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Environmental, Metabolic, and Inflammatory Factors Converge in the Pathogenesis of Moderate Acute Malnutrition in Children: An Observational Cohort Study

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Abstract. Acute malnutrition affects more than 50 million children worldwide. These children are at an increased risk of morbidity and mortality from infectious disease. However, the pathogenesis of acute malnutrition and mechanisms underlying the increased risk and poor outcomes from infection are not well understood. Our objective was to identify differences in inflammation and inflammatory responses between children with moderate acute malnutrition (MAM) and healthy controls (HCs), and search for environmental, pathophysiological, and metabolic factors that may influence this response. Sixteen children with MAM and 16 HCs aged 18–36 months were studied in Nairobi, Kenya. None of the children had symptoms of an infectious disease (fever, diarrhea, or cough) in the 2 weeks before enrollment and sample collection. Demographic and health data were provided by their primary caregivers. Blood samples were collected to measure various biomarkers and the response to an inflammatory stimulus. Children with MAM were more frequently from households with contaminated water, crowding, and unstable income sources. They also had increases in basal inflammation, circulating bacterial lipopolysaccharide (LPS), markers of intestinal damage, and an exaggerated whole blood inflammatory response to LPS. Metabolic changes in children with MAM led to increased plasma levels of long-chain fatty acids, which were found to contribute to the pro-inflammatory state. 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 pathogenesis of MAM.

INTRODUCTION

Acute malnutrition (wasting) affects more than 50 million children younger than 5 years.[1] The WHO defines two levels of severity of acute malnutrition, moderate (moderate acute malnutrition [MAM]) and severe acute malnutrition (SAM), as identified by the weight-for-height *Z* score (WHZ) and/or mid-upper arm circumference (MUAC).[2] Moderate acute malnutrition comprises approximately two-thirds of all cases of childhood acute malnutrition.[3]

Severe acute malnutrition and MAM increase a child's odds of dying by approximately 9-fold and 3-fold, respectively, compared with healthy children.[4] Most of the malnutrition-related increase in childhood mortality is due to an increased susceptibly to a broad array of pathogens.[5–7] 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 *Escherichia coli* and subsequent growth impairment. This is likely due to intestinal damage, increased nutrient loss, malabsorption, and diversion of energy to the immune response.[8] These mechanisms parallel the effects of environmental enteric dysfunction (EED), a subclinical condition characterized by physiological, anatomical, and functional changes to the small intestine that are associated with frequent exposure to enteropathogens.[9] Environmental enteric dysfunction is highly prevalent in regions with elevated rates of malnutrition and can mediate both stunting and wasting, although it has not been causally linked to malnutrition.[10]

There is a high prevalence of bacteremia in children hospitalized with SAM. Two studies found that approximately 17% of children admitted with SAM also had bacteremia.[11,12] Most cases were due to Gram-negative enteric species such as *Salmonella* and *E. coli*.[11,12] Severe acute malnutrition-associated bacteremia 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 antibacterial phagocytic cells.[5–7] Systemic infection with enteric bacteria carries high morbidity and mortality in malnourished children.[5] Intestinal damage in children with SAM also creates chronic systemic exposure to lipopolysaccharide (LPS) and other bacterial products.[13] In a study of children with SAM, the level of endotoxemia was inversely correlated with activation status of dendritic cells,[14] akin to the dampened inflammatory response seen in endotoxin tolerance.[15] This is likely a protective mechanism in malnourished children to mitigate the deleterious effects of chronic exposure to LPS. However, this anergic response may contribute to increased susceptibility to some pathogens.

Few studies describe the pathology and pathophysiology of MAM in children. No markers of host defense or inflammatory status have been consistently associated with MAM. Circulating inflammatory cytokines (e.g., tumor necrosis factor [TNF] and interleukin-6 [IL-6]) and acute-phase response proteins have been inconsistently elevated in children with MAM.[5,6] Differences in study populations and variable presence of overt or subclinical infections make comparison of results between studies difficult.

Although there are currently no globally standardized approaches for treatment of MAM,[16,17] the WHO recommends that children with MAM should be given locally sourced energy-dense supplementary food and monitored for growth recovery.[18] However, with this approach, approximately 20–30% of children

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fail to recover or relapse within a year after recovery.[19–21] Persistent alteration of the intestinal microbiome appears to be a driver of relapse in children who have recovered from SAM.[22] The metabolic profile of children with SAM is markedly different from that of healthy children and does not normalize after nutritional stabilization.[23] Importantly, growth recovery from SAM does not appear to reduce the incidence and mortality of infectious disease,[24] suggesting that recovery of host defense may not parallel growth recovery. Better understanding of the pathophysiology and immunological deficits in SAM and MAM will enable targeted therapeutic nutritional interventions to achieve better clinical outcomes.

In this study, we identified sociodemographic and environmental factors and biomarkers of immune function and inflammation associated with MAM in a cohort of children from an urban slum in Nairobi, Kenya. We correlated measures of inflammation, markers of intestinal damage, and plasma metabolites with environmental and sociodemographic data to provide an exploratory model from which hypotheses can be generated and tested in future studies. In addition to contributing to a better mechanistic understanding of the pathogenesis of MAM, these markers can be explored as potential correlates of recovery after treatment for MAM.

MATERIALS AND METHODS

Subject enrollment and evaluation. The present study was an exploratory cross-sectional study of cohorts of 16 children with MAM and 16 healthy controls (HCs) residing in the same urban slum environment in Nairobi, Kenya. This study was nested within a larger longitudinal study designed to identify risk factors for infection and relapse in 60 children with MAM. Children from the Gatwikira and Soweto villages in the Kibera slum were identified for possible inclusion in the study by community health workers of the Centre for Clinical Research (CCR) at the Kenya Medical Research Institute (KEMRI). Possible subjects were referred for screening evaluation and enrollment at the KEMRI CCR. An algorithm of study procedures is provided in Supplemental Figure 1. Inclusion criteria for the parent study were age 6–60 months and presence of MAM as defined by MUAC equal to or less than 12.5 cm but greater than 11.5 cm, and/or WHZ greater than –3 but less than or equal to –2. Children were excluded if they had SAM as defined by WHZ less than or equal to –3; had chronic severe malnutrition as defined by height-for-age *z* score (HAZ) less than or equal to –3; had severe anemia (hemoglobin < 6.0); had chronic illness such as AIDS, tuberculosis, or malignancy; were HIV+; had active infection in the 2 weeks before enrollment, such as pneumonia, gastroenteritis, urinary tract infections, or meningitis; or had a temperature > 37.5°C.

