeability and systemic inflammation. Children with MAM in our cohort exhibited elevated concentrations of acylcarnitines C16 and C18:1. This suggests impaired import of long-chain fatty acids into the mitochondria for fatty acid oxidation. Incomplete long-chain fatty acid beta-oxidation is linked to insulin resistance [274]. Elevated levels of these acylcarnitines have also been associated with increased gut

permeability and reduced citrulline levels in children with EED [275]. Long-chain acylcarnitines and saturated LCFAs can also induce inflammatory cytokine production in a variety of cell types [274,276,277]. Our data from *ex vivo* stimulated PBMCs from a healthy donor suggests that elevated circulating saturated LCFAs in children with MAM can increase baseline inflammation and, more importantly, dramatically increase response of these cells to LPS. There is little information on SFA proportion in children with MAM. One study found SFA to be elevated in children with MAM [278], though the proportion of SFA was decreased in children with SAM [279,280]. In the cohort from this study, children with MAM had a higher ratio of SFA:PUFA than healthy controls (data not shown). Thus, it is feasible that buildup of saturated LCFAs and acylcarnitines in circulation due to deficits in LCFA oxidation may contribute to the increased inflammatory response observed in children with MAM.

One aim of this study was to identify new markers that could be used as potential correlates of malnutrition or immunological/metabolic health over the course of recovery from malnutrition. LBP and iFABP warrant further investigation as potential tools to track non-weight recovery from malnutrition. Their ties to liver function and intestinal health as well as their strong correlation with anthropometric measures suggest they may be key indicators or mediators of the progression of malnutrition. Future studies could examine change in LBP and iFABP over course of recovery from MAM and up to a year after MAM to see if either of these two markers can predict relapse or sustained recovery. To our knowledge, neither marker has been so strongly associated with MAM in previous studies. iFABP has been negatively associated with WAZ in a study of children with high rates of stunting and moderate to severe wasting [281]. A study of children with SAM and persistent diarrhea found correlations between iFABP and MUAC and LBP and markers of intestinal damage [282].

One difficulty in comparing results of similar cohort studies of MAM is the use of different anthropometric measures of malnutrition to define the study population. MUAC,

WHZ, and WAZ are frequently used to diagnose acute malnutrition but they regularly define different groups within the same population [8]. In our study, MUAC and WAZ were the two most strongly associated anthropometric measures, but WAZ had weaker associations with other variables associated with MUAC. In comparison, MUAC and WHZ were also closely correlated in this cohort and had very similar profiles of correlation with nearly all other variables. However, only three of the sixteen children diagnosed with MAM by MUAC would have been diagnosed with MAM by WHZ. An additional child was eligible to be diagnosed with SAM by WHZ. This large discrepancy in diagnosis between measures means that there are likely children in the community that would not be diagnosed by WHZ but already exhibit some of the signs of MAM such as intestinal damage, metabolic changes, and low-level inflammation. Though the use of MUAC versus WHZ in screening in the community is under debate, this study demonstrates that children with low MUAC but normal WHZ should still be treated for MAM in hopes of preventing any further intestinal, metabolic, or immunological damage.

This study has several limitations. Due to the cross-sectional nature of the data, we cannot predict direction of certain associations. For example, intestinal damage as measured by iFABP and citrulline may be both a cause and a result of malnutrition. Also, the small sample size in this study limits the number of variables we can include in regression modeling, limiting us to mainly univariate analysis. The small sample size also means that results are exploratory and are to be used for hypothesis generation. Strengths of this study include breadth of data gathered on subjects, the fact that subjects all come from the same relatively small geographic area, and our success excluding children with acute or chronic infections, limiting immunological confounders.

In conclusion, this study provides a snapshot of metabolic, immunological, and intestinal differences between children with and without MAM in urban Kenya. This study adds to the literature on inflammatory cytokine expression in children with malnutrition and provides novel data on inflammatory response to LPS and other potential markers of

health such as LBP and iFABP. Data from this study should be used to inform future investigation into the pathogenesis of malnutrition in children as well as studies of the process of recovery from malnutrition.

INTESTINAL MICROBIOTA AND INFLAMMATORY RESPONSE IN A MOUSE MODEL OF MAM

Chapter 3. A Proinflammatory Intestinal Microbiota Promotes an Exaggerated Systemic Inflammatory Response in a Mouse Model of Moderate Acute Malnutrition

ABSTRACT

Acute malnutrition, or wasting, is implicated in over half of all deaths in children under five and increases risk of infectious disease. Studies in humans and preclinical models have demonstrated that malnutrition is linked to an immature intestinal microbiota characterized by increased prevalence of Enterobacteriaceae. This immature microbiota can cause intestinal damage, poor nutrient harvest, and increased susceptibility to enteropathogens. Observational studies in humans have also observed heightened systemic inflammation and signs of increased systemic endotoxin exposure in children with moderate acute malnutrition. However, few studies have attempted to uncover the mechanisms linking intestinal dysbiosis to the systemic inflammatory state observed in children with MAM. Understanding these mechanisms would provide insight on how to better target the microbiota to ensure not only nutritional but immunological recovery. Here we use a mouse model of MAM to investigate baseline inflammatory state and inflammatory response. We also use selective antibiotics to investigate the role of specific intestinal bacterial groups on systemic inflammation and weight loss. We found that moderately malnourished mice exhibit increased inflammation at baseline and a heightened inflammatory response to stimulus that can lead to even further weight loss. This

inflammatory phenotype and weight loss can be ameliorated in part by administration of gut-specific antibiotics that are active against Gammaproteobacteria.

INTRODUCTION

Acute malnutrition affects 50.5 million children under the age of five worldwide [254]. Children with severe acute malnutrition (SAM) are nine times more likely than healthy children to die, and those with moderate acute malnutrition (MAM) are three times more likely [16]. Much of this increased risk of death is due to increased risk of infectious disease, which is implicated in both the development and perpetuation of malnutrition [18]. Despite the existence of well-established protocols to treat severe acute malnutrition (SAM), relapse occurs in up to 37% of treated children [283] and it is unclear why some children response well to treatment while others do not [284]. WASH and nutritional interventions have had underwhelming impacts on malnutrition rates at the current level of implementation, suggesting that additional contributors to growth faltering are not understood and remain untargeted [284-286]. Many descriptive studies of immunological deficits in children with acute malnutrition exist, but little is understood about the mechanistic links between these systemic deficits and malnutrition [18].

Intestinal dysbiosis is both a cause and consequence of malnutrition. Studies of children with SAM and MAM show that malnourished children have "immature" intestinal microbiotas that do not develop along normal timelines [166,171]. The immature microbiota is characterized by low diversity, increased prevalence of aerotolerant species from Enterobacteriaceae [166,168,172], and decreased prevalence of beneficial commensal species [86]. This stunted microbiota hampers energy harvest and allows enteropathogens to flourish, driving impaired barrier function and intestinal inflammation [168,171]. Transplantation of fecal microbiotas from children with SAM into gnotobiotic mice demonstrated that the combination of a malnourished diet and malnourished microbiota can cause weight loss, intestinal permeability, and intestinal damage [95]. A study of

bacterial species targeted by IgA in children with SAM identified members of Enterobacteriaceae as the main drivers of weight loss and disrupted intestinal barrier function [97]. Preclinical studies have shown that the contribution of a nutrient-deficient diet and altered intestinal microbiota to growth faltering is amplified when the host is repeatedly exposed to an intestinal pathogen [86,98].

The inflammatory response is especially energetically costly for malnourished children and diverts already scarce nutrients to acute phase and immune protein production [255]. Observational studies in children with SAM have linked malnutrition, intestinal damage, and elevated circulating LPS [158]. However, few studies have attempted to identify any mechanisms underlying the effects of intestinal bacteria on systemic inflammation in malnutrition. One study of complicated MAM found that though intestinal inflammation, systemic inflammation, and low SCFA production were correlated with mortality, this was not mediated by presence of intestinal pathogens. The authors suggest that pathobionts or loss of SCFA-producing bacteria drive the observed negative outcomes [77]. Other studies have identified bacterial translocation and chronic inflammation as outcomes of stunting and environmental enteric dysfunction (EED) [287]. There are parallels between malnutrition-driven dysbiosis and the dysbiosis observed in other disease states such as colitis, metabolic syndrome, and EED [153]. The link between dysbiosis and systemic inflammation has been better characterized in these disorders and may suggest potential mechanisms for the increased systemic inflammation observed in MAM. For example, Type 2 diabetes is characterized by an increased proportion of intestinal gram-negative bacteria, leading to leakage of LPS and endotoxemia [288,289]. Further, all bacteria that correlated with IL-6 expression belonged to Proteobacteria [290]. Thus, we aim to investigate pathways from these related disease profiles in our effort to identify MAM-related systemic inflammation and how it relates to translocation, intestinal immunity, and microbiota.

In this study, we used a mouse model of childhood protein energy malnutrition with selected micronutrient deficiency to investigate the links between systemic inflammatory response and intestinal microbiota. We used selective, nonabsorbable antibiotics to probe the effects of depletion of different bacterial groups on inflammation and weight loss. We found that MAM produces a heightened inflammatory response to bacterial LPS that may drive poor outcomes such as growth faltering. This heightened response may be a result of increased systemic exposure to endotoxin from Gammaproteobacteria, which are elevated in the cecal contents and MLN of malnourished mice. Depletion of this group by COL treatment reduced weight loss.

MATERIALS AND METHODS

Mouse Diet

Weanling female BALB/C mice were obtained from Harlan Laboratories. Mice were grouped in cages of 5 and had free access to water. Mice were weaned to either a standard mouse chow (Teklad diet number 99103) or an isocaloric but protein, iron, and zinc deficient malnourished diet (Teklad diet number 99075) for 28 days. Mice were pair-fed every 48-72 hrs and weighed every 7 days. Malnourished mice received 90% volume by weight of food that well-nourished mice did. This was calculated by determining g/mouse/day for the previous feeding period for well-nourished mice and then determining the necessary amount per cage for MN mice.

Intradermal Delivery of Inflammatory Stimulus

Well-nourished and malnourished mice (5 mice per group) were injected intradermally in the skin of the dorsal foot with 20 μL of either LPS (10 μg) (*Escherichia coli*, Sigma-Aldrich, L4391-1MG), LTA (50 μg) (*Staphylococcus aureus*, Sigma-Aldrich, L2515-5MG), or PBS (control). Mice were sacrificed 24 hours post-injection via cervical dislocation while under isofluorane anesthesia.

Tissue Expression of Cytokines.

Skin at the site of injection, liver, and ilea were removed and stored in RNAlater Stabilization Solution (Thermofisher) at -80°C until RNA extraction and quantification. Samples were thawed on ice and thirty mg or less of each sample (skin, ileum, and/or liver) was removed from RNAlater, homogenized in lysis buffer (Qiagen) + B-mercaptoethanol (Sigma) via either tissue homogenizer (IKA Utra-Turrax T18 Homogenizer) or bead mill (VWR 4-Place Mini Bead Mill Homogenizer), and spun down to pellet remaining tissue. RNA was extracted from the supernatant (RNeasy, Qiagen) and treated with DNAse (Turbo DNAse, Ambion). All RNA samples were quantified using a Thermo Scientific NanoDrop™ Spectrophotometer and stored at -80°C. cDNA was generated from each sample using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) (Veriti 96 Well Thermal Cycler, Applied Biosystems). Cytokine mRNA was quantified using 20-40 ng cDNA, SYBR Green Master Mix (ThermoFisher), and primers (see Supplemental Table 3.1) on a Viia 7 Real-Time PCR System (Applied Biosystems). CT values were used to calculate fold change in comparison to the mean of WN naïve samples.

Peritoneal Macrophage Isolation and Stimulation

Peritoneal cells were isolated from WN and MN mice in approximately 5 mL chilled RPMI with 10% HIFBS, 50 μM B-mercaptoethanol (Sigma). Cells were pooled into WN and MN groups and spun down at 300g for 7 minutes at 4°C before resuspension in 5 mL 10% RPMI. Viable cells were counted adjusted to 0.5*10^6 / mL. The cell suspensions were plated in 1 ml aliquots in a 24 well plate and allowed to adhere for 2 hours at 37°C and 5% CO_2. Cells were washed twice with 2% RPMI to remove non-adherent cells. Cells were then stimulated with either 20 μg/mL LTA, 10 ng/mL LPS, or 2% RPMI as control. There were 4 replicates per condition. After 4 hours incubation at 37°C and 5% CO_2, 2 mL of supernatants were collected and stored at -80°C.

TNF-A ELISA

Supernatants from macrophage stimulation were thawed on ice and centrifuged at full speed for 10 minutes to pellet any remaining cells. Samples were diluted 1:1 in Standard Diluent Buffer (Invitrogen) prior to cytokine level measurement via ELISA (Invitrogen). Plate was read on a FLUOstar Omega plate reader (BMG Laboratories) and OMEGA software (BMG Laboratories) was used to generate a four-parameter logistic curve to calculate protein concentrations.

Quantification of Bacterial Burden in Mouse Tissue

Spleen, liver, and mesenteric lymph nodes (MLN) were used to determine bacterial burden. The liver was cut into thirds and the anterior portion of the middle section was collected. The spleen was cut in half and the anterior half was collected. Spleen (10-35 mg), liver (40-90 mg), and MLN (3-12 mg) samples were stored in a 10x volume of PBS (ex. 50 mg tissue, 500 μl PBS) in a 2 ml Eppendorf tube. Samples were homogenized in the collection tube with a rubber pestle. Each sample was serially diluted out to 10^-6 in PBS, generating a range of tissue concentrations from 10 mg/μl to 10 ng/μl. 50 μl aliquots of each dilution were plated on Brain Heart Infusion (BHI) Agar (Sigma-Aldrich) plates and incubated aerobically at 37°C. Plates were incubated 3 days and colonies counted on both days 2 and 3. Colony counts and dilution values were used to calculate CFU/g for each sample.

Intestinal Permeability

WN and MN mice were fasted overnight and underwent oral gavage with a mixed suspension of 100 mg/mL of 4 kDA FITC-dextran (46944, Sigma) and 100 mg/mL of 70 kDA rhodamine B isothiocyanate-dextran (R9379, Sigma) in PBS. Mice were given 6 μl of suspension per gram of body weight, corresponding to 600 mg/kg of each dextran. After one hour of transit time to allow the suspension to reach the ileum [291], blood was collected

via cardiac puncture under isofluorane anesthesia. Blood was centrifuged at 2000g for 10 minutes at 4°C and plasma collected. Centrifugation was repeated once and plasma was diluted 1:1 in PBS. Diluted serum and mixed dextran standards were plated on a 96 well plate in duplicate and read on a FLUOstar Omega plate reader (BMG Laboratories). An excitation of 485 nm and emission of 520 nm was used to measure FITC-Dextran concentration and an excitation of 544 nm and emission of 590 nm was used to measure rhodamine B isothiocyanate-dextran concentration. OMEGA software (BMG Laboratories) was used to generate a linear regression fit standard curve.

Peyer's Patch Collection and Analysis

Ilea were collected from mice on day 28 of diet as described above. Ilea were flushed with ice cold PBS + 2% Penicillin/Streptomycin (P/S) and Peyer's patches (PPs) removed with curved forceps and scissors. For qRT-PCR, PPs were stored in RNALater at -80°C prior to RNA extraction and quantification as described above. For flow cytometry, PPs were placed in 1 mL ice cold RPMI + 2% P/S. Collected PPs were mechanically dissociated and filtered through a 40 μm cell strainer three times in ice cold RPMI + 2% P/S. Cell suspension was washed three times and incubated at 37°C and 5% CO2 before stimulation with 100 ng/mL LPS for 12 hours. Golgi block was added at 0.66 μl/mL for the final 6 hours of the incubation period. After stimulation, cells were washed and resuspended in staining buffer. Cells were stained with surface and intracellular antibodies listed in Supplemental Table 3.2. After staining, cells were analyzed on a BD LSRFortessa cell analyzer and data analyzed using FlowJo (v10.6.1).

Initial Microbiota Collection and Analysis

Cecal contents and mesenteric lymph nodes were collected from WN and MN mice and transported on dry ice to the Alkek Center for Metagenomics and Microbiome Research at Baylor College of Medicine for 16s rRNA sequencing and analysis. DNA was

extracted from cecal contents with the PowerSoil DNA Isolation kit (MoBio) and from mesenteric lymph nodes with the UltraClean Tissue and Cells DNA Isolation kit (MoBio). DNA underwent 16Sv4 PCR [292] followed by MiSeq 2x250 bp sequencing and the CMMR-generated 16S analysis pipeline.

Antibiotic Treatment

Mice were put on either a well-nourished or malnourished diet as previously described. After 14 days on the diet antibiotics were administered in their drinking water for the final 14 days of the diet. Mice were given either 0.7 mg/mL of vancomycin hydrochloride (*Streptomyces orientalis*, Sigma Aldrich, V8138), 7,500 U/mL colistin sulfate salt (Sigma Aldrich, C4461), or normal water as control. Water bottles were replaced every 3-4 days. Actual dosages calculated from average water consumption were approximately 5 mg/day of vancomycin and 33,750 U/day colistin. After 14 days on antibiotics, mice were euthanized and skin, blood, and cecal matter were collected and stored at -80°C. Liver, spleen, and mesenteric lymph nodes were also collected and used to quantify bacterial burden as described above. mRNA was isolated from skin samples and quantified as listed previously.

To determine the microbiota composition in the antibiotic-treated mice, DNA was isolated from cecal matter using the QIAamp DNA Stool Mini Kit (QIAGEN) according to manufacturer's protocol and shipped on dry ice to LCSciences (Houston, Texas, USA) for 16S rDNA V3+V4 sequencing and analysis. Bacterial DNA was amplified with primers targeted to the V3 and V4 regions of 16S rDNA. Sequencing adaptors and barcodes were added in further amplification steps and the prepared library was sequenced on the MiSeq platform. Resultant paired-end reads were merged into tags and then grouped into clusters that represent a single operational taxonomic unit (OTU) (minimum 97% sequence similarity). Analysis of OTUs were conducted including measures of alpha diversity, beta

diversity, and taxonomy annotation. Databases for taxonomy analysis included RDP (version date 2016.9.30) and NT-16S (version date 2016.10.29).

Systemic LPS Challenge

Mice were weaned to either a well-nourished or malnourished diet as previously described. After 28 days on the diet, mice were injected intraperitoneally with 4 mg/kg of LPS (*Escherichia coli* O55:B5, Sigma-Aldrich, L2880) in PBS. Mice were given food and water ad libitum and monitored hourly. At six hours post injection, mice were weighed. Mice were weighed again at 24 hours post injection and euthanized via cardiac puncture under isofluorane anesthesia. Blood was taken with a heparinized needle and a liver samples collected for cytokine expression as described above.

Statistics

Unless otherwise specified, groups were compared via Mann-Whitney T test. Outliers were detected via ROUT method with coefficient Q 1% [293] and removed from analysis where appropriate. All analyses were conducted using GraphPad Prism 8 for Mac OS X (Graphpad Software, San Diego California USA).

RESULTS

Malnourished mice exhibit an increased inflammatory response compared to well-nourished mice.

To explore the effects of MAM on inflammation, we used a mouse model of macro-nutrient (protein) and micro-nutrient (iron and zinc) deficiency that mimics the diet that often leads to childhood MAM [294,295]. Experimental diets were otherwise identical except that they were made to be isocaloric by increasing the carbohydrates in the malnourished diet to replace the calories lost in the low protein chow. The mice were pair-fed and the MN mice received 10% less total amount of chow than WN mice, generating a modest energy deficit.

At time of randomized weaning to the experimental diets, mice in the WN and MN group were indistinguishable by weight (mean 14.0g and 13.5g, respectively) (Supplemental Figure 3.1). Mice on the WN diet reached their adult weight (mean=19.35 g) by day 28 of the diet and gained 37.95% of their starting bodyweight. MN mice at day 28 averaged 10.58 g and had lost 21% of their starting bodyweight.