Children from the larger longitudinal study were selected for inclusion in the present cross-sectional sub-study at the time of enrollment if they were aged 18–36 months. The first 16 children within this age-group were enrolled in the sub-study. Sixteen age- and gender-matched HC subjects were recruited simultaneously from the screened study population. Inclusion and exclusion criteria remained the same, with the exception that HC subjects had MUAC > 12.5 and a WHZ > –1. All sub-study procedures and data collection were completed before initiation of treatment according to the Kenyan Ministry of Health guidelines (http://guidelines.health.go.ke:8000/media/IMAM_Guideline_Kenya_June09.pdf).

Caregivers of subjects who met the criteria for enrollment gave their informed consent for inclusion during the initial screening and enrollment visit. Following enrollment, caregivers 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 during the same visit. Biological samples for all tests in this study were obtained from all participants during their enrollment visit.

Consent forms were available in Kiswahili and English and were 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 KEMRI (SERU/SSC Protocol 2871), Nairobi, Kenya. Blood was collected from the healthy adult blood donor after informed consent under protocol no. 11–285 approved by the Institutional Review Board of the University of Texas Medical Branch.

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: 336 Seca) to a precision of 0.01 kg. The MUAC was measured at the midpoint of the left arm using a non-stretch insertion tape to a precision of 0.1 cm. At least two readings of each measure were taken and averaged. Measures differing by more than 0.3 cm were discarded and measurements retaken. The anthropometric measures were expressed as *z* scores based on WHO child growth standards.[25]

Stool microscopy. Fresh stool was collected from the 16 HCs and 16 subjects with MAM for qualitative microscopy to determine the presence of ova and cysts of intestinal parasites. Stool samples were collected in a plain collection vial (AlphaTec, Vancouver, WA) and a vial containing preservative (ProtofixTMCLR, AlphaTec, Vancouver, WA). Each stool sample was immediately processed and evaluated using wet-preparation, qualitative Kato–Katz and formol-ether concentration techniques by an experienced laboratory technician.

Blood collection and whole blood assay. We collected blood samples from the 16 HCs and 16 subjects with MAM for analysis of plasma biomarkers and determination of the whole blood leukocyte response to bacterial LPS. 1.0 mL whole blood was collected in a tube containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant for complete blood count and leukocyte differential. Anemia was defined according to WHO standards with correction for the altitude of Nairobi.[26] An additional 5.0-mL heparinized blood was collected and aliquoted for immune/inflammatory biomarker analysis as follows: 3 mL of the heparinized whole blood was centrifuged, and the naive plasma was 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 LPS from *E. coli* O55:B5 (Sigma-Aldrich, St. Louis, MO) in PBS as stimulation. After incubation, the whole blood was centrifuged, and the plasma was collected and stored at –80°C. Each of the three types of plasma samples (naive, incubated-unstimulated, and incubated-LPS-stimulated) were then analyzed for expression of 10 cytokines via Quantibody array (Ray Biotech, Peachtree Corners, GA). Concentrations were calculated from mean fluorescent intensity (MFI) using a log–log regression curve. Concentrations below the limit of detection (LOD) 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), IgM antibody against endotoxin core (EndoCAb) (Hycult Biotech, Wayne, PA), haptoglobin (Hycult Biotech), LPS-binding protein (LBP) (Abcam, Cambridge, MA), intestinal fatty acid-binding protein 2 (iFABP or FABP2; Abcam), and soluble CD14 (sCD14) (ThermoFisher Scientific, Waltham, MA) in naive plasma samples. Magnetic Luminex Assays (R&D Systems, Minneapolis, MN) were used to measure C-reactive protein (CRP), procalcitonin, growth hormone, leptin, insulin, and adiponectin in naive plasma samples. Data were acquired on a Bio-Plex 200 system (Bio-Rad, Hercules, CA), and the standard curve was calculated via the five-parameter logistic curve.

Endotoxin activity was measured in naive plasma samples using the HEK-Blue LPS Detection Kit 2 (InvivoGen, San Diego, CA) as per the manufacturer's protocol. Samples were diluted 1:3 in assay diluent before quantification.

Plasma metabolite measurement and analysis. One hundred eighty plasma metabolites including amino acids, biogenic amines, phospholipids, sphingolipids, glycerophospholipids, and acylcarnitines were measured by Biocrates Life Sciences (Biocrates, Innsbruck, Austria) in naive plasma samples. Amino acids and biogenic amines were measured by liquid chromatography–mass spectrometry, whereas 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 LOD 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 before univariate statistical analysis. Microsoft Excel (Microsoft, Redmond, WA) and *R* (R Foundation for Statistical Computing, Vienna, Austria) were used to perform metabolite analysis.

Peripheral blood mononuclear cell (PBMC) isolation and fatty acid stimulation. Peripheral blood mononuclear cells were isolated from a healthy adult volunteer for stimulation with saturated long-chain fatty acids (LCFAs) 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 400 g for 40 minutes at room temperature. Cells were transferred from the gradient ring to a new tube and washed in MEM twice and culture medium (Roswell Park Memorial Institute [RPMI] medium, with L-glutamine and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 10% fetal bovine serum [FBS], and 1% penicillin/streptomycin) once. Wells in a 96-well plate were seeded with 200,000 viable 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) were prepared with fatty acid–free bovine serum albumin (AK8909; Akron Biotech, Boca Raton, FL) as described previously.[27] These solutions (or vehicle control) were then added to cells to produce a final concentration of 200 μM. Some cell cultures 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, Hercules, CA). Data were acquired on a Bio-Plex 200 System and standard curve calculated via the five-parameter logistic curve. Concentrations above the upper limit of quantification (ULOQ) were given the value of the ULOQ.

Peripheral blood mononuclear cell stimulation with plasma and LPS. Peripheral blood mononuclear cells were isolated, seeded at 200,000 cells per well, and rested as described earlier. Plasma from four randomly selected children with MAM and four randomly selected HCs was added to duplicate wells at a final well concentration of 20%. Half of all wells also received LPS at a final concentration of 1 ng/mL immediately after addition of plasma. Negative (media only) and positive (media and LPS only) control conditions were conducted in quadruplicate. 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, IL-1β, and TNF-α as described earlier.