In a previous study, MAM was associated with chronic, low grade inflammation and altered response to infection in humans (see Chapter 2). To determine the impact of malnutrition on sterile inflammation, we intradermally injected WN and MN mice with PBS in the dorsal skin of both feet. MN mice demonstrated a stronger response to sterile injury as determined by mRNA levels of inflammatory cytokines (Figure 3.1). Twenty-four hours post injection, MN mice demonstrated significantly increased mRNA expression of neutrophil chemoattractants *Cxcl1* and *Cxcl2*, pro-inflammatory *Il1b*, and monocyte chemoattractant *Ccl2* compared to similarly treated WN mice.

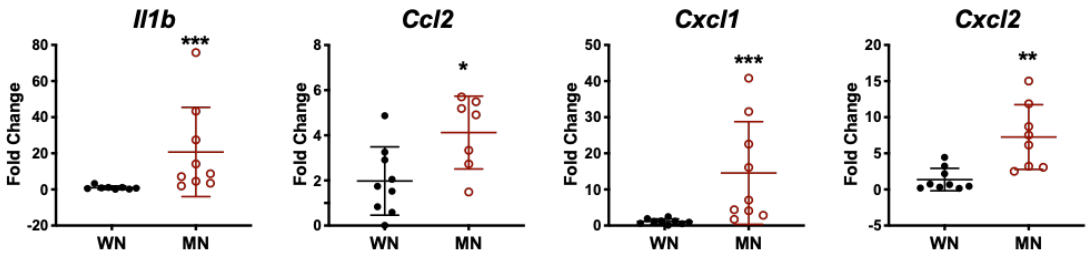


Figure 3.1. Malnourished mice exhibit heightened baseline skin inflammation.

Cytokine mRNA expression in skin from the footpad of WN (n=5) and MN mice (n=5) 24 hours post injection with 20 μL PBS. Values determined via qRT-PCR. WN samples used as baseline to determine fold change. Data is from two experiments. All P values determined by Mann-Whitney U-test (*p<0.05 ** p<0.01 *** p<0.001 ****p<0.0001).

To investigate whether malnutrition impacts the response to an inflammatory stimulus, we injected the skin of MN and WN mice with bacterial lipopolysaccharide (LPS;

from gram-negative bacteria) or lipotechoic acid (LTA; from gram-positive bacteria). Twenty-four hours post injection, mRNA levels of *Il1b*, *Ccxl1*, and *Cxcl2* were significantly upregulated in the skin of MN mice compared to WN controls (Figure 3.2A). The response to LPS was considerably greater than the response to LTA, which showed little difference between WN and MN mice, (Figure 3.2B).

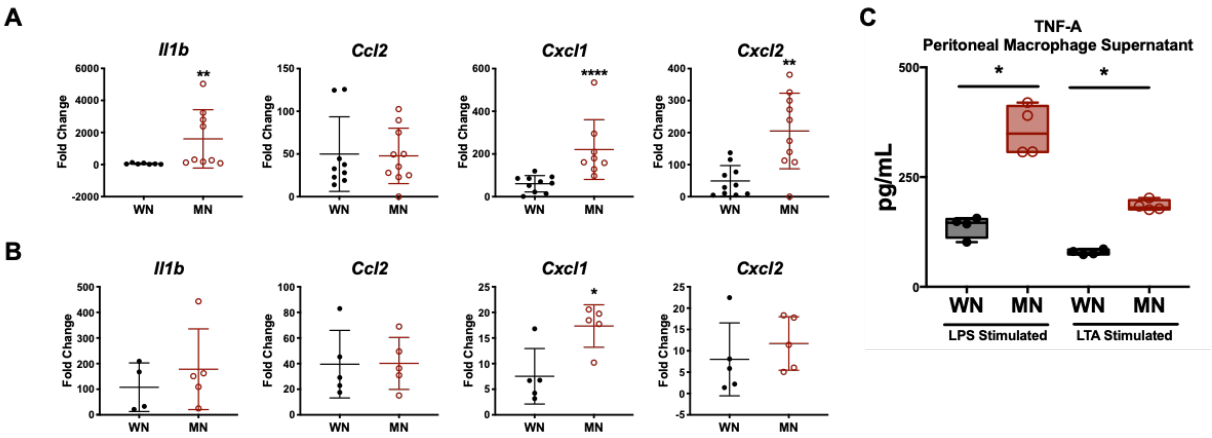


Figure 3.2. Malnourished mice exhibit heightened inflammatory response to bacterial ligands in the skin and circulation.

Cytokine mRNA expression in skin of WN (n=5) and MN mice (n=5) 24 hours post injection with 20 μl of either 500 μg/mL LPS **(A)** or 2,500 μg/mL LTA **(B)**. Values determined via qRT-PCR. WN PBS samples used as baseline to determine fold change. LPS data is from two experiments, LTA data is from one. **(C)** TNF-A protein expression in supernatant of peritoneal cells from WN and MN mice cultured for 4 hours with either 10 ng/mL LPS or 20 μg/mL LTA. All P values determined by Mann-Whitney U-test (*p<0.05 ** p<0.01 *** p<0.001 ****p<0.0001).

To determine if specific immune cell populations in MN mice also had an exaggerated response to bacterial stimuli, we exposed peritoneal macrophages from WN and MN mice to LPS and LTA. Peritoneal macrophages isolated from MN mice and stimulated with LPS produced greater quantities of TNF-α (349 pg/ml) than peritoneal

macrophages isolated from WN mice (149 pg/ml) (Figure 3.2C). When stimulated with LTA, macrophages from MN mice again produced more TNF-α (175 vs 75 pg/ml). TNF-α levels were below the limit of detection in the supernatant of untreated cells. Collectively, these data indicate that malnutrition results in increased inflammatory response to bacterial products or sterile injury.

Malnourished mice experience greater physiological impact from the heightened inflammatory response to systemic LPS challenge.

To explore the effect of malnutrition on the physiological response to an inflammatory stimulus, we injected WN and MN mice intraperitoneally with LPS. After

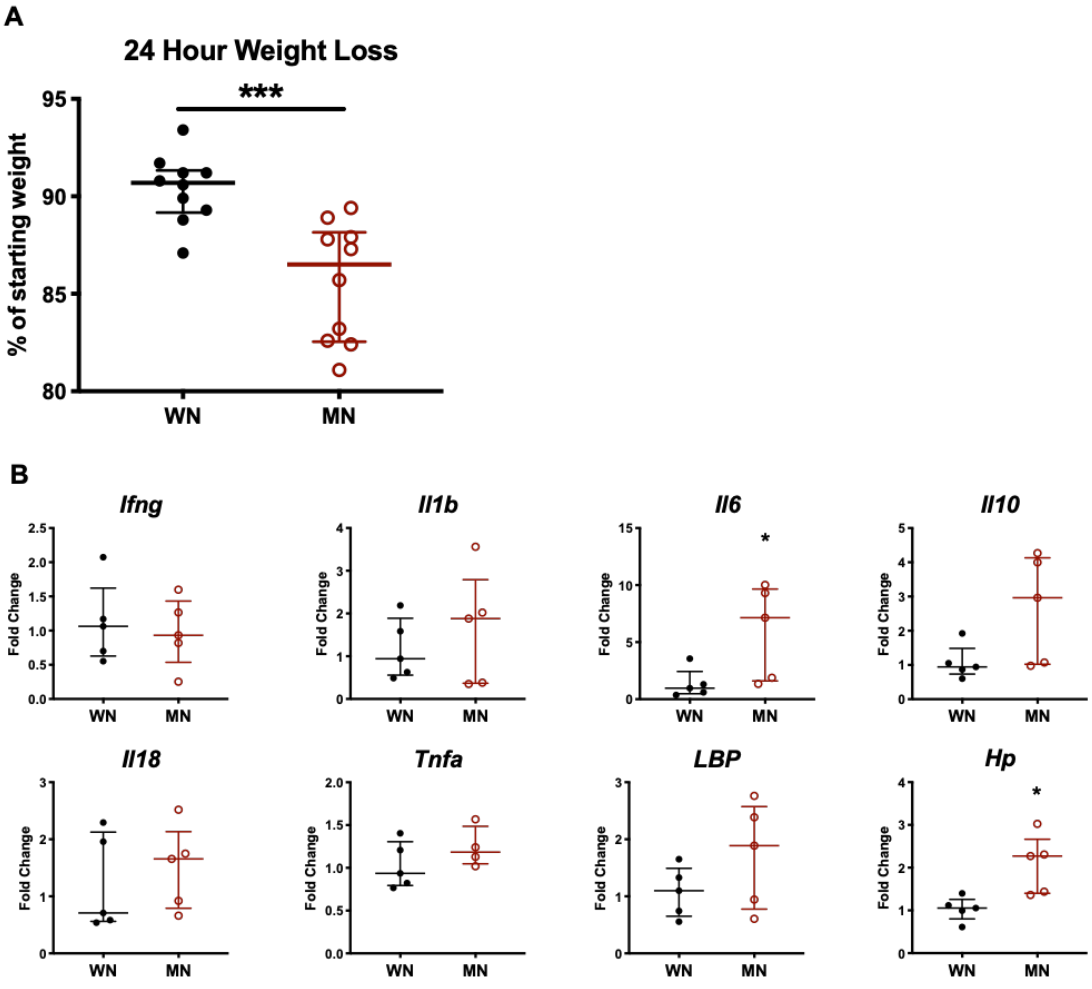


Figure 3.3. Malnourished mice lose more weight than WN mice during LPS challenge.

WN and MN mice were injected intraperitoneally with 4 mg/kg LPS and bled at 6 and 24 hours. Data is from two experiments **(A)** Percentage of starting weight at 24 hours post injection. **(B)** mRNA expression in liver samples at 24 hours post injection. All P values determined by Mann-Whitney U-test (*p<0.05 ** p<0.01 *** p<0.001 ****p<0.0001).

24 hours, MN mice had lost 13.5% of their starting body weight, while WN mice lost 9.3% of their starting weight (p = 0.0007) (Figure 3.3A). Liver samples from MN mice at 24 hours post injection has elevated mRNA levels of *Il6* and the acute phase protein haptoglobin (Figure 3.3B). These data indicate that malnutrition magnifies the systemic inflammatory response and makes the host more vulnerable to weight loss upon exposure to bacterial LPS.

Malnourished mice have evidence of increased bacterial translocation and altered intestinal immunity.

We hypothesized that the heightened baseline inflammation and response to bacterial stimuli observed in MN mice may be a result of exposure to bacteria translocating from the gut. To test this, we examined bacterial burden in the mesenteric lymph nodes, spleens, and livers of WN and MN mice (Figure 3.4). We observed increased numbers of culturable bacteria in the MLN, spleens, and livers of MN mice compared to WN mice. A random sample of aerobic bacterial colonies from both WN and MN mice were identified via mass spectrometry. Only one of 23 colonies isolated was gram-negative (*Eschericia coli*), and the remaining 22 colonies belonged to phyla Firmicutes and included *Staphylococcus sciuri*, *Aerococcus viridans, Enterococcus facaelis,* and *Staphylococcus aereus*. These data demonstrate that malnourished mice experience greater systemic exposure to bacteria, as evidenced by increased bacterial burdens in the spleen, liver, and

lymph nodes. The majority of culturable species collected from these organs are gram-positive members of phyla Firmicutes.

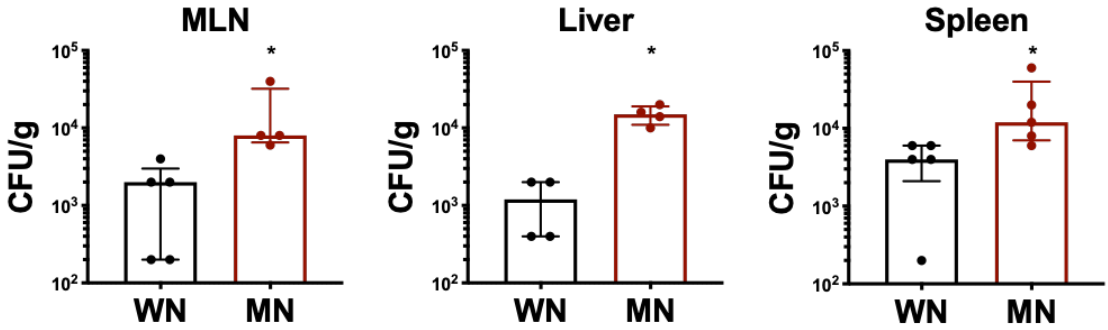


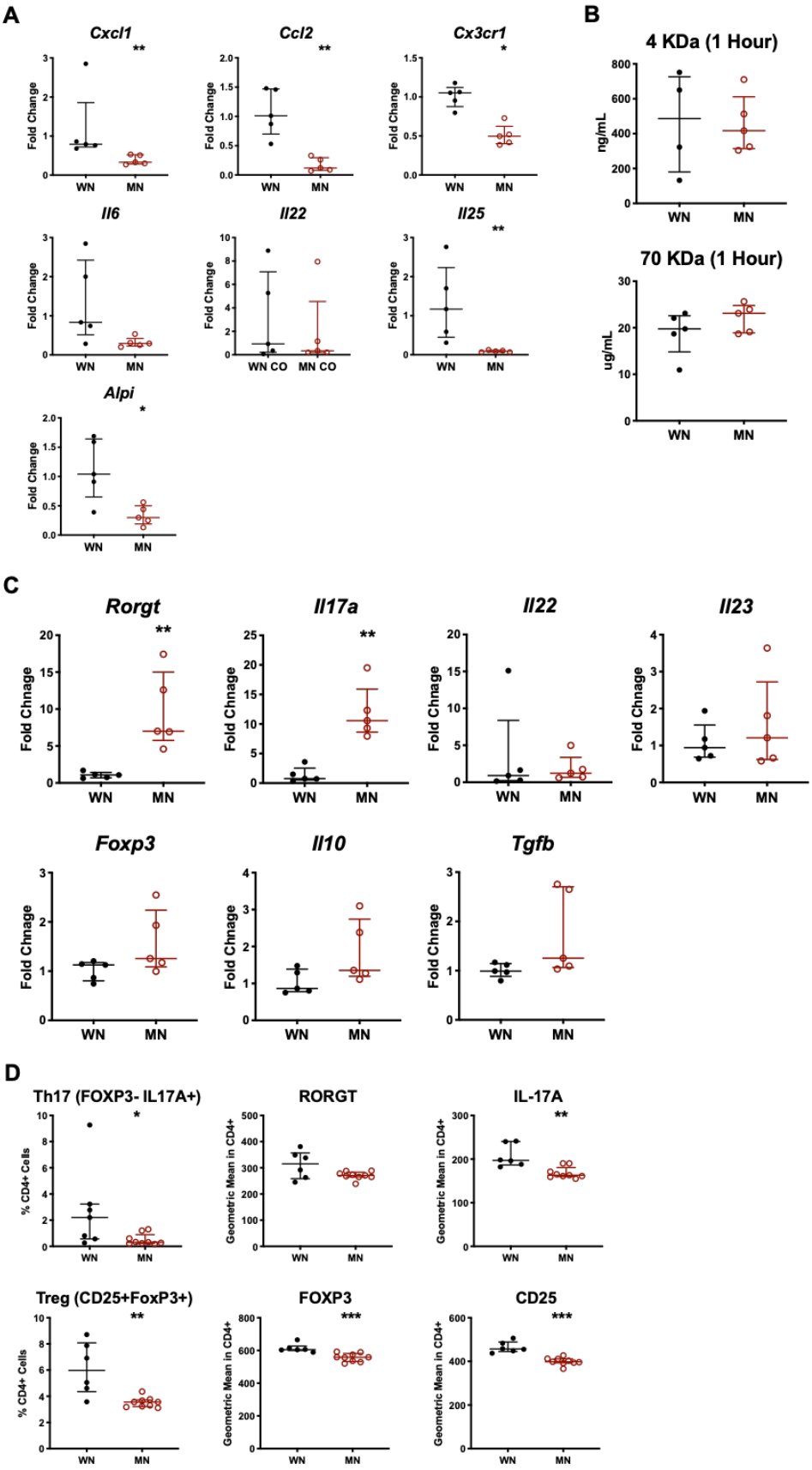
Figure 3.4. Malnutrition promotes bacterial translocation.

Quantification of bacteria in the mesenteric lymph nodes, liver, and spleen of well-nourished and malnourished mice. Live bacteria were quantified by plating tissue homogenates on BHI agar. All P values determined by Mann-Whitney U-test (*p<0.05 ** p<0.01 *** p<0.001 ****p<0.0001).

To determine if this increased extra-intestinal bacterial burden was due to impaired intestinal immunity and barrier function, we examined mRNA expression in the distal third of the small intestine (Figure 3.5A). We observed decreased expression of *Il25*, *Cx3cr1*, *Cxcl1*, *Ccl2*, and *Alpi*. Together, these data suggest decreased immune activation and decreased barrier function in the small intestine of MN mice. However, we observed no differences in paracellular intestinal permeability in WN and MN mice, determined by measuring serum concentration following oral gavage with 4kD FITC-dextran and 70kD rhodamine-dextran (Figure 3.5B).

Peyer's patches (PPs) are the primary site in the small intestine where bacterial antigens and intact bacteria are sampled [296] and an immune response generated. Impaired antibacterial function of PPs can therefore contribute to elevated bacterial translocation [297]. To further investigate immune activation in the small intestine of MN mice, we measured mRNA expression and T cell populations in PPs. Surprisingly, we observed

increased expression of *Rorgt* (Th17 cell transcription factor) and *Il17a* (but not *Il22 or Il23*) in PPs of MN mice. We found no difference in expression of *Foxp3* (Treg cell transcription factor) and T-reg related *Il10* and *Tgfb* in WN and MN mice (Figure 3.5C). This suggests that the cytokine milieu within MN PPs is skewed toward inflammation rather than tolerance. However, flow cytometry of PP cells revealed than MN mice had lower levels of both Treg (CD4+CD25+ FOXP3+) and Th17 (CD4+ FOXP3- IL17A+) cells among CD4+ cells compared to WN mice (Figure 3.5D). Further, intensity of expression of CD25, FOXP3, and IL17A (but not RORGT) was significantly weaker in CD4+ cells from MN mice. These data suggest that CD4+ cells from MN mice may have reduced activation and cytokine production and the heightened *Il17a* production may arise from another source such as Type 3 innate lymphoid cells.


A
Cxcl1
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Il10
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D
Th17 (FOXP3- IL17A+)
RORGT
IL-17A
Treg (CD25+FoxP3+)
FOXP3
CD25
% CD4+ Cells
Geometric Mean in CD4+

Figure 3.5. Malnutrition alters intestinal immune function but not permeability.

(A) mRNA levels in ileal tissue of WN and MN mice. (B) Concentration of 4 KDa FITC-Dextran and 70 KDa Rhodamine-Dextran in blood of WN and MN mice one hour after oral gavage. (C) Cytokine expression in Peyer's patches (D) CD4+ cell proportions in Peyer's patches and expression intensity. All P values determined by Mann-Whitney U-test (*p<0.05 ** p<0.01 *** p<0.001 ****p<0.0001).

Intestinal microbiota from MN mice is characterized by increased proportions of Firmicutes and Proteobacteria and decreased Bacteroidetes.

The increased bacterial translocation and altered intestinal cytokine and chemokine expression observed in MN mice may be due in part to changes in the intestinal microbiota. Therefore, we characterized the bacterial composition of cecal contents and mesenteric lymph nodes in WN and MN mice. In both cecal contents and MLN, MN mice exhibited a decrease in overall proportion of Bacteroidetes and an increase in Proteobacteria and Firmicutes (Figure 3.6A). These changes were significant for cecal contents, but merely a trend in MLN. The cecal microbiota of WN and MN mice showed no difference in alpha diversity (Figure 3.6B) but was distinct in beta diversity (Figure 3.6C). OTUs enriched in the cecal contents of MN mice compared to WN mice include two Proteobacteria OTUs (*Escherichia*/*Shigella*, Desulfovibrio) and seven Firmicutes OTUs (including *Blautia*, *Roseburia*, *Allobaculum*, and *Enterococcus*) (Figure 3.6D). OTUs reduced in the cecal contents of MN mice included four Bacteroidetes (including *Odoribacter* and *Bacteroides*) and five Firmicutes. All significant OTU-level changes in the MLN of MN mice consisted of an increased proportion; there were no significantly decreased OTUs in the MLN of MN mice. Both Proteobacteria OTUs that were increased in proportion in the MN cecal contents were also increased in the MN MLN. All but two of the Firmicutes OTUs that were increased in the cecal contents of MN mice were also increased in the MLN of MN

mice. Thus, WN and MN mice have striking differences in the cecal microbiota composition that are replicated in part in the MLN.