Data management. De-identified study data were collected and managed under a unique subject ID using the Research Electronic Data Capture (REDCap) tool hosted at the University of Texas Medical Branch.[28,29]

For this study, only data from the 32 participants (16 HCs and 16 MAM) from the immune function sub-study were analyzed. The dataset was exported from REDCap into Excel where it was cleaned and formatted for analysis in SAS (SAS Institute, Inc., Cary, NC). Graphics were generated in GraphPad Prism (version 8.1.2 for macOS, GraphPad Prism Software, La Jolla, CA) 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, SD, median, and interquartile 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. The Wilcoxon rank-sum test was used to compare continuous variables between groups. Protein expression levels were compared between HC and MAM groups via the Wilcoxon rank-sum test. We used Spearman's correlation to identify any correlations between biomarker expression and demographic or anthropometric variables; pairwise deletion was used where data were missing. All tests conducted in the aforementioned analysis were two-sided. A P-value of < 0.05 was deemed statistically significant. All analyses were explorative and were performed using SAS software (SAS Institute, Inc.). For sociodemographic data missing in the comparison of the HC and MAM groups, the missing data were omitted and the remaining data analyzed. Missing data are identified in the footnotes to Table 1.

RESULTS

Subject characteristics. Subject characteristics that were significantly different between groups are shown in Table 1, and the full dataset is found in Supplemental Table 1. Mid-upper arm circumference was used to classify subjects into HC (≥ 12.5 cm) and MAM (< 12.5 cm) groups. Children with MAM were significantly younger (740 versus 851 days, $P = 0.026$) than the HC group. Children with MAM were significantly more wasted by WHZ (–1.6 versus –0.2, $P < 0.001$) and underweight (weight-for-age Z score; WAZ –2.2 versus –0.9, $P < 0.001$). However, only three of 16 children in the MAM group met the criteria for MAM by WHZ. The median HAZ was similar between groups (–1.9 versus –1.7, $P = 0.137$).

TABLE 1
Demographics, anthropometrics, and health metrics at enrollment

	Healthy controls (n = 16)	MAM (n = 16)	P-value (HC–MAM)
Demographics/socioeconomic status			
Gender: female, n (%)	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*	4 (1–7)	5 (2–8)	**0.045**
Caregiver age	26 (22–28)	26 (22–31)	0.484
Caregiver's source of income,† n (%)			0.073
Husband	11 (73)	6 (38)	
Other‡	4 (27)	10 (63)	
Father's source of income,§ n (%)			**0.024**
Wife/relatives/friends	0 (0)	4 (27%) 4/15	
Employment	11 (73)	4 (27%) 4/15	
Self-employment/business	4 (27)	7 (47%) 4/15	
Appliances owned (TV/radio/phone), n (%)			**0.014**
One	4 (25)	8 (50)	
Two	5 (31)	6 (38)	
Three	7 (44)	2 (13)	
Water source, n (%)			**0.007**
Piped	16 (100)	9 (56)	
Purchased	0 (0)	7 (44)	
Water treatment,‖ n (%)			**0.044**
Chlorine	6 (38)	3 (20)	
Boiling	9 (56)	5 (33)	
No treatment	1 (6)	7 (47)	
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 (−0.5–1.0)	−1.6 (−1.3–−2.1)	**< 0.001**
Not malnourished, n (%)	16 (100)¶	12 (75)	0.101
Moderately malnourished, n (%)	0 (0)	3 (19)	
Severely malnourished,# n (%)	0 (0)	1 (6)	
Height-for-age z score	−1.7 (−1.8–−0.9)	−1.9 (−2.3–−1.6)	0.180
Not stunted, n (%)	14 (88)	10 (63)	0.356
Moderately stunted, n (%)	1 (6)	4 (25)	
Severely stunted, n (%)	1 (6)	2 (13)	
WAZ	−0.9 (−1.4–0.3)	−2.2 (−2.7–−1.6)	**0.003**
Not underweight, n (%)	13 (81)¶	6 (38)	**0.013**
Moderately underweight, n (%)	3 (19)	7 (44)	
Severely underweight, n (%)	0 (0)	3 (1 9)	
Health metrics			
Hemoglobin (g/dL) (elevation adjusted)**	10.7 (9.6–11.6)	10.2 (9.5–10.9)	0.071
Anemia (Hb < 11.0 g/dl),†† n (%)	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
% 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**

MAM = moderate acute malnutrition; MUAC = mid-upper arm circumference; WHZ = weight-for-height Z score. Median (interquartile) or number (%). P-value by the Wilcoxon rank-sum test for continuous variables, and Fisher's exact (two-sided Pr ≤ P) for categorical variables. Statistically significant values are shown in bold.

* Crowding score is calculated by dividing the number of people residing in the home by the number of rooms in the home.

† Missing data from one child (HC).

‡ "Other" sources of caregiver income reported include self-employment, business, employment, other relatives or friends, or other sources.

§ Missing data from two children (1 HC and 1 MAM).

‖ Missing data from one child (MAM).

¶ One child had an WHZ of 2.68 and WAZ of 2.56, which categorizes them as overweight.

One child in the study was found to be severely malnourished when classified by WHZ, although they were considered moderately malnourished when classified by MUAC.

** Because of Nairobi's high elevation, all hemoglobin levels were adjusted by subtracting 0.5 based on WHO recommendations.

†† Anemia cutoffs taken from the WHO.[27]

‡‡ Missing data from two children (MAM).

All children in this cohort presented at enrollment with normal temperature, heart rate, and pulse. None showed any sign of current infection. Stool tests showed no evidence of current parasitic infection, although malnourished children had significantly increased percentages of eosinophils in their blood (1.5% versus 0%, P = 0.003). It should be noted that the area where the subjects live is a site of frequent antiparasitic drug administration campaigns by uncoordinated volunteer groups. We were unable to obtain an accurate history of recent antiparasitic drug administration to our subjects. Malnourished children also had decreased percentages of circulating monocytes (4% versus 7%, P = 0.047). The frequency of anemia (50% versus 31%, P = 0.473) and elevated platelet counts (509 versus 485, P = 0.867) were high across the cohort, but did not differ significantly between groups.