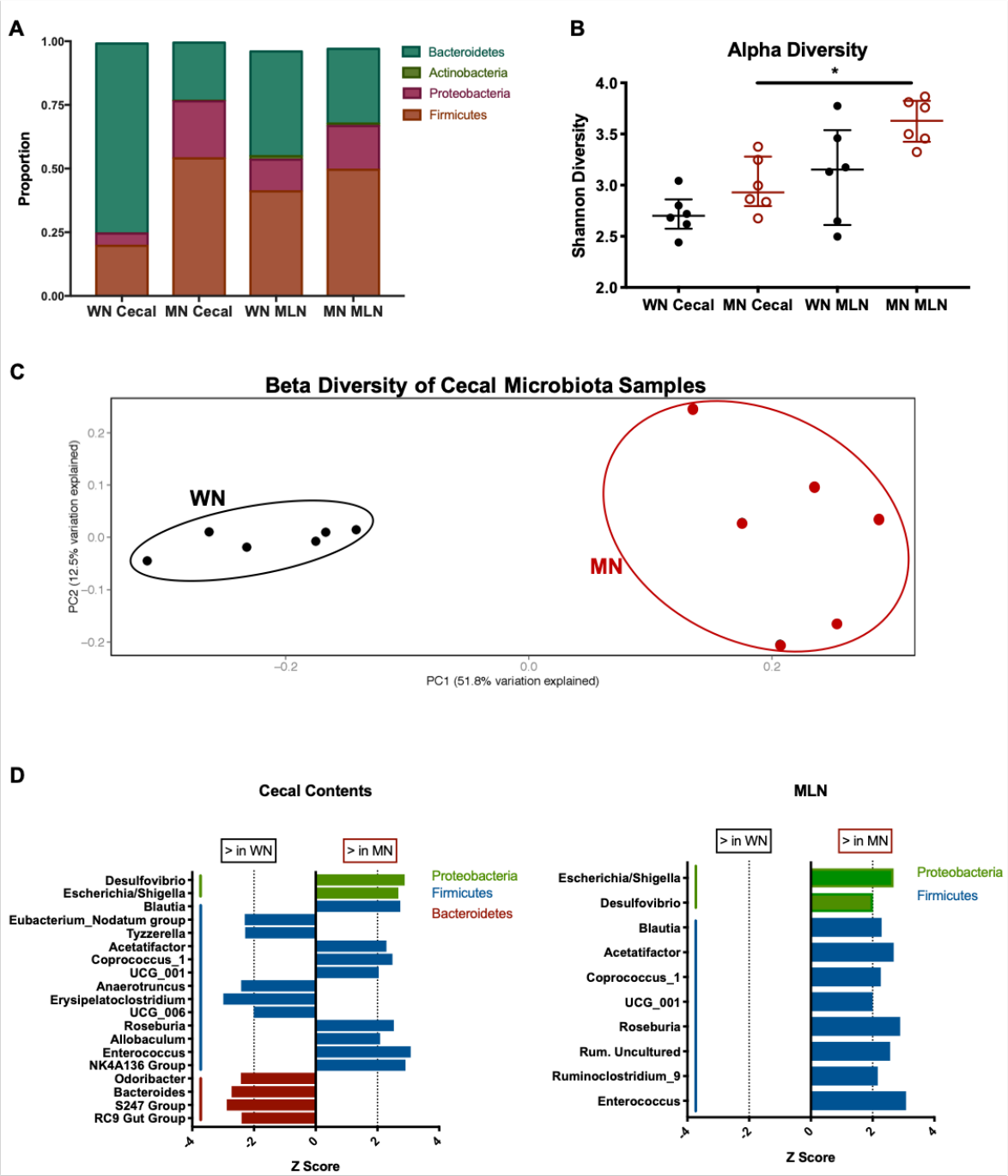


Figure 3.6. Malnutrition increases proportions of Proteobacteria and Firmicutes in cecal and MLN microbiota.

Bacterial 16S rRNA was sequenced from cecal stool and MLN samples from 5 WN and 5 MN mice. **(A)** Average proportions of four most common phyla in cecal stool and MLN samples. **(B)** Alpha diversity for WN and MN cecal stool and MLN samples. Cecal and MLN samples were compared by Mann-Whitney U-test (*$p<0.05$ ** $p<0.01$ *** $p<0.001$ ****$p<0.0001$). (**C**) Beta diversity for WN and MN cecal content microbiota **(D)** Significant ($p < 0.05$, Wilcoxon signed-rank test) genus-level differences between WN and MN cecal stool microbiotas and WN and MN MLN microbiotas. Z score indicates difference in proportion in either the cecal microbiota or MLN microbiota between WN and MN mice.

Oral colistin reduces Gammaproteobacteria and modulates inflammation and weight loss in malnourished mice.

To determine if the changes observed in the cecal microbiota of MN mice were responsible for the increased systemic inflammation and heightened response to bacterial LPS, we selectively depleted specific bacterial groups by oral administration of non-absorbable antibiotics. For the last 14 days of their 28-day diet, MN mice were given vancomycin (VANC), colistin (COL), or no antibiotic in their drinking water (Figure 3.7A). These antibiotics have negligible absorption from oral administration so should have had no direct impact on bacteria outside the gastrointestinal tract. VANC is selectively active against gram-positive bacteria and COL is selectively active against gram-negative bacilli (primarily Gammaproteobacteria). To determine the effects of the oral antibiotics, we determined the microbiota composition of cecal contents of WN and MN mice given no antibiotic, and MN mice given either VANC or COL. COL-treated mice had reduced levels of targeted bacteria, most significantly Gamma and Betaproteobacteria, to < 0.01% (Figure 3.7C). The overall proportion of Proteobacteria was increased in COL-treated mice

compared to MN controls (Figure 3.7B), but this increase was due to increases in Deltaproteobacteria and unclassified Proteobacteria (Figure 3.7C). COL-treated MN mice also had expansion of Bacteroidetes and reduction of Firmicutes in comparison to MN controls. Conversely, the cecal microbiota of VANC-treated mice consisted of over 95% Proteobacteria. Three OTUs accounted for 98% of reads in 4 of 5 VANC mice: Sutterellaceae, Enterobacteriaceae, and *Escherichia* (Figure 3.7D). VANC-treated mice also had an average of 0.02% prevalence of Bacteroidetes and 0.64% Firmicutes compared to 12% and 78.07% in MN mice. Collectively, these data indicate that COL treatment depleted targeted Gamma- and Betaproteobacteria, and VANC treatment depleted targeted

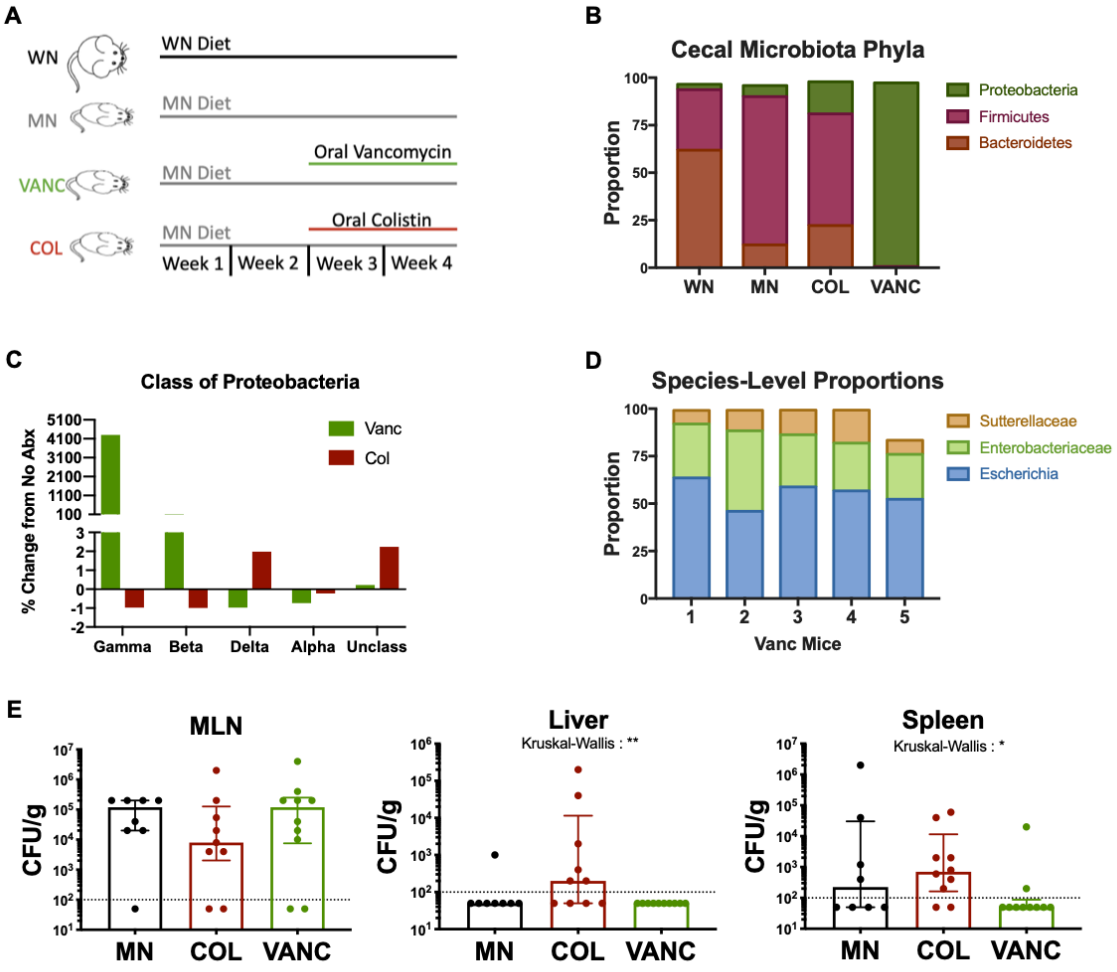


Figure 3.7. Antibiotic treatment alters microbiota and translocation.

Antibiotics selective for gram-negative or gram-positive intestinal bacteria have different effects on bacteria and translocation in malnourished mice. (A) Graphic of experimental design in which MN mice were given either vancomycin or colistin in drinking water over last two weeks of diet. Bacterial16S rDNA from cecal stool was sequenced and analyzed at (B) phyla, (C) class, and (D) species level. (E) Live bacteria were quantified by plating homogenates of mesenteric lymph node, liver, and spleen on BHI agar and statistically compared by Kruskal-Wallis (*$p<0.05$ ** $p<0.01$ *** $p<0.001$ ****$p<0.0001$).

gram-positive intestinal species. Strikingly, depletion of gram-positive bacteria by oral VANC led to an overwhelming expansion of the Gammaproteobacteria.

To determine the impact of antibiotic-mediated change in cecal microbiota composition on bacterial translocation, we quantified culturable bacteria in the MLN, spleen, and liver of antibiotic-treated mice. Consistent with our previous finding that most culturable bacteria translocated to the liver and spleen were gram-positive species, VANC-treated MN mice had significantly reduced levels of culturable bacteria in the spleen and liver compared to control MN mice (Figure 3.7E). Conversely, COL-treated mice exhibited similar or elevated rates of translocation.

To determine the impact of antibiotic-mediated change in cecal microbiota composition on the inflammatory response, we challenged mice with LPS using the skin injection model described previously. COL mice had comparable levels of inflammatory cytokines compared to control MN mice (Figure 3.8). However, VANC-treated MN mice, which showed dramatic expansion of the Gammaproteobacteria, had significantly

increased LPS-induced expression of *Il1b*, *Il6*, *Il17a*, and *Cxcl1* compared to either COL mice and/or MN mice.

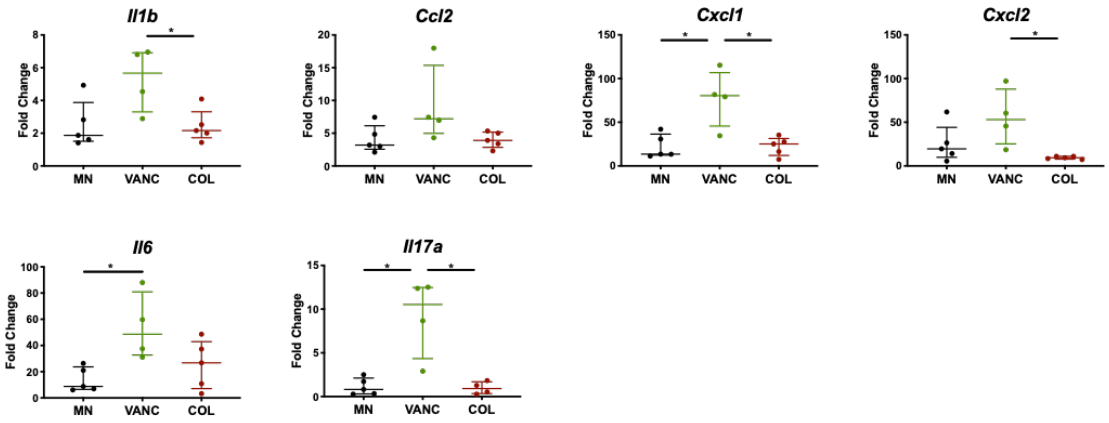


Figure 3.8. Vancomycin enhances response to LPS in skin of MN mice.

Response to footpad LPS injection in malnourished mice with and without antibiotic treatment. Cytokine expression in footpad skin as quantified by qRT-PCR. All P values determined by Mann-Whitney U-test (*p<0.05 ** p<0.01 *** p<0.001 ****p<0.0001).

Lastly, we examined weight change in mice over the two-week course of antibiotics to see how depletion of specific bacterial groups in the intestine impacted weight change. While VANC-treated MN mice, which had expansion of the gut Proteobacteria, exhibited similar weight loss to MN control mice, COL-treated mice, which had depletion of the Gammaproteobacteria, were able to maintain their body weight (Figure 3.9).

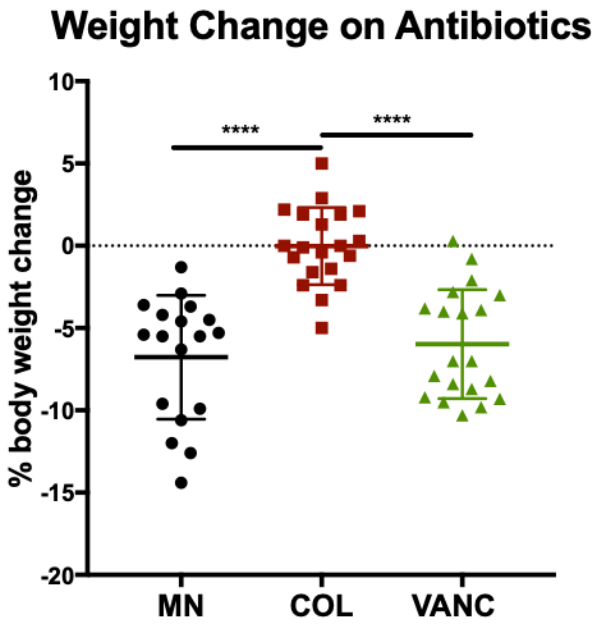


Figure 3.9. Colistin halts weight loss in malnourished mice.

Change in weight in malnourished mice over two weeks with or without antibiotic treatment. P values determined by Mann-Whitney U-test (*p<0.05 ** p<0.01 *** p<0.001 ****p<0.0001).

DISCUSSION

Acute malnutrition increases a child's risk of infection and death from infectious disease [16], but the mechanism(s) that drive this have not been fully defined. A number of descriptive studies document changes in immunity in children with varying degrees of malnutrition [18], but relatively few studies have focused on MAM and the mechanisms underlying these changes. Some human and animal studies have identified dysbiosis and altered gut permeability in MAM, however, the connection between gut microbiota, intestinal barrier function and systemic inflammation has received little attention. In this study, we used weanling mice fed a protein-energy and micronutrient deficient diet to mimic childhood MAM. We found that MAM leads to a heightened inflammatory response to bacterial stimuli. This exaggerated inflammatory response occurs in the setting of increased bacterial translocation from the gut, and reduced expression of cytokines and

chemokines related to intestinal immune defense and barrier function. Increased proportions of Gammaproteobacteria in the intestine and MLN of MN appear to drive the inflammatory phenotype. Administration of non-absorbable antibiotics that reduced (COL) or expanded (VANC) intestinal Gammaproteobacteria modulated the systemic inflammatory response and weight loss.

The hyper-inflammatory phenotype we observed in MN mice is at odds with the majority of data from children with SAM, who typically display a reduced inflammatory response to infection and decreased circulating levels of inflammatory cytokines in absence of infection [18,130]. However, there are also instances in which children with SAM [298] or MAM [299] (see Chapter 2) exhibit increased baseline expression of these cytokines and increased inflammatory cytokine production in response to LPS stimulation. A study in malnourished mice observed that interepithelial lymphocytes could be primed by a consortium of intestinal pathobionts to secrete more inflammatory cytokines after stimulation [98], supporting the notion that immune cells may be primed for a more vigorous response by changes in the intestinal microbiota. Our work here details hyper-responsiveness in the skin and leukocytes of MN mice. These results suggest that in moderate malnutrition, immune cells are primed to have greater production of cytokines. This chronic immune stimulation may contribute to poor outcomes from infection or exacerbate growth faltering due to increased energy cost [287].

The underlying mechanism driving the apparent immune priming is unclear, but is likely due to chronic, low level exposure to endotoxin or other bacterial products. Children with SAM have measurably elevated circulating LPS and evidence of endotoxin tolerance [136], but they also have intestinal architecture disruption that is not common to MAM [158]. There is little evidence for this in children with uncomplicated MAM, but underweight infants (WAZ <-2, >-3) had elevated EndoCAb IgG, a marker of increased exposure to circulating endotoxin, that correlated with increased intestinal permeability [66]. However, a study of slightly older children found no difference in EndoCAb IgG or sCD14 levels

between children with MAM and healthy controls (See Chapter 2). We did not directly measured endotoxin in this mouse study, but the TNF-A response of peritoneal cells to LPS and LTA challenge in this study is suggestive of low-dose endotoxin priming [261] (and possibly cross-priming) [300].

Intestinal damage and altered permeability have been shown in children with SAM and EED. It is likely that similar mechanisms contribute to mild intestinal barrier dysfunction in MAM, but this has not been well studied. Children with MAM may also have EED, intestinal infection, or are taking antibiotics/antiparasitic drugs, making it difficult to isolate the effects of MAM on intestinal permeability. One study of children with uncomplicated MAM found elevated plasma iFABP and reduced citrulline, both of which were correlated with MUAC and indicate intestinal damage (see Chapter 2). Studies in mouse models of MAM have shown a range of mild histopathological changes that are dependent on level of protein restriction [153]. These models also display increased intestinal permeability as measured by FITC-dextran test and altered mRNA expression of tight junction proteins and claudins. We did not observe an increase in intestinal permeability to 4 and 70 kDa dextrans but we did observe signs of depressed intestinal immune function. Transcripts that were downregulated in the malnourished ileum may indicate reduced monocyte and neutrophil recruitment (*Ccl2, Cxcl1*), reduced capacity to modulate gut microbiota and dephosphorylate LPS (*Alpi*) [301-303], and increased bacterial translocation to the MLN (*Cx3cr1*) [304]. Results from the PPs of MN mice also support the idea of dampened intestinal immunity. Despite increased *Il17a* and *Rorgt*, MN mice exhibited reduced proportions of both Treg and Th17 cells among CD4+ cells. Even among CD4+ cells that produce IL17A and IL21, the intensity of expression was reduced in cells from MN mice. Intensity of CD25, which is a marker of T cell activation in addition to a marker of Tregs, was also reduced in MN cells. This suggests that malnutrition reduces activation and cytokine production in T cells, a result that has been observed previously in peripheral T cells from fasted mice [141].

While there was no evidence of increased paracellular intestinal permeability, dampened intestinal immunity may still allow transcellular translocation of bacteria and bacterial products such as LPS. The increased bacterial translocation to the spleen and liver observed in MN mice in this study may also be due to failure of systems in the intestine, MLN, and liver that control and clear bacteria and endotoxin from circulation [264]. In a previous study, the skin-draining LN of malnourished mice exhibited reduced numbers of phagocytic cells, diminished barrier function, and increased dissemination of an intracellular pathogen. [305]. The barrier capacity of mesenteric LNs and defense against bacterial translocation may be similarly compromised. The liver is critical for removal of bacteria and LPS from circulation and failure of this firewall can result in systemic immune response [306,307]. Undernourished neonatal mice develop liver injury, autophagy, oxidative stress, inflammation, and altered bile acid metabolism that undoubtedly affect immunological function in this organ [148]. Protein-deprived adult mice exhibited reduced number and proliferation of Kupffer cells [308]. Thus, reduced immune function and phagocytosis in the MLN and liver coupled with increased bacterial translocation from the intestine may contribute to increased circulating endotoxin and subsequent immunostimulation in MN mice.

Gut microbiota play a large role in modulating intestinal integrity and immune function. Studies of dysbiosis in children with malnutrition differ depending on the geographic location, age group, local diet, and other factors. However, loss of microbial diversity, decreases in Bacteroidetes, and increases in Proteobacteria are common themes [86,168,170,309]. In this study, we observed no significant difference in alpha diversity of the cecal microbiota between WN and MN mice, but there was a significant increase in alpha diversity in the MLN compared to cecum in MN mice, suggesting bacterial translocation to the MLN is not a random process. At phyla level, we observed a reduction of Bacteroidetes and increase in Proteobacteria in MN mice, similar to other studies of malnutrition [86,168]. Loss of Bacteroidetes species such as those in genus *Bacteroides*

indicate a loss of commensals that contribute to enterocyte health, digestion of plant matter, and production of short chain fatty acids (SCFA), vitamin K, and potassium. Increase in Firmicutes genera such as *Enterococcus* may signal an increase of pathobionts. Expansion of Proteobacteria such as those from the Escherichia/Shigella group (as seen in this study) is also commonly observed in malnutrition [168] and has been linked to development of intestinal and systemic inflammation [290]. Proteobacterial blooms occur concurrently with the development of colitis in a variety of mouse models and are over-represented in the intestinal microbiota of humans with metabolic disorder and IBD [309]. Further, LPS from Proteobacteria and Bacteroidales have different effects on TLR4 signaling [310]. LPS from Bacteroidales species comprises up the majority of fecal LPS in a healthy person and silences TLR4 signaling. Reduced expression of TNF-A and IL-1B was observed in PBMCs stimulated with Bacteroides LPS alone or with equal parts Bacteroides LPS to *E. coli* LPS. Thus, the decrease *in E. coli* and rise in Bacteroides in COL-treated mice may explain in part why increased bacterial translocation did not increase the inflammatory response. Conversely, loss of inflammation-silencing Bacteroides in VANC-treated mice may contribute to the increase in inflammatory response observed upon LPS stimulation.

VANC treatment in MN mice effectively eliminated targeted gram-positive species but had the surprising effect of allowing three OTUs from Gamma and Beta Proteobacteria (Sutterellaceae, Enterobacteriaceae, and Escherichia) to expand to make up over 95% of bacteria in the cecum. This severe drop in diversity and loss of commensal species undoubtedly would affect nutrient harvesting and bacterial metabolite processing. Further, many species from Gamma and Betaproteobacteria have been linked to reduced intestinal barrier function and subsequent liver inflammation [288]. A study in another malnourished mouse model reported increased tissue-adherent bacteria in the small intestine of malnourished mice, especially among Gammaproteobacteria as compared to Bacteroidetes or Firmicutes [98]. These mice also experienced an influx in intestinal gamma-delta T cells and exhibited heightened intestinal and systemic response to *Salmonella* Typhimurium

challenge. The close contact of Gammaproteobacteria with immune cells in the crypts of the small intestine likely drives many of these immunological outcomes.

The massive increase in Gammaproteobacteria in VANC-treated mice is likely to have promoted leakage of LPS into the circulation, priming leukocytes for the heightened inflammatory response we observed. Since there was decreased translocation of live bacteria to the spleen and liver in VANC-treated mice (because VANC eliminated gram-positive aerobes, which comprised the majority of the translocated bacteria), translocated bacteria are not likely to be the sole cause of the systemic inflammation and immune priming. Furthermore VANC treatment of malnourished mice did not ameliorate or exacerbate weight loss, despite loss of commensal species involved in nutrient absorption [311] and expansion of enteropathogens associated with energetically costly inflammation [309]. Many factors that we did not quantify also contribute to metabolic homeostasis and may explain why VANC-treated mice did not experience the expected weight change.

There are several potential mechanisms that could contribute to the amelioration of weight loss in COL-treated MN mice. Compared to untreated MN mice, COL-treated MN mice had greater proportions of bacterial genera known to enhance nutrient harvesting (*Bacteroides*, *Parabacteroides*, *Butyvibrio*) [311] and reduced proportions of genera that have been associated with malnutrition [168] and inflammation [288]. Additionally, colistin's ability to bind and inactivate LPS [312] may directly reduce exposure to LPS in the intestine and its leakage into the circulation. Depletion of bacteria with highly immunogenic LPS may also reduce immune activation [310] and the consequent energy consumption [313,314]. The reduced weight loss of COL-treated mice occurred despite increased translocation to the spleen and liver, again suggesting that translocated gram-positive bacteria do not play a primary role in the inflammation and weight loss of malnutrition. As discussed previously, it is likely that certain bacteria are better suited to translocation and survival outside the gut. Enterococcus, a genus isolated from spleen and liver of MN mice previously, was enriched in the cecal microbiota of COL mice. This also

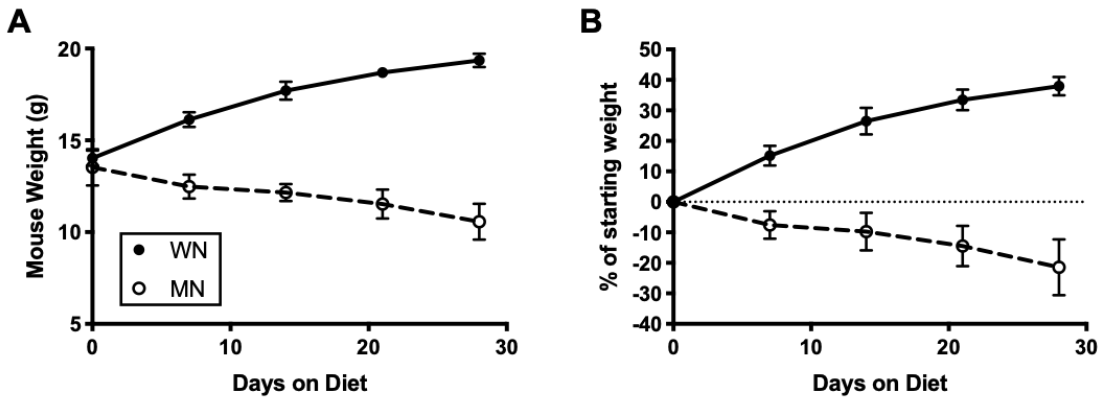
suggests that the detrimental effects of systemic exposure to bacteria is dependent on the species.

This study has several limitations. There are many models of childhood malnutrition and while this model of protein-energy and micronutrient malnutrition has been validated to represent childhood MAM [294], it does not incorporate other environmental stressors that contribute to MAM in children. Second, while we did not observe an increase in intestinal permeability in MN mice, that does not mean that increased permeability would not be observed by using a more rigorous method such as an Ussing chamber. Third, our view of bacterial burden in the MLN, spleen, and liver is likely incomplete since non-culturable bacteria were not evaluated. Aerobic culture of tissue homogenates on BHI agar is a valid and often-used technique but may result in certain species of bacteria going uncounted [315].

The results of this study suggest several avenues for future work. While there appears to be evidence of immune priming in MN mice, we have yet to directly measure circulating LPS levels. The repetition of some of these experiments in gnotobiotic mice on a MN diet could determine if the hyper-responsiveness is due to exposure to LPS or other MN driven changes. It is also unclear how much of the effect of COL treatment is due to changes in microbiota and how much is due to the effect of COL detoxifying LPS in the intestinal lumen. Transplantation of microbiotas from COL and VANC-treated mice into gnotobiotic mice may help answer that question. Further, while we assume that bacteria colonizing the liver and spleen originate in the intestines, we have yet to conduct experiments to that effect. We could orally gavage labelled bacteria (both species known to translocate and those that don't) and track them over time. This would allow us to interrogate the route (lymphatics or blood) taken by any bacteria that wind up outside the intestine. Finally, liver function is known to be decreased in children with MAM and impacts metabolism, acute phase response, and bacterial clearance. Future studies to

examine liver pathology and Kupffer cell killing and phagocytic ability could help explain the altered immune response observed in MN mice.

In sum, our work describes a baseline state of systemic inflammation and heightened systemic inflammatory response in a model of MAM that occurs in the context of muted intestinal immunity, intestinal dysbiosis, and increased extra-intestinal bacterial burden. Modulation of the intestinal microbiota with antibiotics most strikingly alters the proportion of Gammaproteobacteria, the increase of which exacerbates inflammation and reduction of which slows weight loss. These data suggest that even in absence of overt histological intestinal damage and permeability, dysbiosis in MAM can drive systemic bacterial exposure and chronic immune stimulation and exacerbated weight loss. Therefore, selective reduction of intestinal bacterial groups may be beneficial in ameliorating weight loss while also reducing inflammation. Ultimately, an understanding of the connections between gut health, immune function, and metabolism will be required to develop monitoring and treatment programs than ensure holistic, long term recovery from childhood MAM. Further exploration into these mechanisms and potential anti-inflammatory therapies should be pursued as we work towards elimination of childhood malnutrition.



Supplemental Figure 3.1. Representative chart of mouse weight

Supplemental Table 3.1 qRT-PCR primers used in this study

Target	Primer	Sequence (5'-3')	Amplicon Size
18S	18SF 18SR	**Got from lab	
Akp3	AkpF AkpR	ACATTGCTACACAACTCATCTCC TCCTGCCATCCAATCTGGTTC	141
Ccl2	CCL2 For CCL2 Rev	GCTCAGCCAGATGCAGTTAACGC TGGGGTCAGCACAGACCTCTCT	169
Ccl7	CCL7F CCL7R	GCTGCTTTCAGCATCCAAGTG CCAGGGACACCGACTACTG	135
Cldn3	Cldn3 For Cldn3 Rev	ACCAACTGCGTACAAGACGAG CAGAGCCGCCAACAGGAAA	78
Cox2	COX2F COX2R	AACCCAGGGGATCGAGTGT CGCAGCTCAGTGTTTGGGAT	143
Cx3cr1	CX3CR1 For CX3CR1 Rev	GAGTATGACGATTCTGCTGAGG CAGACCGAACGTGAAGACGAG	102
Cxcl1	CXCL1F CXCL1R	ATCCAGAGCTTGAAGGTGTTG GTCTGTCTTCTTTCTCCGTTACTT	171
Cxcl2	CXCL2F CXCL2R	CCAGACAGAAGTCATAGCCACT GGTTCTTCCGTTGAGGGACA	160
Foxp3	FOXP3_F FOXP3_R	ACTCGCATGTTCGCCTACTTCAGA TGGCTCCTCTTCTTGCGAAACTCA	155
Hp	Hapt For Hapt Rev	GCTATGTGGAGCACTTGGTTC CACCCATTGCTTCTCGTCGTT	101
Il1b	IL1BF IL1BR	TTGACGGACCCCAAAAGATG AGAAGGTGCTCATGTCCTCAT	204
Il10	IL10f IL10r	CCCTGGGTGAGAAGCTGAAGACC ACCTGCTCCACTGCCTTGCTCTTA	94
Il17a	IL17A For IL17A Rev	ACCAGCTGATCAGGACGCGC CCAGGCTCAGCAGCAGCAACA	84
Il18	IL-18 For IL-18 Rev	AGAAAGCCGCCTCAAACCTTCCA TGACGCAAGAGTCTTCTGACATGGC	80
Il22	IL-22 For IL-22 Rev	AAACTGTTCCGAGGAGTCAGTGCT GCTGAGCTGATTGCTGAGTTTGGT	159

Il23	IL23f	CTCTCGGAATCTCTGCATGCT	129
	IL23r	TCACAACCATCTTCACACTGGAT	
Il25	IL-25 For	ACAGGGACTTGAATCGGGTC	121
	IL-25 Rev	TGGTAAAGTGGGACGGAGTTG	
Il6	IL-6 For	AGACAAAGCCAGAGTCCTTCAGAGA	146
	IL-6 Rev	GCCACTCCTTCTGTGACTCCAGC	
LBP	LBP_F	GATCACCGACAAGGGCCTG	143
	LBP_R	GGCTATGAAACTCGTACTGCC	
Rorc	RORGT_F	GACCCACACCTCACAAATTGA	137
	RORGT_R	AGTAGGCCACATTACACTGCT	
Tgfb	TGF-Bf	GCGTGCTAATGGTGGACCGCA	108
	TGF-Br	CGGGCACTGCTTCCCGAATGT	
Tnfa	TNFa For	AGCCGATGGGTTGTACCTTGTCTA	103
	TNFa Rev	TGAGATAGCAAATCGGCTGACGGT	

Supplemental Table 3.2 Flow cytometry antibodies used in this study

Name	Region	Fluorescence	Brand	Clone	Ab (*μ*g per 2 million cells)
Live/Dead		Zombie Aqua	Lifetech		
CD4	Surface	APC-Cy7	BD Biosciences	GK1.5	0.5
CD25	Surface	PE-Cy7	Tonbo	PC61.5	0.5
FOXP3	Intracellular	PE-Cy5	eBiosciences	FJK-16s	1
IL-12 p35	Intracellular	eFluor 660	Invitrogen	4D10p35	0.125
IL17A	Intracellular	BV421	BD Horizon	TC11-18H10	0.5
IL-21	Intracellular	PE	Thermofisher	FFA21	0.25
RORGT	Intracellular	eFluor 610	Thermofisher	B2D	0.25

EFFECTS OF OMEGA-3 LONG CHAIN PUFA ON INFLAMMATION DURING MALNUTRITION

Chapter 4. Increased Dietary Omega-3 PUFA Reduces Inflammation in a Model of Moderate Acute Malnutrition

ABSTRACT

Despite concerted global efforts to eradicate acute malnutrition, 7.3% of all children worldwide are wasted. Ready to use therapeutic foods (RUTF) and supplemental foods (RUSF) for treatment of severe acute malnutrition (SAM) and moderate acute malnutrition (MAM) come in the form of macro and micronutrient dense pastes, often based on peanut or corn oil. These treatments provide the high energy, protein and micronutrients needed to support growth recovery, but interventions to address other important aspects of childhood malnutrition such as impaired intestinal function, altered intestinal microbiota, and increased systemic inflammation have not been considered. These dysfunctions do not necessarily resolve upon weight recovery and should be addressed to achieve complete and long-lasting recovery. Omega-3 long-chain polyunsaturated fatty acids (LC-PUFAs) docosahexaenoic acid (DHA) and eicosapentaenoic (EPA) commonly found in fish oil are beneficial in reducing inflammation in inflammatory bowel disease (IBD) and cardiac conditions and are necessary for brain development. Traditional RUTFs and RUSFs contain negligible amounts of omega-3 PUFAs and their inclusion could theoretically reduce inflammation and maladaptive heightened inflammatory response. In this study we assess the impact of dietary fish oil on inflammation, intestinal microbiota, and metabolism in a mouse model of MAM. We observed dampened systemic inflammation and bacterial translocation, evidence of increased intestinal barrier function, reduction of pathobionts in the cecal microbiota, and altered fatty acid metabolism. Furthermore, consumption of fish

oil was protective against weight loss upon systemic challenge with bacterial LPS. These results suggest that inclusion of fish oil (or free DHA and EPA) in treatments for MAM may have beneficial effects in reducing of malnutrition-related inflammation.

INTRODUCTION

In 2018, 49.5 million children suffered from undernutrition [14]. Despite the heightened investment in development and deployment of interventions to treat malnutrition in recent years, prevalence of malnutrition is not dropping at a rate required to meet Sustainable Development Goals and WHO Global Targets [284,316]. The sustained prevalence of malnutrition will continue to drive childhood mortality, disability-adjusted life years (DALYs), and decline of national GDP, especially in Asia and Africa [16,22]. Many factors including poor treatment coverage, low accessibility, and lack of education drive the failure to meet these global goals [242,317,318], but a more complete understanding of the pathology of malnutrition is also required to reach the goal of eradication.

Acute malnutrition, or wasting, can occur as a result of an intense period of nutrient intake or utilization that is not sufficient to maintain normal health. Acute malnutrition can be moderate (MAM) or severe (SAM) and is diagnosed by mid-upper arm circumference (MUAC) or weight-for-height Z score (WHZ). Treatment for SAM includes inpatient stabilization and milk-based therapeutic feeds, after which patients can be treated at home with ready-to-use therapeutic foods (RUTFs). Children with MAM are given ready-to-use supplementary foods (RUSFs) that are complementary to their regular diets and monitored for recovery. However, there is a high variability of response to treatment among children with MAM and SAM [319] and relapse after recovery varies from 0-37% [283].

Acute malnutrition is a complex condition with many contributing factors and complications, including systemic inflammation, reduced intestinal barrier function, and altered immunity [18]. Malnutrition can cause DC [136] and T cell dysfunction [137] while also increasing baseline inflammatory cytokine production [130]. Impaired intestinal barrier

function may contribute to this phenotype by increasing systemic exposure to enteric bacteria and bacterial products. Finally, children with malnutrition experience metabolic derangement due in part to liver dysfunction and steatosis, altered bile acid synthesis, and impaired fatty acid oxidation [260]. Current treatments for malnutrition such as RUTFs and RUSFs were not designed with these complications in mind, likely due to limited understanding of the pathophysiology of malnutrition. However, to reduce rates of treatment failure and relapse, new interventions should be developed that target not only nutrient deficiencies but also inflammation, dysbiosis, and/or metabolic derangement during malnutrition.

RUTFs and RUSFs were designed to promote weight gain and provide key nutrients by providing a micronutrient- and protein-rich and energy-dense supplement for treating acute malnutrition. RUTFs have a long shelf life, are packaged in single servings, and do not require cooking or dilution, making them convenient to stock and distribute as needed. While they can be produced from a variety of components based on availability and cultural acceptance, UNICEF dictates requirements for nutrient content, purity, and stability that must be met [199]. Required nutrient composition for RUTFs states that 45-60% of total energy should come from lipids and 10-12% from protein. Common ingredients include milk powder, peanuts, and a limited range of oils (palm, rapeseed, soy, or sunflower). Compared to other dietary treatments for malnutrition, standard RUTF probably improves recovery and reduces relapse, but there is little evidence of reduced mortality [320]. While delivery of any specially formulated food improved recovery from MAM, lipid-based supplements such as RUSFs were not superior in reduction of mortality or progression to SAM [205]. The unacceptably high treatment failure and relapse relates may be due in part to the single-minded design of current therapeutic foods. These foods were designed to correct energy and nutrient deficiencies while little thought was given to address other pathophysiological features [321,322]. Trials of new RUTF and RUSF formulations focused on weight gain, safety, and cost but did not assess effects on intestinal

microbiota, inflammation, or metabolism [323-325]. In fact, many of the common ingredients of RUTFs and RUSFs are rich in pro-inflammatory omega-6 polyunsaturated fatty acids (PUFAs), include peanuts and palm or other vegetable oils.