Children with MAM have evidence of damage to the small intestine. We measured markers of intestinal damage

and bacterial translocation in naive plasma samples (Figure 1). We found that children with MAM had elevated concentrations of iFABP (2053 versus 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 versus 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 HCs. However, we found significantly increased plasma LPS in children with MAM compared with HCs (0.55 versus 0.09 endotoxin units [EU]/mL, $P = 0.020$).

Children with MAM have elevated basal markers of systemic inflammation and an exaggerated inflammatory response to bacterial LPS. We measured eight inflammatory cytokines in naive plasma (Supplemental Table 2) and found significantly elevated levels of IL-6 (40.3 versus 8.4 pg/mL, $P = 0.009$), IL-1β (7.8 versus 2.6 pg/mL, $P = 0.047$), and IFN-γ (69.2 versus 35.9 pg/mL, $P = 0.005$) in children with MAM (Figure 2A). The concentrations of acute-phase proteins such as CRP, procalcitonin, and haptoglobin were not significantly different in children with MAM and HCs (Figure 2B). Lipopolysaccharide binding protein, an acute-phase protein that has dual functions of facilitating LPS-mediated activation of toll-like receptor 4 (TLR4) signaling and detoxifying circulating LPS,[30,31] was significantly lower in children with MAM (0.44 versus 8.09 pg/mL, $P < 0.0001$).

To examine the response to a bacterial inflammatory stimulus, we exposed whole blood to bacterial LPS for 20 hours and measured plasma cytokine levels (Table 2). Blood from children with MAM exhibited increased IL-6 (4,000 versus 3,287 pg/mL, $P = 0.018$) production and a trend toward increased IFN-γ (694 versus 470 pg/mL, $P = 0.051$).

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 versus 0.11 μM, $P = 0.049$) and C18:1 (0.14 versus 0.10 μM, $P = 0.007$) were significantly elevated in children with MAM (Figure 3A). 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 versus 0.16, $P = 0.039$). The metabolic hormones leptin (2,423 versus 3,325 pg/mL, $P = 0.005$) and insulin (341 versus 693 pg/mL, $P = 0.022$) were significantly lower in the MAM group, whereas adiponectin levels were similar between groups (Figure 3B).

Associations between anthropometric measures and variables of intestinal health, inflammation, and metabolism. We conducted Spearman's correlation to examine the relationships between different measures of malnutrition and the variables that showed significant differences between children with MAM and HCs. Significant correlations are displayed in Figure 4, and variables are arranged by hierarchical clustering. Mid-upper arm circumference, WHZ, and to a lesser degree WAZ had similar profiles of correlation. Height-for-age *z* score 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 naive plasma IFN-γ. Whereas both LBP and iFABP strongly correlated with measures of MAM, LBP alone is inversely correlated with both naive and LPS-stimulated cytokine concentrations (Figure 4). Also, although leptin levels are strongly correlated with measures of MAM, they are not correlated with iFABP or LBP. Finally, although circulating LPS concentration exhibited a strong negative correlation with anthropometric measures, there was little correlation with other variables. Thus, effects of circulating LPS on inflammation

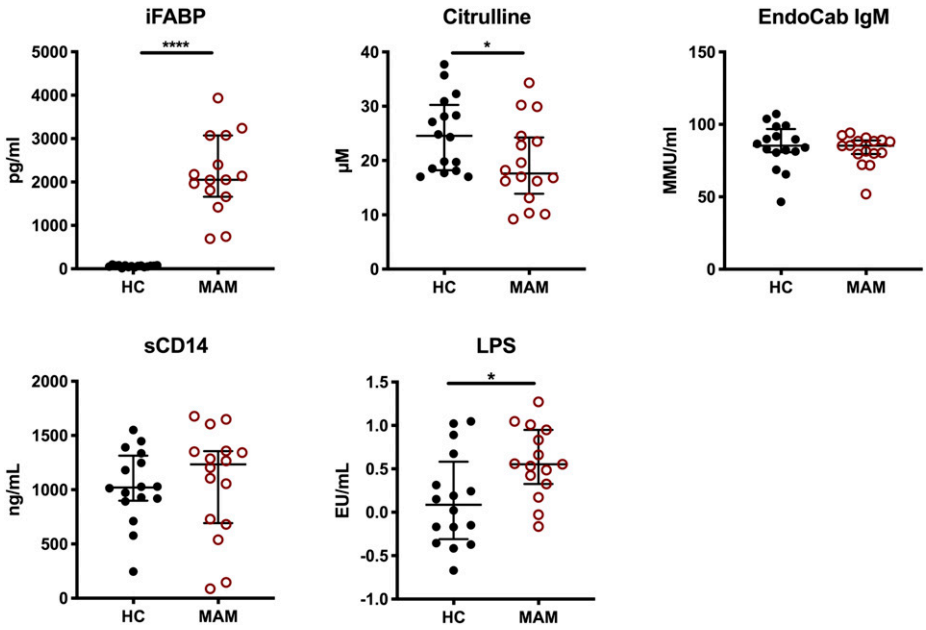

FIGURE 1. Concentrations of markers of intestinal health, bacterial translocation, and inflammation in naive plasma from children with MAM ($n = 16$) and healthy controls (HCs) ($n = 16$). Data missing for lipopolysaccharide value for one child with MAM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. MAM = moderate acute malnutrition. This figure appears in color at www.ajtmh.org.

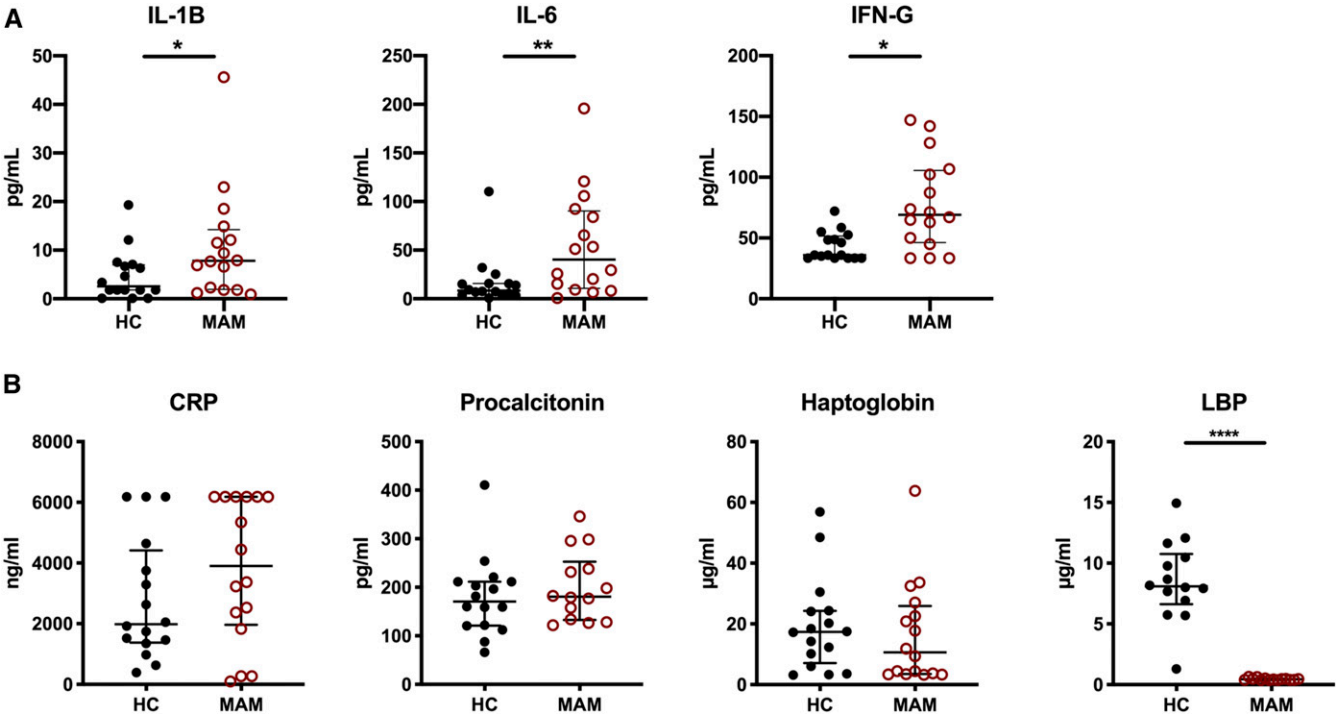

FIGURE 2. Concentration of inflammatory cytokines (**A**) and acute-phase response proteins (**B**) in naive plasma from children with MAM ($n = 16$) and healthy controls (HCs) ($n = 16$). Data missing for LPS-binding protein (LBP) value for two children with MAM and two HCs. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. MAM = moderate acute malnutrition. This figure appears in color at www.ajtmh.org.

and inflammatory response may be indirect and/or mediated through a more complex system.

Saturated long-chain fatty acids induce inflammatory cytokine expression in PBMCs. Children with MAM had increased levels of plasma LCFAs, either through increased absorption or impaired consumption (beta-oxidation) (Figure 1). Because previous studies demonstrated that saturated medium and LCFAs induced inflammatory cytokine production by human and mouse macrophage cell lines,[32,33] 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 5). We observed significant increases in TNF-α, IL-6, and IL-1β production in cells treated with C16 and C18 compared with controls, and increased IL-1β production 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 circulating LPS, can promote inflammatory cytokine production by leukocytes.

Plasma from children with MAM amplifies the inflammatory response to LPS. Malnutrition may drive increased inflammation and inflammatory responses by changing the function of circulating immune cells and the concentration of pro-inflammatory and anti-inflammatory factors in circulation (Figure 1). To determine if factors in plasma alone can induce a MAM-like profile of cytokine expression in healthy cells, we incubated PBMCs from a healthy adult volunteer with plasma from children with MAM or HCs (Figure 6). Some cell cultures were concurrently exposed to low-dose LPS (1 ng/mL). Peripheral blood mononuclear cells incubated with plasma from children with MAM exhibited significantly greater secretion of IL-1B, IL-6, and TNF than PBMCs incubated with plasma from HCs. This pattern was also observed in cells treated with LPS, although exposure to either HC or MAM plasma attenuated the cytokine response. These data indicate that factors in plasma from MAM children contribute to both the increased baseline inflammation and increased inflammatory response to LPS. Although LBP, inflammatory cytokines, LCFAs, and LPS could all contribute to this outcome, the exact factors responsible for this effect have not been defined.

DISCUSSION

In a cohort of children with MAM from an urban slum in Nairobi, Kenya, we evaluated environmental, sociodemographic, and physiological variables with the goal of developing a model of pathogenesis from which new hypotheses could be generated and tested. The cohort of HCs was from the same community. Children with MAM who had caregiver-reported symptoms of an infection within the 2 weeks before enrollment in the study were excluded from the study to avoid confounding of the analysis of inflammatory markers. Our data suggest that low socioeconomic status, crowding within the household, and environmental exposure to non-purified (likely contaminated) water are associated with MAM. Children with MAM had increased markers of intestinal damage, translocation of bacterial LPS to the systemic circulation, evidence of increased inflammation in the basal state, and a heightened whole blood inflammatory response to bacterial

TABLE 2
Median (interquartile) cytokine concentration (pg/mL) in plasma from lipopolysaccharide-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)	**3,287** (2,756.8–4,000)	**223** (155.3–511.9)	**301** (232.3–356.4)	**470** (325.2–653.0)	**620** (350.3–1,225.3)	**823** (470.5–1,374.4)
Moderate acute malnutrition	**314** (160.5–509.0)	**820** (771.9–1,036.7)	**4,000** (3,592.6–4,000)	**205** (153.0–607.7)	**258** (177.2–442.6)	**694** (473.6–1,184.1)	**612** (411.5–895.5)	**814** (651.0–1,077.9)
P-value	0.224	0.402	*0.018*	0.956	0.838	0.051	0.867	0.956

The median values are shown in bold.

LPS. Metabolic changes were associated with MAM. In particular, elevated plasma LCFAs were found to promote systemic inflammation.