The essential fatty acids omega-6 and omega-3 PUFA have opposing effects on inflammation and energy homeostasis [326]. Omega-6 PUFA arachidonic acid (AA) is a precursor for inflammatory eicosanoids and leukotrienes and drives increased production of inflammatory cytokines. Excess AA also dysregulates energy homeostasis and is implicated in development of insulin and leptin resistance, hepatic steatosis, and appetite deregulation. Conversely, omega-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are precursors for anti-inflammatory eicosanoids and leukotrienes and inflammation-resolving resolvins and protectins. EPA and DHA also regulate insulin and leptin sensitivity, increase beta-oxidation, and regulate adipokine secretion. The anti-inflammatory action of DHA and EPA is mediated via inhibition of inflammatory signaling pathways and reduction of omega-6 PUFA availability [327]. The production of AA-derived or EPA and DHA-derived mediators is dependent on the ratio of omega-6: omega-3 PUFAs incorporated into the cellular membranes of erythrocytes, monocytes, neutrophils, and other cell types. This ratio is reflective of the dietary intake ratio, which has been heavily skewed towards omega-6 PUFAs in modern times. To experience the anti-inflammatory and energy regulating effects of omega-3 PUFAs, the dietary ratio of omega-6: omega-3 PUFA should be $\leq 4:1$. However, modern diets typically have ratios $> 15:1$, potentially contributing to increasing rates of obesity and diabetes observed worldwide. Further, RUTFs and RUSFs also have high ratios of omega-6: omega-3 PUFAs and are required to contain a minimum 0.3% omega-3 PUFA and up to 10% omega-6 PUFA as a proportion of total energy [199]. Thus, use of omega-6 PUFA rich RUTF as the only source of food during treatment for SAM may exacerbate malnutrition-related inflammation and metabolic derangement. In fact, low levels of omega-3 PUFAs were demonstrated in children with MAM and were inversely correlated with markers of

inflammation [278]. Alternate forms of RUTF or RUSF with reduced omega-6:omega-3 PUFA ratios (or supplementation with omega-3 PUFA-rich fish oil) may reduce inflammation in children with SAM and MAM, thereby improving recovery rates.

To our knowledge, no studies currently exist on the effects of omega-3 PUFA supplementation in preclinical models of malnutrition and the two clinical trials of omega-3 PUFA treatment for MAM were limited in scope [328-330]. Both trials were designed to examine the safety of modified RUTFs with elevated omega-3 PUFAs and did not assess their effects on recovery, inflammation, or metabolism. However, omega-3 PUFAs were successful in reducing intestinal inflammation and promoting recovery in inflammatory bowel disease, which shares aspects of intestinal pathophysiology with acute malnutrition [153,331,332]. Further studies on the effects of omega-3 PUFAs on weight, inflammation, microbiota, and metabolism during malnutrition are required to evaluate its appropriateness as a treatment for SAM and MAM.

In this study, we altered dietary PUFA ratios in a murine model of MAM by substituting fish oil for corn oil as lipid source in a nutrient-deficient diet. We hypothesized that increased consumption of omega-3 PUFAs will reduce malnutrition-driven inflammation by altering the intestinal microbiota, improving intestinal defense, and reducing bacterial translocation. We investigated a number of indicators known to be affected by malnutrition in this model, including correlates of systemic immunity, bacterial translocation, intestinal function, metabolism, intestinal microbiota, and response to infection. We found that fish oil consumption reduces inflammation, potentially by reducing exposure to endotoxin and/or by reducing generation of inflammatory mediators. Fish oil was protective against weight loss during LPS challenge and not harmful during enteric infection, suggesting it could be safe for use for malnourished children who have increased susceptibility to infection.

MATERIALS AND METHODS

Mouse Diet

Weanling female BALB/C mice were obtained from Harlan Laboratories. Mice were grouped in cages of 5 and had free access to water. Mice were weaned to one of three diets for 28 days (Teklad): (i) a standard mouse chow (WN) (Teklad diet number 99103) (ii) an isocaloric but protein, iron, and zinc deficient malnourished (MNCO) chow (Teklad diet number 99075), or (iii) a chow identical to the MNCO chow, but formulated with fish oil in place of corn oil (MNFO) (Teklad diet number 160285). The fish oil used was comprised of approximately 33% omega-3 FA (13-16% EPA, 10-11% DHA, 6-9% other omega-3 FA). Further details of diet composition are given in Supplemental Table 1 and in previous studies using this model [294]. Mice were pair-fed every 48-72 hours and weighed every 7 days. MNCO and MNFO mice received 90% volume by weight of food that WN mice did. This was calculated by determining food consumption (grams/mouse/day) for the previous feeding period for well-nourished mice and then determining the necessary amount per cage for MNCO and MNFO mice. Mice were studied after 28 days on the diet, and then euthanized by cervical dislocation while under isofluorane anesthesia.

Systemic LPS Challenge

Mice were weaned to either a WN, MN, or MNFO diet as previously described. After 28 days on the diet, mice were injected intraperitoneally with 4 mg/kg of LPS (*Escherichia coli* O55:B5, Sigma-Aldrich, L2880) in PBS. Mice were given food and water ad libitum and monitored hourly. At six hours post injection, Mice were weighed at 6 and 24 hours post injection and euthanized via cardiac puncture under isofluorane anesthesia. Blood was taken with a heparinized needle and plasma isolated and stored at -80°C.

RNA Isolation and qRT-PCR

Skin from the dorsal foot was removed with a scalpel and stored in RNAlater Stabilization Solution (Thermofisher) at -80°C. Ileal samples were taken as the distal third of the small intestine, cut into 1 cm pieces, and stored in RNAlater Stabilization Solution (Thermofisher) at -80°C. Samples were thawed on ice and thirty mg or less of either skin or intestine was removed and homogenized in lysis buffer (Qiagen) + B-mercaptoethanol (Sigma) via bead mill (VWR 4-Place Mini Bead Mill Homogenizer) and spun down to pellet remaining tissue matter. RNA was extracted from the supernatant (RNeasy, Qiagen) and treated with DNAse (Turbo DNAse, Ambion). All RNA samples were quantified using a Thermo Scientific NanoDrop™ Spectrophotometer and stored at -80°C. cDNA was generated from each sample using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) (Veriti 96 Well Thermal Cycler, Applied Biosystems). Cytokine mRNA was quantified using 20-40 ng cDNA, SYBR Green Master Mix (ThermoFisher), and primers (Supplemental Table 2) on a Viia 7 Real-Time PCR System (Applied Biosystems). CT values were used to calculate fold-change in comparison to the mean of WN naïve samples.

Quantification of Bacterial Burden in Mouse Tissue

Spleen, liver, and mesenteric lymph nodes were used to determine bacterial burden. The liver was cut into thirds and the anterior portion of the middle section was collected. The spleen was cut in half and the anterior half was collected. The MLN were collected and used for bacterial quantification. Spleen (10-30 mg), liver (40-90 mg), and MLN (3-12 mg) samples were stored in a 10x volume of PBS (ex. 50 mg tissue, 500 μl PBS) in a 2 ml Eppendorf tube. Samples were homogenized in the collection tube with a rubber pestle. Each sample was serially diluted out to 10^{-6} in PBS, generating a range of tissue concentrations from 10 mg/μl to 10 ng/μl. 50 μl aliquots of each dilution were plated on Brain Heart Infusion (BHI) Agar (Sigma-Aldrich) plates and incubated aerobically at 37°C.

Plates were incubated 3 days and colonies counted on both days 2 and 3. Colony counts and dilution values were used to calculate CFU/g for each sample.

Determination of Intestinal Microbiota Composition

Cecal contents from WN, MNCO, and MNFO mice were collected immediately after euthanasia and snap-frozen at -80°C. DNA was isolated from cecal contents using the QIAamp DNA Stool Mini Kit (QIAGEN) according to manufacturer's protocol and shipped to LCSciences (Houston, Texas, USA) for 16S rDNA V3+V4 sequencing and analysis. Bacterial DNA was amplified with primers targeted to the V3 and V4 regions of 16S rDNA. Sequencing adaptors and barcodes were added in further amplification steps and the prepared library was sequenced on the MiSeq platform. Resultant paired-end reads were merged into tags and then grouped into clusters that represent a single operational taxonomic unit (OTU) (minimum 97% sequence similarity). Analysis of OTUs were conducted including measures of alpha diversity, beta diversity, and taxonomy annotation. Databases for taxonomy analysis included RDP (version date 2016.9.30) and NT-16S (version date 2016.10.29).

Metabolomics

Blood was collected from WN, MN, and MNFO mice after overnight fast via cardiac puncture and plasma collected after centrifugation at 2000g for 10 minutes at 4°C. Plasma was shipped on ice to Biocrates Life Sciences (Biocrates, Innsbruck, Austria) where the concentrations of 180 metabolites including hexose, amino acids, biogenic amines, acylcarnitines, glycerophospholipids, and sphingolipids were determined. Amino acids and biogenic amines were measured by liquid chromatography-mass spectrometry, while other metabolites were measured by flow-injection mass spectrometry. Metabolites were reported as μM concentration. The dataset was cleaned, and metabolites that were measured below the limit of detection in greater than 20% of samples were removed from

further analysis. Remaining missing values were imputed using a Logspline imputation method. The imputed dataset was then log2 transformed prior to univariate statistical analysis. Microsoft Excel, R, and GraphPad Prism were used to perform metabolite analysis.

Citrobacter rodentium Challenge

Citrobacter rodentium (DBS100, ATCC® 51459, Virginia, USA) was grown overnight in Luria Broth at 37°C while shaking. Subcultures were made by diluting 200 μl of overnight culture in 10 mL LB and incubating at 37°C with shaking for 4 hours. Subcultures were pooled, washed with PBS, and resuspended in PBS. The culture density was measured via spectrophotometer and adjusted to 5 x 10^{10} bacteria/μL. Mice were orally gavaged with 200 μl, resulting in a dose of 1x10^{10} bacteria. Dilutions of the inoculum were plated on MacConkey agar (M7408, Sigma-Aldrich, Missouri, USA) to verify bacterial count. Mice were monitored for thirty minutes after gavage and daily for the next three days. Diets and monitoring were continued as before, with the exception that water bottles were replaced with HydroGel packs (ClearH_2O, Maine, USA). Body weight and at least two fresh fecal pellets per mouse were collected by applying gentle abdominal pressure on days 0, 3, 5, 7, 10, and 14. Pellets were weighed and homogenized in PBS before dilution and plating on MacConkey agar. Colonies of *C. rodentium* were counted and CFU/g of fecal matter was used to calculate bacterial burden. On day 14 post gavage, mice were euthanized under isofluorane anesthesia by cardiac puncture and the colon tissue collected, washed, and stored in RNAlater at -80°C.

Statistics

Unless otherwise specified, groups were compared via Mann-Whitney U test. Outliers were detected and removed from analysis where appropriate. All analyses were

conducted using GraphPad Prism 8 for Mac OS X (Graphpad Software, San Diego California USA) or R 3.4.3.

RESULTS

Dietary fish oil promotes maintenance of body mass in malnourished mice

To determine the effect of dietary fish oil we monitored food consumption and body weight over the course of 28 days for mice fed a normal diet (WN), or a diet deficient in protein, iron and zinc that included either corn oil (MNCO) or fish oil (MNFO) as the dietary lipid. The nutrient analysis of the different diets are detailed in Supplemental Table 4.1. Replacement of corn oil with fish oil in the malnourished diets did not alter weight loss over the course of the experiment (Figure 1A, B). However, MNFO mice were able to maintain similar weights and rate of weight change to MNCO mice while consuming significantly less food overall (p = 0.0461) and when calculated as a percentage of bodyweight (p = 0.006) (Figure 1C). This suggests that MNFO mice are either reducing their energy expenditure or increasing nutrient harvesting such that they are able to maintain a similar weight to MNCO mice on fewer calories.

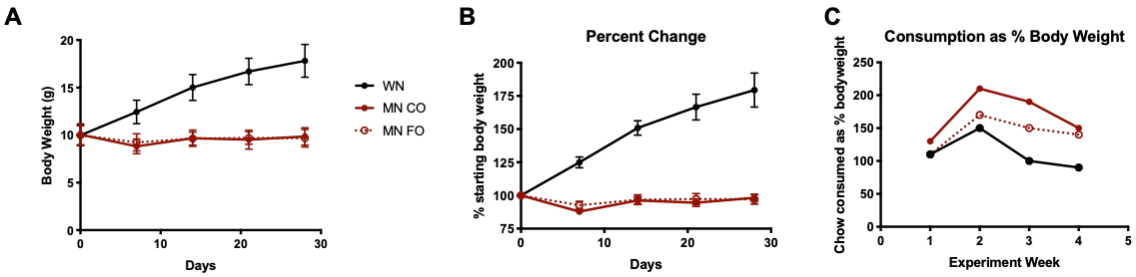


Figure 4.1. Weight change and food consumption over the diet period.

Weight in grams **(A)** or as a percentage of starting weight **(B)**. Weight of chow consumed by a cage of five mice in one week as percentage of the weight of the mice at the end of the week (**C**).

Dietary fish oil reduces systemic inflammation and extra-intestinal bacterial burden in MN mice

We previously demonstrated increased basal inflammation and exaggerated inflammatory responses to bacterial LPS in this mouse model of moderate acute malnutrition (see Chapter 3). Since dietary fish oil was found to reduce systemic inflammation in several disease models, we hypothesized that it could ameliorate the inflammation associated with MAM. We first determined the effects of fish oil on expression of inflammatory mediators in the skin of WN, MNCO, and MNFO mice at baseline. Compared to MNCO mice, MNFO mice had reduced mRNA expression of neutrophil chemoattractant *Cxcl2* and inflammatory cytokine *IL17f* and similar trends for *Il1b*, *Ccl2*, and *Cxcl1* (Figure 4.2).

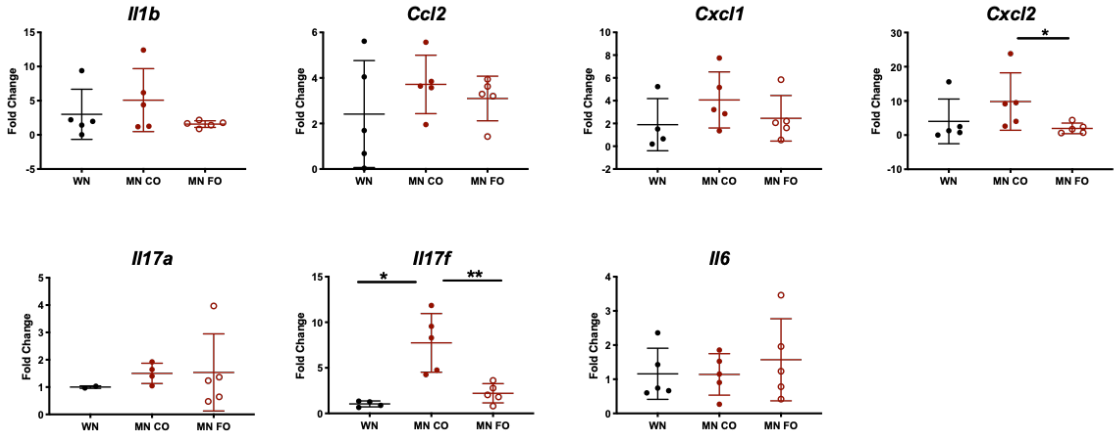


Figure 4.2. Dietary fish oil reduces baseline mRNA expression of *Cxcl2* and *IL17f* in skin.

Values determined via qRT-PCR. WN samples used as baseline to determine fold change. All P values determined by Mann-Whitney U-test (*p<0.05 ** p<0.01 *** p<0.001 ****p<0.0001).

To explore the effect of malnutrition on the physiological response to an inflammatory stimulus, we injected WN, MNCO, and MNFO mice intraperitoneally with

bacterial LPS. At six hours post challenge, MNFO mice had lost the least weight of any group, with mice weighing a median 96.2% of their starting weight (Figure 4.3A). WN mice weighed a similar 95.8% of their starting weight, while MNCO mice lost significantly more than either group (median 92.1% of starting weight). At 24 hours post-challenge the MNCO mice showed a significant drop in body weight compared to WN mice, but the MNFO were no different from the WN mice and showed as trend toward less weight loss than the MNCO mice ($p = 0.119$) (Figure 4.3B).

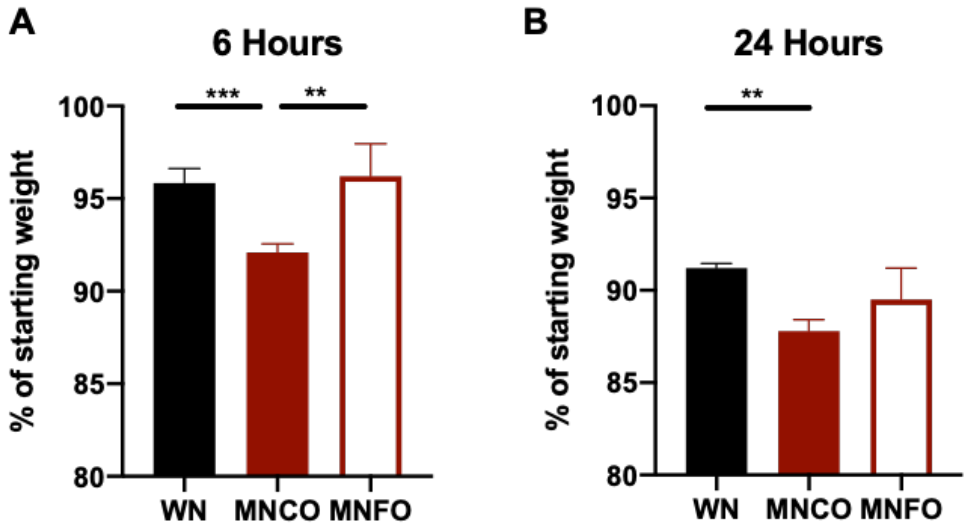


Figure 4.3. Consumption of fish oil protects against weight loss in MN mice injected with LPS.

Malnourished mice given fish oil are partially protected from weight loss at 6 (A) and 24 (B) hours post IP injection of LPS. All P values determined by Mann-Whitney U-test (*p<0.05 ** p<0.01 *** p<0.001 ****p<0.0001).

In our previous studies, malnutrition led to increased translocation of bacteria from the gut to the visceral organs (See Chapter 3). Here we found that MNFO mice had significantly less bacteria in the liver compared to MNCO mice ($9.6*10^3$ vs. $2.4*10^5$ CFU/g, $p = 0.016$) and an insignificant decrease in bacteria in the spleen ($4.7*10^4$ vs.

1.6*10^6 CFU/g $p = 0.333$) (Figure 4.4). However, MNFO mice exhibited a similar bacterial burden in the MLN compared to MNCO mice (4.00*10^6 vs 1.67*10^6, $p = 0.389$).

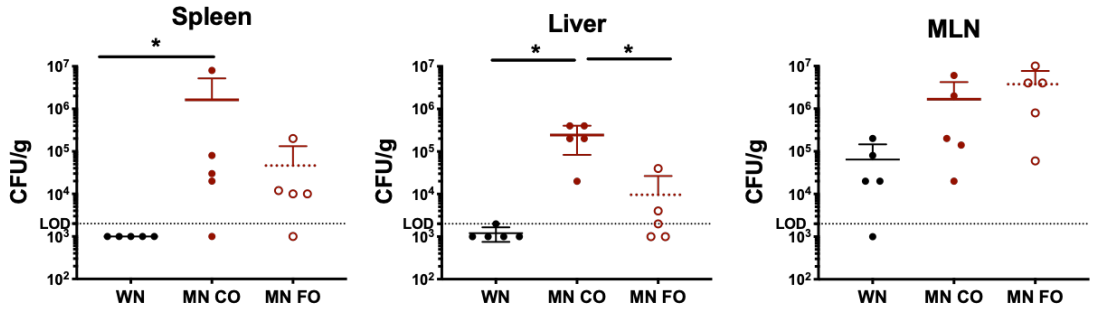


Figure 4.4. Reduced bacterial burden in spleen and liver of MNFO mice.

Dietary fish oil reduces bacterial burden in the liver. Live bacteria were quantified by plating tissue homogenates on BHI agar. All P values determined by Mann-Whitney U-test (*p<0.05 ** p<0.01 *** p<0.001 ****p<0.0001).