Comparison of children with MAM with healthy children 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 is consistent with larger population-based studies.[34,35]

Water source and water treatment had the strongest association with MAM among environmental variables. The purity of the water sold by street vendors from open containers has not been studied, but it is generally considered to have more bacterial contaminants than piped water. Water quality has been linked to stunting, EED, and wasting in cohorts around the world.[10,36,37] Children enrolled in the subset used for this study had no symptoms of diarrheal disease within the 2 weeks before sample collection. However, repeated exposure to enteric pathogens over time, even if it is not associated with clinical symptoms, perhaps through exposure to contaminated water, food, or environment, may result in persistent intestinal damage and weight faltering.[38] In a recent study of children with EED, a consortium of bacteria not typically defined as enteropathogens mediated duodenal inflammation and damage, leading to systemic circulation of intestinal bacteria.[39] There also may be nonpathogenic 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.[40]

Although 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. Intestinal fatty acid-binding protein 2 is a marker of recent intestinal damage as it enters the circulation when the tips of villi in the small intestine are damaged.[41] Citrulline is produced by healthy enterocytes in the small intestine, and low concentrations correlate with several acute and chronic intestinal pathologies.[42] Together, these markers suggest that children with MAM experience intestinal damage that could contribute to poor nutrient absorption, reduced intestinal barrier function, 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,[43] 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.[44] We observed significantly increased levels of bacterial endotoxin in plasma from children with MAM. 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. The level of LPS leakage may be too low to trigger IgM or sCD14 production. However, the elevated levels of IFN-γ, IL-6, and IL-1β observed in naive plasma of children with MAM suggest that even in the absence of overt infection, there is chronic, low level inflammation.

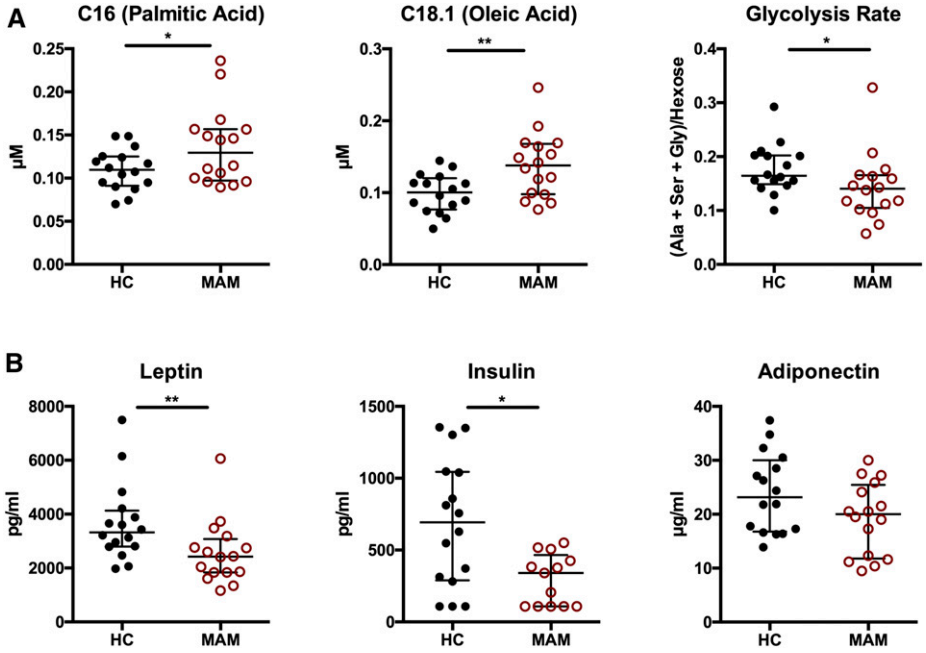

FIGURE 3. Concentrations of metabolites (**A**) and metabolic regulators (**B**) in naive plasma from children with moderate acute malnutrition (MAM) (n = 16) and healthy controls (HCs) (n = 16). Glycolysis rate is estimated from amino acid concentrations ([alanine + glycine + serine]/hexose). *P < 0.05 and **P < 0.01. This figure appears in color at www.ajtmh.org.

The increased levels of plasma iFABP and LPS, and reduced LBP suggest possible pathophysiologic mechanisms that may contribute to the development of MAM. Their ties to intestinal health and liver function as well as their strong correlation with anthropometric measures suggest they may be key mediators of the progression of malnutrition. To our knowledge, neither marker has been previously associated with MAM. Intestinal fatty acid-binding protein 2 inversely correlated with WAZ in a study of children with high rates of stunting and moderate to severe wasting.[45] In a longitudinal study of Peruvian infants, iFABP levels at baseline were elevated in the group of infants who later became stunted.[46] In children with SAM and persistent diarrhea, there were positive correlations between iFABP and MUAC and LBP and markers of intestinal damage.[47] LPS-binding protein is produced by hepatocytes and intestinal cells,[30] so dysfunction of either may drive the low LBP concentrations observed in children with MAM. LPS-binding protein regulates the cellular response to LPS in a concentration-dependent manner. At high concentrations, LBP binds LPS and facilitates its transfer to plasma lipoproteins, leading to blunting of the leukocyte inflammatory response.[30,31] However, at low concentrations, LBP facilitates transfer of LPS to CD14, amplifying TLR4-mediated activation and inflammatory cytokine production.[30] Thus, the low LBP in our subjects with MAM would be expected to lead to amplified inflammatory response to LPS. This is consistent with the inverse correlation between levels of LBP and IL6 in naive and LPS-stimulated plasma in our cohort. Our finding of an amplified response to exogenous LPS in PBMCs exposed to plasma from children with MAM may be a consequence of the elevated plasma LPS, reduced LBP, or a combination of both. Exposure to low levels of LPS can lead to "endotoxin priming,"[15] in which a second LPS exposure leads to a potentially detrimental exaggerated inflammatory response. Exposure of leukocytes to low level of circulating LPS may lead to endotoxin priming, which might explain the exaggerated LPS-induced cytokine production we found in children with MAM. More importantly, the exaggerated cytokine response, whether from endotoxin priming or low LBP, may contribute to the poor outcome of sepsis caused by Gram-negative enteric bacteria in malnourished children.[5] The elevated baseline levels of IFN-γ (such as those observed in children with MAM in this cohort) can also sensitize cells to pathogenic stimuli such as endotoxin.[44] This may explain the correlation we see between naive IFN-γ levels and LPS-induced IL-6 response and contribute to the overall pattern of systemic inflammation observed in children with MAM.