Dietary fish oil improves intestinal immune function and cecal microbiota diversity

To determine the effect of dietary fish oil on intestinal immunity, barrier function, and overall health, we measured mediators of barrier function and host defense the distal third of the small intestine. MNFO mice displayed increased mRNA levels of antimicrobial proteins *Reg3b* and *Reg3g* and tight junction protein *Cldn3* (Figure 4.5A). MNFO mice also exhibited reduced levels of *Hapt*, which is the precursor of zonulin (a protein that regulates tight junctions to increase intestinal permeability) [333]. The increased *Cldn3* and the reduced *Hapt* in the MNFO mice indicate reduced intestinal permeability. Finally, MNFO mice had significantly increased expression of *Il17f* and *Cxcl1* and a trend towards increased *Il17a* (Figure 4.5B). In sum, these data suggest that MNFO mice experience increased intestinal barrier function and host defense as well as reduced extra-intestinal bacterial burden.

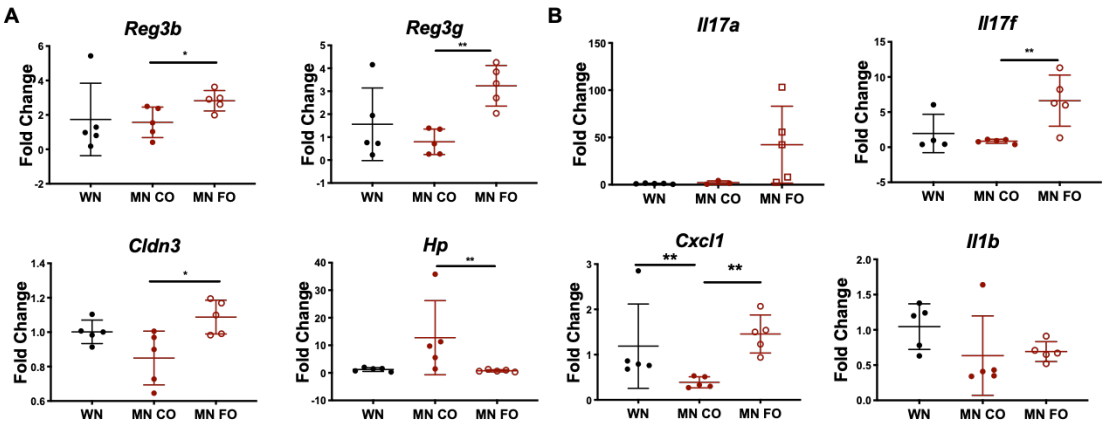


Figure 4.5. Altered mRNA expression of proteins involved in antimicrobial defense and intestinal immunity in MNFO mice.

Dietary fish oil alters mRNA expression of proteins involved in intestinal barrier function and antimicrobial defense (A) and increases mRNA expression of certain intestinal cytokines and chemokines (B). All P values determined by Mann-Whitney U-test (*p<0.05 ** p<0.01 *** p<0.001 ****p<0.0001).

The changes in systemic inflammation, bacterial translocation, and intestinal function in MNFO mice are likely intimately related to changes in the intestinal microbiota. Therefore, we sequenced 16S DNA from cecal contents of WN, MNCO, and MNFO mice. The MNFO microbiota has significantly greater alpha diversity, measured by Shannon index, than either WN or MNCO mice (Figure 4.6A), and all three groups could be distinguished by principal component analysis (Figure 4.6B). While MNFO samples cluster independently of other samples, they still have more similarity to the MNCO microbiota than the WN microbiota. At the phyla level, we observed a decrease in proportion of Proteobacteria (MNFO 4.9% vs MNCO 9.5%, p = 0.008) (Figure 4.6C), driven almost entirely by reduction of an OTU assigned to Escherichia/Shigella (Figure 4.6D). The only other phylum significantly altered between MNCO and MNFO is Tenericutes, which is present at less than 0.1% in both groups. At the OTU level, there are 15 OTUs that vary significantly between MNCO and MNFO mice (Figure 4.6D).

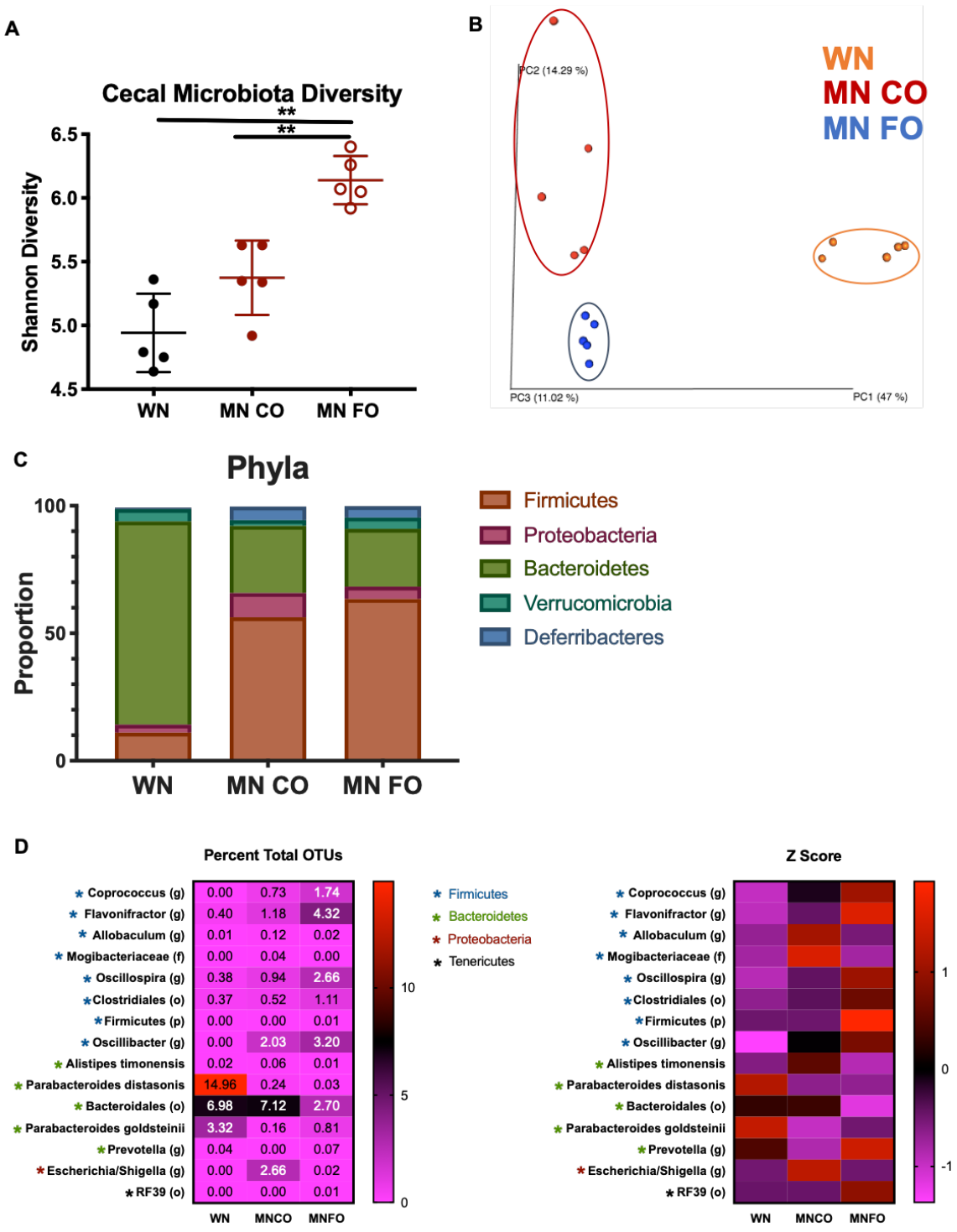


Figure 4.6. Fish oil consumption increases cecal microbiota diversity, reduces proportion of Proteobacteria in MN mice.

Shannon diversity calculated for all groups and compared with Mann-Whitney U-test (A). Principal component analysis was used to examine beta diversity (B). Mean phyla

proportions in cecal microbiota calculated from 5 mice per group (C). Heatmaps of OTUs present at significantly different ($p < 0.05$) proportions in MN CO and MN FO mice displayed as % of population or as Z score (D). Z –scores calculated using mean and std dev for each OTU alone. Heatmaps arranged by phyla and in descending order of statistical significance.

Dietary fish oil alters lipid and amino acid metabolism in MN mice

The substitution of fish oil for corn oil in the chow of malnourished mice reduced the dietary omega-6 PUFA to omega-3 PUFA ratio from 17.5 to 2.4 and increased the saturated fatty acid (SFA) percentage from 14.8% to 20.5% (Supplemental Table 4.1).

Table 4.1 Concentration of discriminatory metabolites.

Metabolite	Class	WN	MNCO	MNFO
PCaeC40.2	PUFA	0.39	0.59	2.92
PCaeC40.6	PUFA	2.31	2.58	12.4
PCaaC36.5	PUFA	2.42	6	173.8
PCaeC44.5	PUFA	0.11	0.14	0.88
PCaeC38.0	SFA	1.13	1.7	8.09
PCaeC38.6	PUFA	1.92	2.77	7.86
PCaaC36.6	PUFA	0.19	0.37	5.62
PCaeC36.0	SFA	0.37	0.5	1.35
PCaeC44.6	PUFA	0.11	0.14	0.53
PCaaC40.6	PUFA	31.97	37.86	148.2
SMC18.0	SM	8.52	21.7	12.3
SMC24.0	SM	7.15	12.4	8.43

SMOHC22.1	SMOH	3.02	5.75	4.04
SMOHC16.1	SMOH	0.58	1.04	0.86
SMC18.1	SM	2.06	4.51	1.95
PCaeC36.3	PUFA	1.03	2.29	0.94
PCaaC36.1	PUFA	15.7	32.93	27.8
PCaaC34.1	PUFA	79.73	148.44	128.4
SMOHC22.2	SMOH	1.34	2.59	2.46
SMC16.0	SM	28.99	44.07	40.84

PC = phosphatidylcholine, SM = sphingomyelin, SMOH = hydroxysphingomyelin, aa=diacyl residue, ae=acyl-alkyl residue, Cxx.y= number of carbon atoms followed by number of double bonds (Ex. C40.2 = 40 carbon atoms and 2 double bonds).

Since fatty acids are precursors for bioactive metabolites and immune mediators, we conducted plasma metabolomics to determine the impact of lipid source on metabolism in MNCO mice. Partial least squares discriminant analysis (PLS-DA) of all metabolites demonstrated that the MNFO metabolome was distinct from WN and MNCO along Component 1 and had greater similarity to the WN than the MNCO group along Component 2 (Figure 4.7A). The WN and MNCO metabolomes exhibit slight overlap and are differentiated primarily along Component 2. Further analysis via sparse PLSDA determined that 10 phosphatidylcholines (8 PUFA) were capable of classifying samples with an overall error rate of 0.375, while addition of 10 more metabolites (7 sphingolipids and 3 phosphatidylcholines) reduced the error to 0.00 (Table 4.1 and Supplemental Table 4.3). Thus, lipid metabolites alone are sufficient to discriminate between WN, MNCO, and MNFO mice.

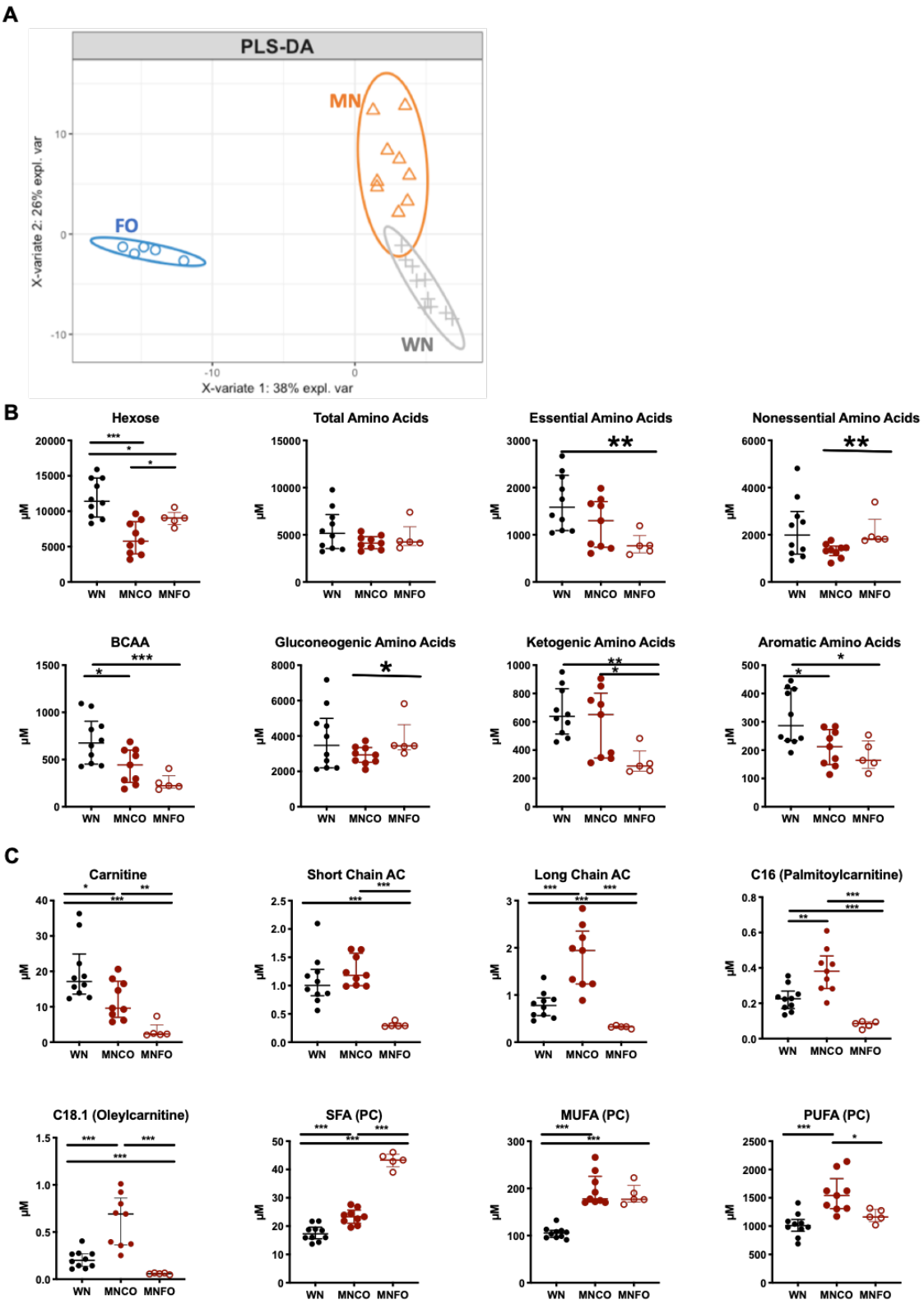

A
PLS-DA
MN
FO
WN
X-variate 2: 26% expl. var
X-variate 1: 38% expl. var
B
Hexose
Total Amino Acids
Essential Amino Acids
Nonessential Amino Acids
BCAA
Gluconeogenic Amino Acids
Ketogenic Amino Acids
Aromatic Amino Acids
C
Carnitine
Short Chain AC
Long Chain AC
C16 (Palmitoylcarnitine)
C18.1 (Oleylcarnitine)
SFA (PC)
MUFA (PC)
PUFA (PC)
µM
WN
MNCO
MNFO

Figure 4.7. Altered carbohydrate, amino acid, and lipid metabolism in MNFO mice.

There is more variation between MNCO mice and MNFO mice than between WN and MNCO mice, as determined by PLS-DA of 180 metabolites (A). Concentrations of select metabolites including hexoses and groups of amino acids (B), acylcarnitines, and fatty acids (phosphotidylcholines) (C). All P values determined by Mann-Whitney U-test (*p<0.05 ** p<0.01 *** p<0.001 ****p<0.0001).

We also analyzed metabolites to broadly assess carbohydrate, protein, and lipid metabolism. The one measure of carbohydrate metabolism, plasma hexose, was elevated in MNFO mice compared to MNCO mice, but both malnourished groups still had lower concentrations than WN mice (Figure 4.7B). This indicates that dietary fish oil can reduce malnutrition-related hypoglycemia, which is a risk factor for mortality during SAM [334]. Total plasma amino acid concentrations were similar between all groups, but the concentration of various groups of amino acids differed between WN, MNCO, and MNFO mice. Essential amino acid concentrations were significantly lower in MNFO than in WN mice. Compared to MNCO mice, MNFO mice also had significantly higher concentrations of nonessential amino acids and gluconeogenic amino acids, but reduced concentration of ketogenic amino acids. Collectively, these data indicate differences in gluconeogenesis and ketogenesis between MNCO and MNFO mice, suggesting that dietary fish oil alters metabolic pathways and substrates utilized during malnutrition.

We also examined acylcarnitine (AC) concentrations and found that MNFO mice had significantly lower concentrations of free carnitine, short chain ACs, and long chain ACs compared to WN and MNCO mice (Figure 4.7C). Also of note, MNCO mice had a significantly higher concentration of long chain AC compared to WN and MNFO mice, suggesting incomplete fatty acid oxidation. Concentrations of LC acylcarnitines C16 and C18.1 are significantly increased in MNCO mice and significantly decreased in MNFO mice. These two LC AC were also elevated in MN children (See Chapter 2) and have been

associated with increased systemic inflammation. Therefore, reduced plasma LC AC in MNFO mice may indicate increased fatty acid oxidation [335] and reduced exposure to inflammatory metabolites.

Finally, we calculated SFA, monounsaturated fatty acid (MUFA), and PUFA plasma concentrations using phosphatidylcholine concentrations. MNFO mice had the highest levels of SFA of all three groups (Figure 4.7C). Both MNCO and MNFO mice had higher concentrations of MUFA than WN mice. MNCO mice had significantly higher levels of PUFAs than WN mice, while MNFO mice fell between the two groups. It is unclear why MNFO mice have elevated concentrations of plasma SFA. While the MNFO diet has increased percentage of SFA compared to the MNCO diet, the difference in intake is much smaller than the difference in plasma concentration of SFA. Regardless, elevated SFA may support membrane integration of omega-3 PUFA and resultant impaired inflammatory action of AA [336]. In sum, these metabolic data suggest that dietary fish oil mitigates malnutrition-related hypoglycemia by altering amino acid metabolism and restoring beta-oxidation.

Dietary fish oil alters intestinal immunity and does not increase susceptibility to *Citrobacter rodentium* infection

Previous research on omega-3 PUFA supplementation in a mouse model of high-fat diet observed reduced inflammation but also compromised control of *Citrobacter rodentium* infection [337]. Fish oil supplementation must not increase susceptibility to enteric infection if it is to be considered a viable treatment for MAM. To determine the impact of fish oil consumption on enteric infection during MAM, we infected mice with *C. rodentium* after 28 days on diet and charted fecal *C. rodentium* burden and weight change until 14 days post-infection, when the immune response is reported to peak [338]. The *C. rodentium* burden in fecal pellets was similar between WN, MNCO, and MNFO mice until day 14, at which point the burden in WN mice dropped significantly below that

of MNCO and MNFO mice (Figure 4.8A). Both MNCO and MNFO mice gained weight over the initial 3 days post-infection while weight of WN mice remained static (Figure 4.8B). All mice experienced a temporary weight loss between 3 and 14 days that coincided with the timing of the immune response as typically observed in mouse models [338]. When we examined colon mRNA expression of host defense-related proteins and cytokines known to be affected by omega-3 PUFA consumption we found few differences between groups. All groups responded to infection with increased *Il17a* and *Il22* production, both of which play a role in clearance of *C. rodentium*. However, MNFO mice alone increased colonic production *Ifng* in response to infection. *Ifng* is critical for control of *C. rodentium* [339], yet both malnourished groups exhibited delayed clearance compared to WN mice. These data indicate that fish oil consumption does not increase susceptibility to *C. rodentium* infection in MN mice, though more investigation must be conducted to determine impact on clearance of this and other pathogens.