Changes in liver function and metabolism may also be 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 fatty acid oxidation in children with MAM, possibly through reduced cellular uptake (random insulin levels were lower) or impaired import of LCFAs into the mitochondria. Elevated levels of these acylcarnitines have been associated with increased gut permeability and reduced citrulline levels in children with EED.[48] Long-chain acylcarnitines and saturated LCFAs can also induce inflammatory cytokine production in a variety of cell types.[49–51] Our data from ex vivo stimulated PBMCs from a healthy donor suggest that elevated circulating saturated LCFAs in children with MAM can increase baseline inflammation and, more importantly, increase response of these cells to LPS. There is little information on saturated fatty acids (SFAs) in children with MAM. One study found SFAs to be elevated in children with MAM,[52] although the proportion of SFA was decreased in children with SAM.[53,54] In the cohort from this study, children with MAM had a higher ratio of SFA:PUFA than HCs. Thus, it is feasible that buildup of saturated LCFAs and acylcarnitines in circulation due to deficits in LCFA oxidation

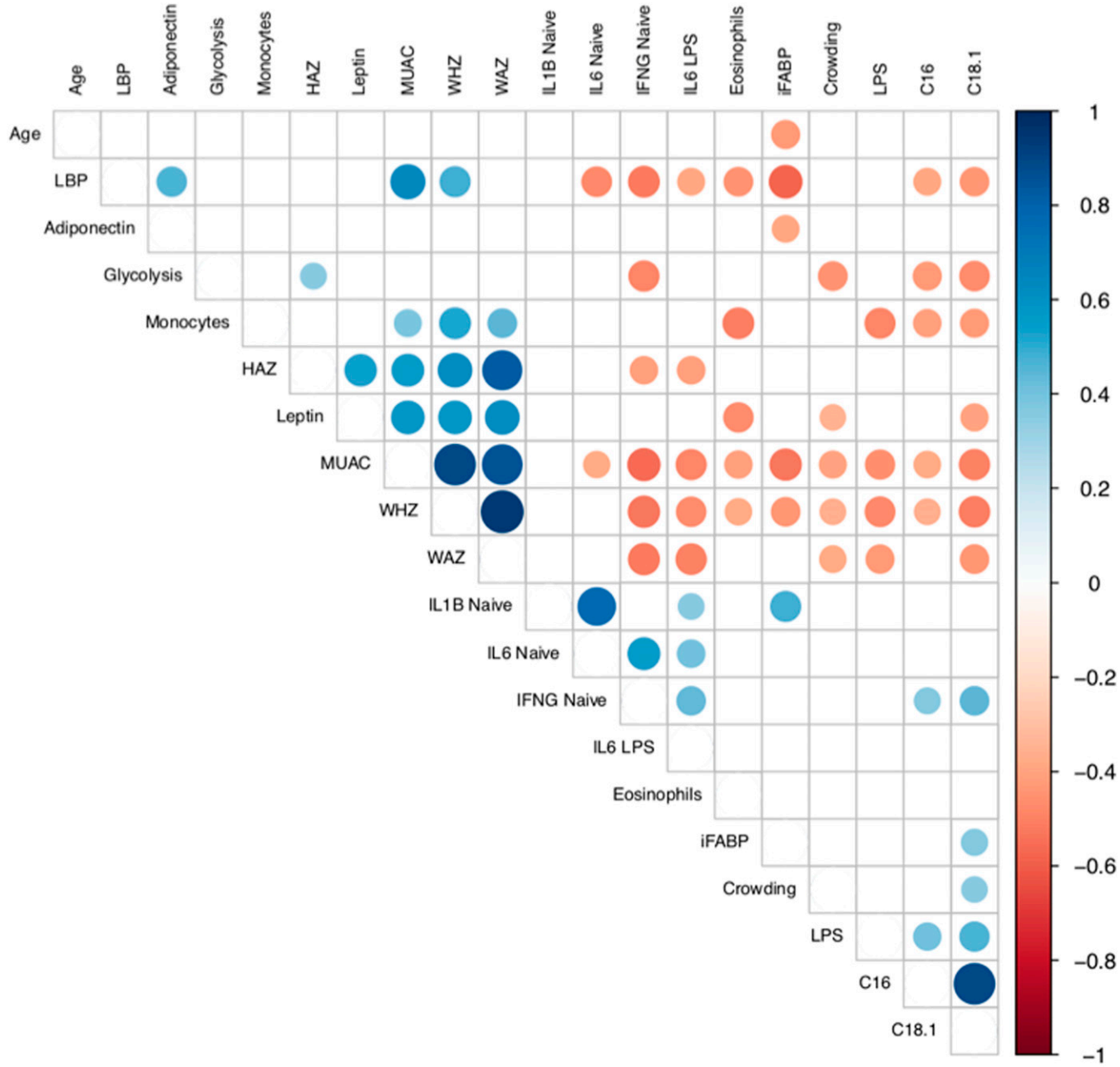

FIGURE 4. Correlations between variables associated with malnutrition. Correlations that were not significant ($P > 0.05$) are shown as empty boxes. Size of dot indicates the level of statistical significance; a larger dot indicates a smaller *P*-value. Color indicates correlation coefficient as indicated 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.

may contribute to the increased inflammatory response observed in children with MAM.

Exposure of healthy PBMCs to plasma from children with MAM produced an inflammatory profile similar to what was observed in whole blood samples. While malnutrition impacts the function of immune cells, this work suggests that factors in circulation also play a role in the stimulation of circulating immune cells. It is unclear which factors are the primary drivers of this response. It is likely that complex interactions and combined effects of multiple factors (including factors not measured here) are required to produce this response. This may explain why the circulating endotoxin level was correlated with anthropometric scores but few other variables, as there may be many indirect pathways and mediators between the exposure and outcome. We found higher concentrations of LPS, long-chain acylcarnitines, and inflammatory cytokines, and lower concentrations of LBP, all of which may contribute to increased response to inflammatory stimuli.