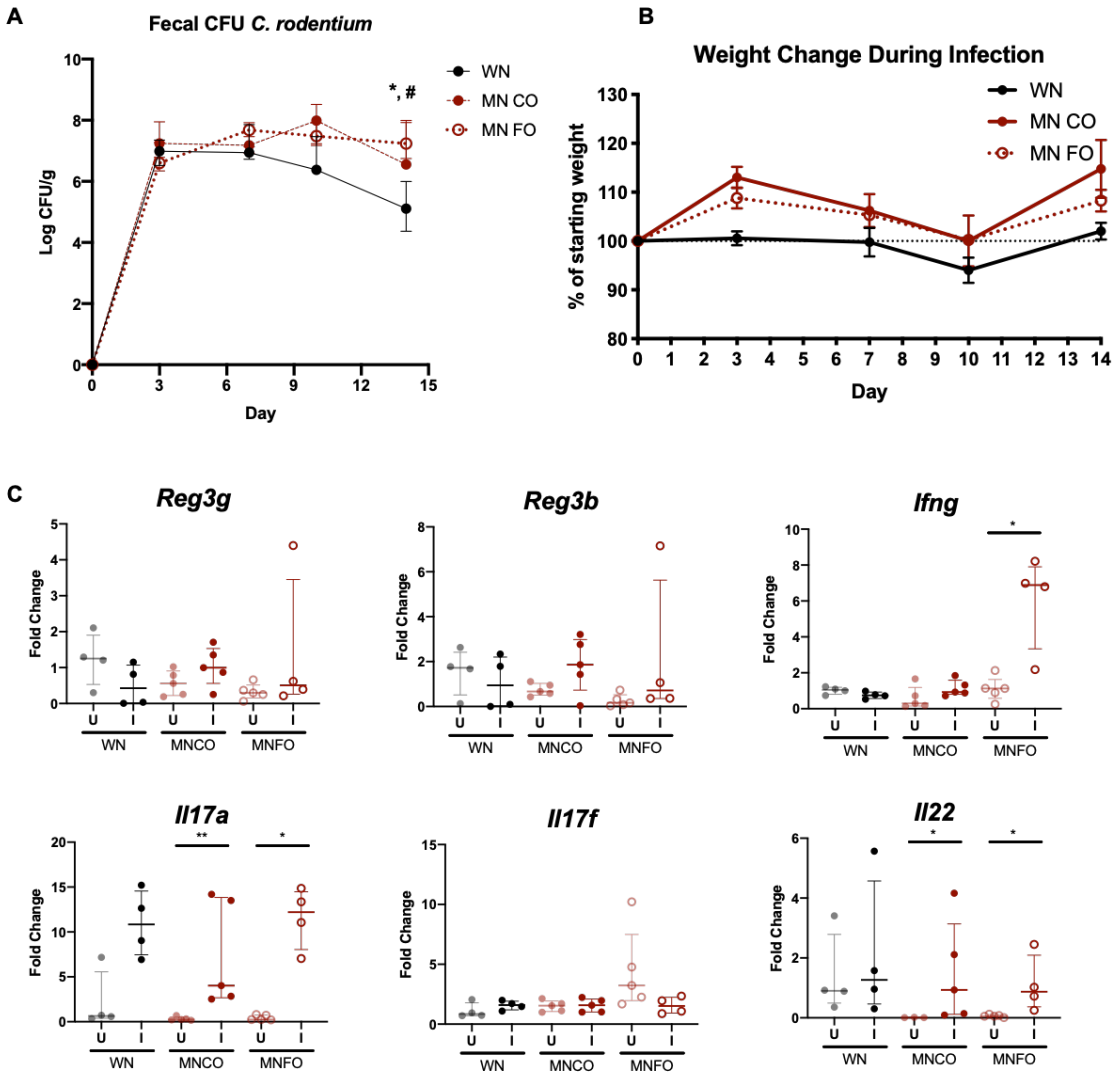


Figure 4.8. *Citrobacter rodentium* persists longer in MN mice, regardless of lipid source.

Omega3 impacts intestinal RNA, but not weight or burden, 14 days post infection. (a) Median and IQR of log CFU/g of fecal matter at 0,3,7,10, and 14 days post infection. * = P<0.05 after FDR from MNCO to WNCO. # = P<0.05 after FDR from MNFO to WNCO. (b) Weight change over the course of the experiment. (C) Cytokine mRNA expression in colons of *C. rodentium* infected mice (day 14 post infection) and noninfected controls. Comparison by Mann-Whitney U-test or Kruskal-Wallis, as appropriate (*p<0.05 ** p<0.01 *** p<0.001 ****p<0.0001).

DISCUSSION

Children with MAM often experience treatment failure or relapse [283], potentially due to failure of current MAM treatments to address systemic inflammation and intestinal dysbiosis. Developing the next generation RUTFs and RUSFs that go beyond simple correction of nutrient deficiencies to target the altered physiological processes of acute malnutrition, such as dysbiosis and inflammation, is critically important to enhance recovery rates [164,169]. Omega-3 LC PUFAs are essential for early child development [340] but are deficient in children with MAM [278] and in traditional RUTFs [328]. Omega-3 LC PUFAs reduce intestinal inflammation in people with IBD and exhibit systemic anti-inflammatory effects through a variety of mechanisms [327]. In this study, we found that increased dietary omega-3 LC-PUFAs reduced systemic inflammation that occurs during MAM, potentially by improving intestinal dysbiosis, strengthening intestinal barrier and immune function, and reducing exposure to translocated bacteria and bacterial products. Importantly, MNFO mice were better able to tolerate systemic challenge with LPS, likely due to omega-3 PUFA's anti-inflammatory and lipolytic effects [326]. Therefore, incorporation of omega-3 LC PUFAs into MAM treatment programs could be beneficial in correcting energy deficits while also mitigating the increased systemic inflammation found in children with MAM. Several studies showed that incorporation omega-3 LC PUFAs in RUTF was safe and supported weight gain and in children with MAM [337,341]. Further information is needed on how omega-3 LC PUFAs affect systemic inflammation, intestinal function, microbiota, and metabolism in the context of MAM.

Curiously, dietary FO enabled MN mice to maintain the same weight as MNCO mice while consuming fewer calories. This suggests that they are better able to harvest nutrients from their food and/or expend less energy. The immunological data collected suggest that MNFO mice may conserve energy by reducing chronic systemic inflammation. The current depth of our metabolic analysis demonstrates differences in

metabolism between MNCO and MNFO mice but cannot address energy expenditure or efficiency. Future studies in metabolic chambers could begin to address this question. A study of mice on a starvation diet found that supplementation with 4% FO improved survival, satiety, and appearance by normalizing neurochemical signaling related to satiety despite slightly increased weight loss in the FO group [342]. Thus, it is possible that in our model MNFO mice consume less food due to the satiety inducing effects of FO on neurochemical signaling. The implications of this information for treatment of childhood MAM are unclear.

Compared to MNCO mice, MNFO mice have reduced translocation of gut bacteria to the liver (and potentially spleen) but similar levels of culturable bacteria in the MLN. This outcome may be driven by i) restored intestinal barrier function, ii) improved MLN barrier function so that fewer bacteria escape the LN, and/or iii) more effective phagocytosis and bacterial killing in the liver and spleen. Our finding of increased transcripts for antimicrobial proteins and intestinal barrier enhancing proteins in the small intestine may indicate ability to reduce the number of bacteria that penetrate the mucosa and reach the MLN. Ghosh et al. [337] found that addition of FO to a high fat diet in mice reduced intestinal damage and translocation during *C. rodentium* infection. The mechanisms behind and relevance of increased expression of *Il17f* and *Cxcl1* in the small intestine are unclear, but elevated colonic expression of *Il17a* and other inflammatory cytokines was observed in uninfected mice by Ghosh et al. as well. The altered microbiota in MNFO mice may encourage appropriate immune response and activity, though further investigation into the immune cell populations of the small intestine are required to fully understand this. Previous studies in this model have indicated that LN barrier function is reduced in MNCO mice due to reduced numbers of phagocytes [305]. Similarly, malnutrition reduces the number and proliferation of splenic cells in mice [18] and drives oxidative stress, inflammation, and autophagy in the livers of neonatal mice [260]. Fish oil consumption increases macrophage phagocytosis [343] and reduces inflammatory cytokine

production in neutrophils, monocytes, and macrophages [327]. These mechanisms may result in improved bacterial capture in the MLN and clearance in the liver of MNFO mice, resulting in the observed decrease in bacterial burden.

Changes to the cecal microbiota of MNFO mice may drive downstream reduced inflammation. While fish oil consumption significantly alters the MN microbiota, it does not necessarily have greater similarity to the microbiota of WN mice. Thus, while the changes observed in the MNFO microbiota may be beneficial, transition to a normal microbiota and recovery from MAM will also require correction of the nutrient deficits (in this case low protein, iron and zinc). One of the most striking findings is that the MNFO cecal microbiota exhibited significantly greater alpha diversity than microbiotas of WN or MNCO mice. This increased diversity of OTUs likely indicates an increased diversity of bacterial genes and functional groups which may afford increased resiliency to perturbation and ability to harvest nutrients from different foods [344]. Despite the differences in diversity, only 15 OTUs differed significantly in proportion between MNFO and MNCO mice. At the phyla level, MNFO mice had a significant reduction of proportion of Proteobacteria compared to MNCO mice. This was largely driven by a significant decrease in an OTU attributed to the Escherichia/Shigella group. Previous studies in this model of MAM have identified associations between elevated Gammaproteobacteria proportion and exaggerated systemic inflammation (See Chapter 3). Studies in humans and other models have also demonstrated the proinflammatory nature of Gammaproteobacteria [288]. Analysis of total human fecal LPS identified Bacteroidales species as the primary source of fecal LPS in healthy adults and found that Bacteroidales-derived LPS is immunoinhibitory while *E. coli*-derived LPS is immunostimulatory [310]. Bacteroidales-derived LPS inhibited inflammatory action of *E. coli*-derived LPS on cultured peripheral blood mononuclear cells until they were stimulated with over 10x as much *E. coli*-derived LPS as Bacteroidales-derived LPS. The aforementioned study by Ghosh et al. [337] also observed a decrease in Enterobacteriaceae proportion in FO supplemented mice. Another

study by Kaliannan et al. [341] found that mice with elevated tissue omega-3 PUFA levels had a reduced proportion of Proteobacteria. The reduction of Proteobacteria and in particular Escherichia/Shigella in MNFO mice may reduce systemic inflammation and inflammatory response by reducing the amount of circulating endotoxin.

Dietary fish oil unsurprisingly had a large impact on metabolism in MN mice. MNFO mice exhibit increased plasma hexose compared to MNCO mice, suggesting better ability to maintain blood glucose levels. While the total amino acid concentration was similar between groups, MNFO mice had significantly lower concentrations of essential amino acids and ketogenic amino acids. It is unclear whether levels are lower due to differences in absorption, storage, and/or metabolism. The plasma metabolome of MNCO mice showed greater similarity to that of WN mice than that of MNFO mice. These differences were driven largely by differences in lipid metabolites. MNFO metabolomes were discriminated from WN and MNCO metabolomes predominantly by a set of 10 phosphotidylcholines, 7 of which contained PUFAs. WN and MNCO metabolomes were further discriminated from each other predominantly by sphingomyelins, which are implicated in chronic low-grade inflammation [345] and are elevated in MNCO mice compared to WN mice (and usually compared to MNFO mice). The effects of SM on inflammation and response to LPS are in part dependent on SM concentration [345]. Plasma concentration of saturated SM such as SMC18.0 and SMC24.0 were also correlated with insulin resistance, liver dysfunction, and altered liver metabolism in obese subjects [346]. These SM were among the top three metabolites differentially expressed between WN and MNCO mice but were less discriminatory between WN and MNFO mice. MNFO mice also had the lowest concentrations of carnitine and all AC among groups, which may indicate increased beta-oxidation and reduced exposure to inflammatory AC [276,347]. Lastly, MNFO mice had elevated concentrations of SFA compared to WN and MNCO mice and lower concentration of PUFAs than in MNCO mice. The MNFO chow had a greater proportion of SFA than the CO-based diets, but the difference is not enough to

account for the concentrations observed in plasma. Regardless, elevated SFA improves incorporation of omega-3 PUFAs into cellular membranes and supports mechanisms that minimize the inflammatory effects of AA [336].

While MNFO did not appear to impact weight change or fecal burden during *C. rodentium* infection during the first 14 days of infection, mRNA level of IFN-γ in the colon suggests MNFO mice might be better able to eventually clear *C. rodentium* than MNCO mice if the infection was allowed to continue. IFN-γ is critical for control of *C. rodentium*, activation of T cell response, and phagocytosis during infection [339]. The data from the *C. rodentium* and LPS challenge experiments suggest that adding dietary fish oil to a malnourished diet is safe and possibly beneficial in subjects exposed to enteric pathogens. Future studies should investigate the effects of different doses and formulations of DHA and EPA to identify the most successful way to incorporate omega-3 LC PUFAs into MAM treatment.

There are several limitations to this present study. We completely substituted fish oil for corn oil in the diet, which is not realistic for treatment of children with MAM in the field. The primary goal was to achieve a ratio of dietary omega-6 to omega-3 PUFAs that could reduce inflammation. In fact, the ratio achieved is similar to what is recommended for human health [326,348]. We also weaned the mice to this diet, on which they remained for 1-2 months. To mimic incorporation of fish oil in a therapeutic supplement, mice would need to be malnourished before initiating the dietary fish oil. Nevertheless, study of this model enabled initial identification of the effects of fish oil consumption in the context of MAM. While we investigated the effects of dietary fish oil on intestinal barrier function, microbiota composition, and host defense, additional studies to define broader physiological effects would be revealing. The metabolic dataset analyzed in this study is useful in generation of hypotheses regarding mechanisms of omega-3 LC PUFA's effects on malnutrition-related inflammation and dysbiosis but is limited in scope. We only

measured metabolites at one timepoint and in plasma alone, not in other compartments such as the liver. We also did not directly measure EPA and DHA levels in blood and liver.

The data collected in this study suggests several paths forward in advancing use of fish oil in the field. More in-depth studies of the effect of fish oil consumption on intestinal function, circulating immunity, and response to infection would be helpful. In the case of LPS challenge, kinetics studies to determine cytokine expression over time would clarify if fish oil consumption protects weight loss by delaying or reducing inflammatory cytokine production. In the case of *C. rodentium* infection, extension of the infection time course would allow researchers to determine if fish oil consumption accelerates clearance relative to MNCO mice. More detailed metabolic studies to examine liver and kidney function as well as levels of DHA and EPA and byproducts such as eicosanoids and prostaglandins in MNFO mice will help elucidate the mechanisms by which fish oil impacts metabolism and immunity. Studies of body composition could determine if energy is stored differently, despite MNCO and MNFO mice remaining similar in weight. Finally, tests of different ratios of fish oil to corn oil ratios given at different times in the course of malnutrition should be conducted to determine the amount required for beneficial effects on recovery from MAM.

In summary, increased dietary intake of omega-3 reduces the inflammatory effects of malnutrition in a mouse model. Increased dietary omega-3 altered the metabolic profile, intestinal microbiota, and intestinal barrier function, all of which could contribute to the reduced inflammation. Dampening inflammation through dietary omega-3 did not compromise host defense against an intestinal pathogen. In fact, several markers of intestinal innate immunity were improved with increased dietary omega-3. We observed no negative consequences of fish oil consumption in our model, adding to the literature on the safety of omega-3 LC PUFAs as a nutritional intervention. Collectively, these data suggest dietary omega-3 LC PUFAs could be beneficial in the treatment of malnutrition. Future studies should corroborate our findings in children with malnutrition.

Supplemental Table 4.1. Diet composition.

	WN	MNCO	MNFO
Protein (%)	17	3	3
Dextrose (%)	643	807	807
Iron (ppm)	100	10	10
Zinc (ppm)	30	1	1
SFA (% FA)	14.8	14.8	20.5
MUFA (% FA)	26.2	26.2	25.1
PUFA (% FA)	59	59	54.5
Omega-3 PUFA (g/kg)	2.6	2.6	12.0
Omega-6 PUFA (g/kg)	44.9	44.9	29.0
Omega-6:Omega-3	17.5	17.5	2.4

Supplemental Table 4.2. qRT-PCR primers used in this study.

Target	Primer	Sequence (5'-3')	Amplicon Size
18S	18SF	**Got from lab	
	18SR		
Ccl2	CCL2 For	GCTCAGCCAGATGCAGTTAACGC	169
	CCL2 Rev	TGGGGTCAGCACAGACCTCTCT	
Cldn3	Cldn3 For	ACCAACTGCGTACAAGACGAG	78
	Cldn3 Rev	CAGAGCCGCCAACAGGAAA	
Cxcl1	CXCL1F	ATCCAGAGCTTGAAGGTGTTG	171
	CXCL1R	GTCTGTCTTCTTTCTCCGTTACTT	
Cxcl2	CXCL2F	CCAGACAGAAGTCATAGCCACT	160
	CXCL2R	GGTTCTTCCGTTGAGGGACA	
Hapt	Hapt For	GCTATGTGGAGCACTTGGTTC	101
	Hapt Rev	CACCCATTGCTTCTCGTCGTT	
Il1b	IL1BF	TTGACGGACCCCAAAAGATG	204
	IL1BR	AGAAGGTGCTCATGTCCTCAT	
Il17a	IL17A For	ACCAGCTGATCAGGACGCGC	84
	IL17A Rev	CCAGGCTCAGCAGCAGCAACA	
Il17f	IL17F For	TGCTACTGTTGATGTTGGGAC	161
	IL17F Rev	AATGCCCTGGTTTTGGTTGAA	
Il22	IL-22 For	AAACTGTTCCGAGGAGTCAGTGCT	159
	IL-22 Rev	GCTGAGCTGATTGCTGAGTTTGGT	
Il6	IL-6 For	AGACAAAGCCAGAGTCCTTCAGAGA	146
	IL-6 Rev	GCCACTCCTTCTGTGACTCCAGC	
Reg3b	Reg3B For	ACTCCCTGAAGAATATACCCTCC	165
	Reg3B Rev	CGCTATTGAGCACAGATACGAG	
Reg3g	Reg3G For	ATGCTTCCCCGTATAACCATCA	201
	Reg3G Rev	GGCCATATCTGCATCATACCAG	

Supplemental Table 4.3. SPLSDA Error Rates and Components.

Overall Error Rate			**BER**		
	Max Dis	**Centroid**		**Max Dis**	**Centroid**
Comp 1	0.375	0.167	**Comp 1**	0.333	0.141
Comp 2	0.000	0.000	**Comp 2**	0.000	0.000

Component 1	**Weight Coefficient**	**Component 2**	**Weight Coefficient**
PCaeC40.2	-0.664	SMC18.0	0.513
PCaeC40.6	-0.423	SMC24.0	0.462
PCaaC36.5	-0.298	SMOHC22.1	0.450
PCaeC44.5	-0.272	SMOHC16.1	0.373
PCaeC38.0	-0.271	SMC18.1	0.329
PCaeC38.6	-0.237	PCaeC36.3	0.190
PCaaC36.6	-0.230	PCaaC36.1	0.139
PCaeC36.0	-0.121	PCaaC34.1	0.115
PCaeC44.6	-0.106	SMOHC22.2	0.057
PCaaC40.6	-0.090	SMC16.0	0.025

Sparse PLSDA calculated using R packages “Mix0mics” and “pls”. PC = phosphatidylcholine, SM = sphingomyelin, SMOH = hydroxysphingomyelin, aa=diacyl residue, ae=acyl-alkyl residue, Cxx.y= number of carbon atoms followed by number of double bonds (Ex. C40.2 = 40 carbon atoms and 2 double bonds).

DISCUSSION

Chapter 5. Implications of Evidence for Inflammation, Reduced Intestinal Barrier Function, and Metabolic Dysfunction in Pathology and Treatment of MAM

Subjects with MAM exhibit systemic low-level inflammation and exaggerated inflammatory response, which may be a result of super-low dose exposure to circulating endotoxin. Malnutrition-driven changes to the intestine and intestinal microbiota likely underlie this increased inflammation. Interventions that reduce exposure to inflammatory stimuli generated by the microbiota and by-products of metabolism are able to reduce malnutrition-induced inflammation and may have potential to improve odds of long-term recovery from MAM.

Inflammation and Inflammatory Response in Moderate Acute Malnutrition

We measured inflammation and inflammatory response in the blood of children with MAM and in the skin and serum of a mouse model of malnutrition. We observed increased baseline expression of IL-6 in blood from children with MAM and increased basal cytokine production in the skin of mice. Both human whole blood and mouse skin and peritoneal macrophages had increased expression of cytokines on stimulation with LPS compared to healthy controls. Further, MN mice had increased weight loss upon systemic LPS challenge. These data support the idea that MAM produces elevated systemic baseline inflammation and an exaggerated inflammatory response to bacterial stimulus.