This study has several limitations. Because of 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. It is not possible in this study to determine whether the observed physiological characteristics of children with MAM

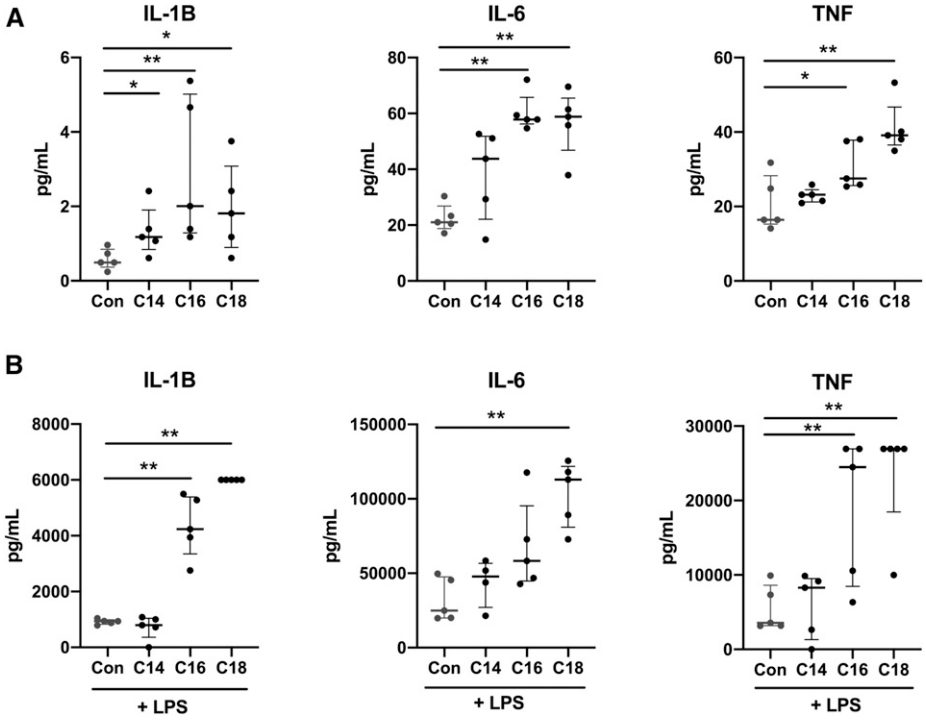

FIGURE 5. Cytokine production by human PBMCs on exposure to free fatty acids and/or lipopolysaccharide. PBMCs were isolated from a healthy adult volunteer and exposed to albumin-bound saturated long-chain fatty acids or vehicle control (con) (**A**), or to albumin-bound saturated long-chain fatty acid or a vehicle control plus 10 ng/mL Lipopolysaccharide (**B**). Long-chain fatty acids included myristic acid (C14), palmitic acid (C16), and steric acid (C18). After 24 hours, cytokines were measured in supernatant by Bio-Plex. Data representative of two independent experiments. *$P < 0.05$ and **$P < 0.01$. PBMC = peripheral blood mononuclear cell.

occurred as a result of the nutritional and psychological stress of undernutrition or whether these physiological characteristics drove subsequent weight loss. The small sample size in this study limits the number of variables that can be included in regression modeling, restricting us to univariate analysis. The small sample size also means that results are exploratory and are to be used for hypothesis generation. There is a possibility of type 1 error due to the large number of comparisons of demographic characteristics and biological measurements between children with MAM and HCs. Comparison of biomarkers with minimal statistically significant differences should be considered with this in mind. However, the pattern of results described herein is feasible and supports current understanding in this field. The absolute values for these measures are also inherently valuable additions to the literature. The timing of blood collection was without regard to the child's last meal, so the metabolic findings beg additional study under more controlled circumstances. 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 exclusion of children with acute or chronic infections that are likely to confound analyses of metabolism and inflammation.

The generalizability of the study results should be cautiously considered. We examined the biological effects of acute malnutrition in a cohort of children in an urban slum in Kenya. The exposures children face in this environment, such as crowding, high levels of pollution, and diet, may differ from the exposures of children in more rural settings. These results may be generalizable to children in

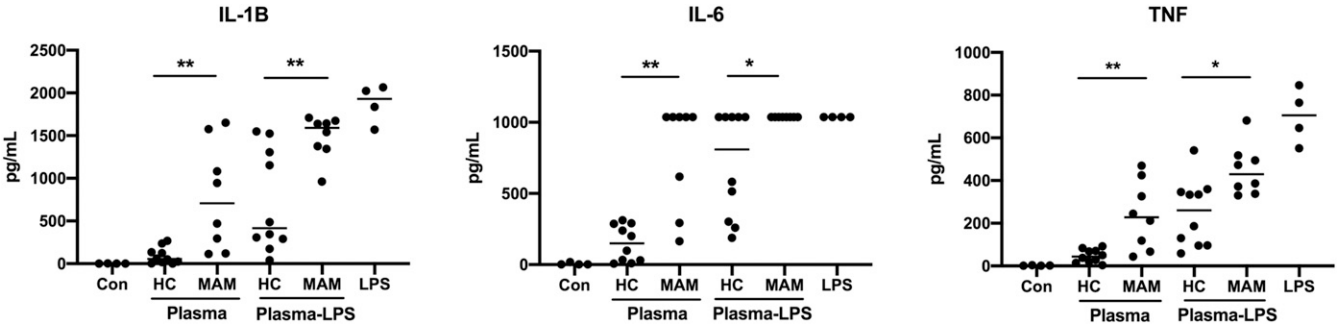

FIGURE 6. Cytokine production by peripheral blood mononuclear cells (PBMCs) from a healthy adult volunteer following exposure to plasma from children with MAM or healthy controls (HCs) with or without simultaneous exposure to LPS. Cytokine concentration was measured in cell supernatants by Bio-Plex. The Mann–Whitney *U* test was used to compare cells exposed to plasma from MAM subjects and HCs, with or without LPS. *$P < 0.05$ and **$P < 0.01$. MAM = moderate acute malnutrition; LPS = lipopolysaccharide.

other large, urban slums with high rates of malnutrition across Africa or Southeast Asia, but this cannot be ascertained without further research, as the literature on childhood MAM is scarce.

In conclusion, this study provides a snapshot of metabolic, inflammatory, and intestinal differences between children with MAM and HCs who reside in the same urban slum. The study demonstrates systemic inflammation in children with MAM in the absence of overt infection. It identifies plasma LPS, LBP, and iFABP as potential quantitative biomarkers and contributors to the pathogenesis of MAM. These biomarkers may also have value as tools to track progression or recovery from malnutrition independent of anthropometrics. Data from this study should be used to inform future studies of the pathogenesis of childhood MAM.

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