However, it is unclear if this phenotype is maladaptive or helpful. Systemic inflammatory response may help to defend against infection but is energetically costly and uses nutrient resources that are already limiting in children with MAM [313]. An effective

immune response requires use of micronutrients such as iron and zinc as enzymatic cofactors, glucose for T cell activation and function, and amino acids for cytokine and effector protein production. Thus, infection exacerbates malnutrition [314] via increased utilization of already scarce nutrients, increased energy expenditure and wasting, inhibition of muscle protein synthesis, and increased loss of micronutrients in urine. Children with complicated SAM who later died had greater systemic inflammation upon admission than children who eventually recovered [77]. In fact, systemic inflammation was a stronger predictor of death in this cohort than intestinal inflammation, diarrhea, or number of enteric pathogens. In MN mice, we observed rapid and drastic body weight loss upon exposure to systemic LPS. These data support the idea that increased mortality in children with acute malnutrition and infectious disease is a physiological consequence of cytokine storm. However, an attenuated response to infection may also be detrimental in a child's ability to clear a pathogen. Children with SAM have predominantly been found to have an attenuated acute phase response (APR) to infection, which may contribute to their increased mortality from infectious disease in other ways [314]. While an attenuated systemic inflammatory response may prevent mortality from cytokine storm, failure to clear infectious pathogens can also be deadly. Examples failed pathogen clearance were detailed in Chapter 1, including reduced clearance of *Mycobacterium tuberculosis* and increased dissemination of *Leishmania donovani*. The danger of attenuated immune response was also observed in children with kwashiorkor, who experience muted APR to infection and increased mortality compared to children with marasmus [313].

MAM and SAM appear to drive different inflammatory responses, but it is unclear what mechanisms underly these effects. Our evidence suggests that MAM induces endotoxin priming in leukocytes, while evidence in the literature suggests children with SAM often develop endotoxin tolerance [136]. There are several dose-dependent ways that circulating leukocytes respond to systemic endotoxin. Endotoxin tolerance occurs when exposure to low dose (1-100 ng/mL) LPS induces an attenuated response upon greater

exposure [261]. This is likely a protective mechanism to attenuate a pathologic inflammatory response to infection. Endotoxin priming occurs when exposure to super low dose (0.05-0.5 ng/mL) endotoxin drives exaggerated inflammatory cytokine release upon a second exposure, particularly of IL-1B, TNF-A, and IL-6. Super low dose endotoxin exposure also induces mild and persistent cytokine exposure, even without secondary stimulus [349]. This priming generally occurs in response to circulating levels of endotoxin, not when the dose of endotoxin is constrained to a particular tissue location [261]. This phenotype of endotoxin priming matches what we have observed in children and mice with MAM, with increased baseline expression of inflammatory cytokines and exaggerated response to LPS. In mice, we observed increased TNF-A production in response to LPS in peritoneal cells in vitro and increased response to intradermal LPS in skin. The response of peritoneal cells is a classic example of endotoxin priming. The response in skin may still represent endotoxin priming, as previous research demonstrated increased local response to intradermal endotoxin in skin after an initial intravenous injection of endotoxin [350]. While we do not know the concentration of circulating endotoxin in serum from mice or children with MAM, previous studies of children with SAM have measured LPS concentrations within the range known to induce tolerance [158,282] and have demonstrated tolerance of dendritic cells to LPS exposure [136]. However, measuring circulating endotoxin levels is still difficult due to high possibility of contamination, the presence of inhibitors in serum, and the need for tests sensitive enough to measure super low dose concentrations in diluted serum.

Further evidence for the role of endotoxin exposure in inflammatory response is the link between intestinal Proteobacteria (particularly *E. coli*) and the inflammatory phenotype. In mice with higher levels of *E. coli*, (MN, VANC-MN), we observed greater levels of baseline inflammatory cytokine expression and/or inflammatory response than in mice with comparatively lower levels of Proteobacteria (WN, MNFO, COL-MN). While we only rarely cultured translocated *E. coli* or other Proteobacteria from the MLN, spleen,

and liver of mice, we did observe increased proportion of *E. coli* DNA in the MLN of MN mice compared to WN mice. This may suggest a mechanism of increased systemic exposure to endotoxin, even if the *E. coli* did not survive long outside the intestine. Other mechanisms may also contribute to potential increases in endotoxin exposure, including minor intestinal damage and dampened LPS dephosphorylation in the intestine, liver, and spleen. In MN mice, we observed decreased intestinal expression of mRNA encoding IAP, a protein that detoxifies LPS and bacterial flagellin and reduces bacterial translocation [303]. Lastly, decreased circulating LBP in children with MAM may exacerbate the inflammatory response to LPS. While high concentrations of LBP inhibit response to LPS, low concentrations accelerate LPS signaling through TLR4 [273].

Endotoxin is not the only potential inflammatory stimulus in children with MAM, as certain classes of lipid metabolites can also induce inflammation. SMs, long chain ACs, and saturated LCFA have all been implicated in promotion of inflammation. They generally induce inflammation by disturbing lipid raft formation or directly binding TLRs and related signaling proteins [345,351]. Sphingolipids can both induce and repress inflammation dependent on context, thus it is difficult to conjecture about the role they play in MN mice [345]. However, SM are substrates for sphingomyelinases whose activity are required for TLR4 lipid raft formation and subsequent inflammatory cytokine production [345]. SMs were generally upregulated in the serum of MN mice compared to WN mice and were prominent components of the PLSDA used to classify metabolomic samples. Both long chain acylcarnitines and saturated LCFA have also been mechanistically linked to inflammation [351] in a variety of cell types. We observed a significant negative correlation between acylcarnitines C16 and C18.1 and measures of wasting and underweight in children with MAM. In PBMCs from a healthy adult volunteer, we observed a synergistic relationship between LPS and saturated LCFA in inducing inflammatory cytokines.

In sum, the data suggest that super low dose exposure to LPS may drive endotoxin priming in children and mice with MAM. Metabolic mediators of inflammation may also contribute to this state in children with MAM. As malnutrition progresses to SAM, increased exposure to LPS and loss of necessary macro and micronutrients may drive endotoxin tolerance and attenuated APR typical of SAM.

INTESTINAL FUNCTION AND BACTERIAL TRANSLOCATION IN MALNUTRITION-RELATED INFLAMMATION

While we have established that circulating immune cells in children and mice with MAM exhibit characteristics of endotoxin priming, we have not discussed potential sources and mechanisms of endotoxin exposure. While we have limited data from our cohort of children with MAM on intestinal damage and microbiota, our studies in mice suggest that MAM dampens antibacterial defenses across multiple organs, leading to increased extra-intestinal bacteria (and likely bacterial products) in absence of overt intestinal permeability.

We have examined intestinal function in WN, MN, and MNFO mice, predominantly via mRNA expression in the distal third of the small intestine. Overall, we observed dampened expression of cytokines involved in immune cell recruitment (*Cxcl1*, *Ccl2)*, intestinal barrier function (*Il25*), prevention of translocation (*Cx3cr1*), and dephosphorylation of LPS (*Alpi*). Analysis of PP mRNA and CD4+ cells also suggests that intestinal CD4+ T cells are anergic in MN mice, potentially due to dysfunctional antigen presenting cells [136,352]. As PPs are the site of antigen sampling in the intestine, reduced innate and adaptive immune function in PP likely contributes to dampened immunity throughout the small intestine. Though we did not observe increased permeability with FITC-Dextran gavage, MN mice exhibited higher mRNA level expression of TJ modulating protein zonulin (*Hapt*). MNFO mice experienced reduced extra-intestinal burden and dampened response to inflammatory stimulus. Intestinal mRNA expression of antimicrobial proteins Reg3G and Reg3B, chemokine CXCL1, and tight junction protein

Claudin 3, combined with decreased *Hapt* expression suggests that MNFO mice have a more active immune defense and mucosal barrier against bacteria in the intestine, resulting in decreased spread of bacteria and bacterial products to other locations.

One feature frequently observed in mice with MAM is increased presence of culturable bacteria in the MLN, liver, and spleen. Extraintestinal burden was quantified via culture of tissue homogenates and was likely not advanced enough to detect strictly anaerobic species. However, of the bacteria we were able to culture, the majority were gram-positive. This was unexpected, considering our hypothesis that circulating immune cells are primed by endotoxin. We observed increased proportions of *E. coli* DNA in the MN cecal contents and MLN compared to WN mice, but this does not necessary translate to increased presence of live bacteria in the liver or spleen. Further, overall proportions of *E. coli* in the microbiota remain minimal (approximately 4% in MN cecal contents, 3% in MN MLN). Despite lack of evidence for translocation of live gram-negative bacteria to the spleen and liver, there are several mechanisms that may contribute to systemic endotoxin exposure. Gammaproteobacteria penetrated deeply into the crypts of the small intestine in mice with experimental EE [98] and are known to modulate intestinal permeability [353]. Close contact with intestinal epithelial cells coupled with dampened intestinal immune function may allow for transcellular passage through enterocytes of live bacteria and bacterial products. Previous studies in rats have observed this phenomenon, even in undamaged enterocytes [353]. Additionally, evidence in animal models and humans with sepsis suggests that once outside the intestinal lumen, bacteria and bacterial products are primarily drained via lymphatics, rather than through the portal vein. Thus, any escaped bacteria or bacterial products will first be trafficked to the MLN. Previous studies in this mouse model of MAM demonstrate dampened barrier function and reduced number of phagocytes in a draining LN [305], which may result in failure to capture and eliminate those bacteria and bacterial products. From the MLN, lymph is eventually returned to the circulatory system via the thoracic duct and subclavian vein [353]. Circulating immune cells

would be exposed to bacteria and bacterial products that, if not cleared, would reach the spleen and liver. This pathway of bacterial translocation suggests that multiple mechanisms for bacterial clearance must fail for colonization of the spleen and liver to occur and is supported by evidence of impaired immune function in the intestine of MN mice in our study and reduced phagocytosis in the MLN [305] and liver [308] of mice with PEM in the literature.

FUTURE OF DIAGNOSTIC MARKERS AND TREATMENTS FOR MALNUTRITION

Biomarkers

There is a need to identify goalposts of recovery from malnutrition beyond changes in anthropometric scores. Recovery from metabolic dysfunction [129] and susceptibility to life threating infectious diseases lag behind weight gain during treatment for SAM [177]. These studies suggest that incomplete recovery may contribute to increased relapse and death. The work detailed in this dissertation has identified plasma biomarkers that are representative of intestinal health, liver function, and metabolism in children with MAM. These biomarkers should be measured over the course of recovery from MAM to determine if they are acceptable proxies for recovery of specific systems that are impaired during MAM. The first of these markers is iFABP. We observed a clear difference in plasma iFABP concentration between children with MAM and healthy children (2163 ± 893.3 vs 61.49 ± 21.18 pg/mL), indicating recent intestinal damage in children with MAM. This data was corroborated by reduced citrulline levels in children with MAM, as citrulline is a measure of enterocyte health and is reduced in villus atrophy syndromes [354]. To my knowledge there are currently no published studies of children with MAM that measure iFABP or demonstrate changes in concentration over course of recovery from wasting. However, iFABP has been measured in plasma of children hospitalized with SAM [158,282], adults with SAM [355], or children with stunting and/or underweight [281,356]. A study of children with SAM observed an inverse correlation between iFABP and MUAC

but not any other anthropometric score [282]. The concentration of iFABP in children in that study was within the range for children with MAM in our study (2938.19 ± 1409.89 pg/mL). Another study of children with SAM and community controls demonstrated both groups had similar concentrations of circulating iFABP (mean 3140 pg/ml and 2010 pg/ml, respectively) [158]. However, both of these studies examined only hospitalized, severely malnourished kids, with persistent diarrhea and only one provided values for community controls. The high concentration of iFABP in community controls also highlights the difficulty of identifying a true "healthy" control group. EED is widespread in regions with high burdens of MAM and likely contributes to elevated iFABP levels. Thus, to use iFABP concentration as a marker of intestinal recovery may require comparison not to healthy community controls, but to a historical reference range or the pre-intervention level in a treated child.

The second potential plasma biomarker of recovery we found during our research was LBP. Surprisingly, circulating LBP concentration was reduced in children with MAM (453 ± 86 ng/mL) compared to healthy children (8499 ± 3290 ng/mL). This acute phase protein is typically increased during systemic inflammation [272]. However, we failed to observe an increase in expression of any other APR proteins in children with MAM, suggesting impaired hepatic protein synthesis may be the major determinant of APR protein levels. Other recorded instances of exceedingly low LBP concentrations occur during acute liver damage [357]. Further, low levels of LBP sensitize circulating immune cells to LPS and induce greater inflammatory cytokine response, while higher levels inhibit LPS transfer to CD14 and attenuate inflammatory response [272]. Compared to normal reference ranges in adult human serum (5000-15000 ng/mL) [358] and in healthy 6 year old American children (8500 ± 2900 ng/mL) [359], plasma LBP concentrations were normal for well-nourished children and low for children with MAM in our study in Kenya. One of the aforementioned studies of hospitalized children with SAM and diarrhea measured a low concentration of LBP in children with SAM (219.37 ± 107.71 ng/mL) but observed no

correlation with iFABP or any anthropometric measures [282]. The lack of correlation may be due to the severity of SAM in all children in the cohort. Another previously mentioned study of children with underweight and stunting observed LBP levels similar to those of the well-nourished children in our cohort in both children with malnutrition and healthy controls [281]. However, these children were only slightly (or not at all) wasted by WHZ standards. While it is not certain that low concentrations of LBP are indicative of liver damage in children with MAM, we do observe a striking difference between concentrations in children with MAM and well-nourished children as well as a high degree of correlation with other markers affected by MAM. Plasma LBP was correlated with markers of intestinal health, inflammatory cytokines, fatty acid metabolism, and measures of wasting (but not stunting or underweight). Thus, plasma LBP concentration may be linked to recovery of multiple systems during treatment for MAM. Future studies should assess LBP levels in relation to other markers and metabolites over the course of recovery from MAM.

Markers of metabolic recovery may be difficult to identify due to variances in local diets, but our research identified several potential metabolite classes that appear to be elevated in children or mice with MAM. Though we observed heightened SM and long chain AC concentrations in MN mice compared to WN, we observed few differences between children with MAM and well-nourished children. This is likely due to a number of confounders that could not be controlled for due to small sample size (fasted state, age, gender, diet). However, of four metabolites present at significantly different concentrations in children with MAM and well-nourished children, two were long chain AC (C16 and C18.1). Two other long chain AC (C18 and C18.1) also exhibited a trend towards increased concentration in children with MAM. Evidence for metabolomic signatures of MAM and recovery is lacking, as metabolomics studies are generally conducted with children hospitalized for SAM who are all on similar diets. One such study used the same metabolomics assay as we did to assess changes in metabolome over the course of admission with SAM and stabilization as compared to healthy controls [129]. They

observed significant changes in metabolome from admission to stabilization, yet the stabilized metabolome was still far more similar to the admission metabolome than to healthy controls. SMs and C18.1 were noted as differences between children with SAM and healthy controls. The increased long chain AC observed in children and mice with MAM likely indicate incomplete fatty acid oxidation. Studies of protein deficiency in mice have observed mitochondrial dysfunction and peroxisome damage, which drive impaired fatty acid oxidation and steatosis [145]. Thus, decreases in circulating long chain AC may be an indicator of return to healthy liver function in children previously suffering from MAM.

In summary, iFABP, LBP, and AC or SM concentrations in plasma may provide correlates of recovery of intestinal health, liver function, and metabolism. However, this data is from a narrowly defined cohort of children with uncomplicated MAM from one geographic region. Efficacy of these biomarkers as potential benchmarks of recovery might be different in populations from other regions or with complicating conditions such as EED or stunting.

Treatments

Due to the influence of microbiota on other biological systems, treatments directed at modulation of the intestinal microbiota can eventually have systemic downstream effects necessary for long lasting recovery from MAM. Our study of fish oil supplementation in mice with MAM suggest that omega-3 LC PUFAs show promise as a treatment that could both directly and indirectly modulate inflammation, metabolism, and microbiota. In MNFO mice, we observed decreased skin inflammation, reduced extra-intestinal bacterial burden, increased expression of intestinal defense proteins, and reduced Proteobacteria in the microbiota. MNFO mice also experienced significant alterations in lipid metabolism, maintained the same weight with less food consumption than MN mice, and were able to better maintain weight during LPS challenge. Finally, increased colonic expression of *Ifng*

on day 14 post *C. rodentium* infection suggests that they might have eventually cleared the infection faster than MN mice. All of these outcomes would be beneficial in children undergoing treatment for MAM and thus far we have not observed any harmful effects of fish oil intake either at baseline or in response in infection. However, many questions remain to be addressed before fish oil can be touted as a treatment for MAM. This study used a 1:1 substitution of fish oil for corn oil in chow. It is not feasible to provide such a high concentration of fish oil to children during MAM treatment, thus other studies should be conducted with varying ratios of omega-3 PUFA to omega-6 PUFA to determine the lowest amount of fish oil that provides necessary benefits when given as a RUSF. As different effects have been observed with different formulations of DHA and EPA, it may be necessary to trial different delivery formats and FA compositions as well [360]. Our study observed effects of fish oil in mice over the course of development of MAM, not as a treatment for MAM. Again, future studies are needed to see what differences in outcomes occur when fish oil is given as a treatment after development of MAM, and the length of time needed to see beneficial results. Finally, shelf stability has been a major obstacle to inclusion of omega-3 LC PUFAs in RUSFs. An experimental LNS was recently produced with flaxseed oil that remained shelf stable for 6 months [361]. However, the form of omega-3 PUFA in flaxseed oil (ALA) is not efficiently converted to DHA or EPA and studies of ALA supplementation have shown little benefit in treating malnutrition [328]. Another options for incorporation of fish oil into MAM treatment include providing it as an additional supplement in either dropper or pill form, depending on the age of the child.

While the selective antibiotic experiment conducted in MN mice was intended to explore the effects of specific bacteria and not as a potential treatment, the study provoked several considerations on the role of antibiotics during treatment for SAM or MAM. Ampicillin, amoxicillin, or other broad-spectrum antibiotics are given as part of the normal protocol for treatment of SAM, regardless of presence or lack of evidence of infection [1]. The benefit of this protocol has been debated extensively [362-364]. A well-designed and

well-conducted study provided evidence to support marginal increases in weight gain and reduced mortality in children treated with antibiotics [365], but these results have not been widely replicated. Mass administration of another broad-spectrum antibiotic (azithromycin) among all children in entire communities reduced all-cause mortality in children under 5, though this effect was only significant among the communities with the highest rates of child mortality [253]. A follow-up study indicated little evidence of reduced efficacy due to antimicrobial resistance after 3 years of azithromycin administration [366], but there was increased prevalence of antimicrobial resistance genes in the microbiota of treated children [367]. It remains to be seen why this protocol was only beneficial in high-risk communities and what mechanisms underlie the reduction in mortality, though long-term reduction of *Campylobacter* species appears to play a role [367]. It is also unclear what effects, if any, this protocol will have on health of these children later in life. Importantly, antibiotic treatment early in life can provoke long-lasting changes to the microbiota that may also prove detrimental [368]. In VANC-treated mice, we observed a massive drop in diversity and an overwhelming expansion of Proteobacteria. It is unclear if this drastic of a change in microbiota occurs in MN children given broad-spectrum antibiotics. To my knowledge no publications have described the effects of antibiotics on the malnourished microbiota. In COL-treated mice, we observed less severe microbiota alterations, mainly reduction of Gammaproteobacteria and Betaproteobacteria. Non-absorbable oral colistin might be a viable option to replace broad spectrum antibiotic use in children with SAM and MAM, as COL-treated mice demonstrate that targeted reduction of pathobionts can improve health without dramatically overhauling the intestinal microbiota. Thus, a more judicious use of antibiotics that target specific pathobionts during treatment for SAM may be worth consideration. Long-term studies of microbiota development in children treated for SAM with and without antibiotics may also help researchers weigh the costs and benefits of routine antibiotic use.

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