Low serum ω-3 and ω-6 polyunsaturated fatty acids and other metabolites are associated with poor linear growth in young children from rural Malawi

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ABSTRACT

Background: Stunting affects ~25% of children <5 y of age and is associated with impaired cognitive and motor development and increased morbidity and mortality. The pathogenesis of stunting is poorly understood.

Objective: The purpose of this study was to identify altered metabolic pathways associated with child stunting.

Design: We measured 677 serum metabolites using liquid chromatography–tandem mass spectrometry in a cross-sectional study of 400 Malawian children aged 12–59 mo, of whom 62% were stunted.

Results: A low height-for-age *z* score (HAZ) was associated with lower serum concentrations of *1*) ω-3 (n–3) and ω-6 (n–6) polyunsaturated fatty acids (PUFAs), *2*) sulfated neurosteroids, which play a role in brain development, *3*) carnitine, a conditionally essential nutrient with an important role in the carnitine shuttle for the metabolism of fatty acids and energy production, and *4*) γ-glutamyl amino acids, which represent an altered γ-glutamyl cycle of glutathione metabolism. A low HAZ was associated with significantly higher serum concentrations of 5 biomarkers related to cigarette smoke exposure.

Conclusions: This metabolomics study shows a cross-sectional association between stunting and low serum ω-3 and ω-6 long-chain PUFAs, which are essential for growth and development; low sulfated neurosteroids, which play a role in brain development; low carnitine, which is essential for β-oxidation of fatty acids; alterations in glutathione metabolism; and increased serum metabolites that are associated with secondhand tobacco smoke exposure. This trial was registered at www.controlled-trials.com as ISRCTN14597012. *Am J Clin Nutr* 2017;106:1490–9.

Keywords: arachidonic acid, carnitine, dehydroepiandrosterone, docosahexaenoic acid, malnutrition, pregnenolone sulfate, stunting

INTRODUCTION

Stunting affects ~25% of children <5 y of age worldwide. Stunting is defined as a height-for-age *z* score (HAZ) >2 SDs below the median (HAZ <−2) and is the best available summary measure of chronic malnutrition in children. There are an estimated 155 million stunted children aged <5 y worldwide (1). Stunting is associated with impaired cognitive and motor development and increased morbidity and mortality. Later in adulthood, those who were stunted have a higher risk of reduced economic productivity and a greater chance of being impoverished (2–6). The World Health Assembly and the UN have a global target to reduce by 40% the number of stunted <5-y-old children by 2025 (7, 8). Recent estimates based on proxy measures of stunting and poverty show that 250 million children <5 y old in low- and middle-income countries are at risk of not reaching their developmental potential (9).

Stunting is attributed to a host of factors, including poor prenatal nutrition, micronutrient deficiencies, insufficient breastfeeding, poor hygiene, and infectious diseases (10). Micronutrient-based supplementation or lipid-based supplements with micronutrients have had a limited impact on reducing stunting in children (11–14). Studies suggest that inadequate dietary intakes of essential amino acids (15, 16) and choline (16, 17) contribute to stunting. The richest dietary sources of essential amino acids and choline are animal-source foods, which are rarely consumed by children from poor families in low-income countries. The pathogenesis of stunting remains poorly understood. The biological mechanisms by which stunting could affect cognition and neurodevelopment of young children are also not well characterized.

We previously examined the relation between serum metabolites and stunting using an assay that was limited to 139

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Supplemental Tables 1 and 2 and Supplemental Figure 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

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Abbreviations used: ARA, arachidonic acid; HAZ, height-for-age *z* score; RSD, relative SD; UPLC-MS/MS, ultra-HPLC–tandem mass spectrometry.

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metabolites (16). To expand these investigations, we conducted a discovery phase metabolomics study by measuring serum metabolites using the Metabolon platform that can measure 600–700 serum metabolites.

METHODS

Study design and participants

This cross-sectional study involved children aged 12–59 mo from 6 villages (Masika, Makhwira, Mitondo, Mibiza, Chamba, and Mayaka) in rural southern Malawi in 2011 (**Supplemental Figure 1**). Eligibility criteria included no congenital or chronic disease or caretaker-reported diarrhea and no current treatment of acute malnutrition. Field staff measured weight to the nearest 5 g using a digital scale (Seca 344) and length to the nearest 0.1 cm using a rigid length board (Seca 417). Using a standardized questionnaire, the parent or guardian was asked about the primary source of water used by the household, i.e., wells, streams, or boreholes. Wells and streams were considered potentially contaminated, whereas boreholes were considered clean. However, no microbiological testing was done to corroborate these assumptions. The parent or guardian was also asked about animals in the household and various indicators of socioeconomic status. Written and oral informed consent from each child's caretaker were obtained by Malawian research nurses before enrollment in the study. Consent for the study on the community level also was obtained from the village chief and local health officials. The study protocol was approved by the College of Medicine Research and Ethics Committee of the University of Malawi, the Human Research Protection Office of Washington University in St. Louis, and the Johns Hopkins School of Medicine Institutional Review Board. The study protocol was conducted in accordance with the 1964 Helsinki Declaration. The trial was registered at www.controlled-trials.com as ISRCTN14597012.

Measurement of serum metabolites

Venous blood was drawn by study nurses and doctors. Serum samples were processed, aliquoted, and snap-frozen in liquid nitrogen in cryovials within 4 h of blood drawing. Cryovials were transferred to storage at −80°C until time of analysis. The serum samples used in this study were previously subjected to 3 freeze-thaw cycles. Preanalytical studies show that 2–4 freeze-thaw cycles have minimal impact on serum metabolites (18, 19). All experimental samples were prepared and analyzed in a masked fashion. Samples were prepared at Metabolon Inc. with the use of their DiscoveryHD4 platform with methods as described elsewhere (20). The staff at Metabolon had no access to the clinical data. In brief, for quality control, recovery standards were added before the first step of the extraction process. Proteins were precipitated with methanol under vigorous shaking for 2 min by using a GenoGrinder 2000 (Glen Mills) followed by centrifugation. The resulting extract was divided into 5 fractions: *1*) early- and *2*) late-eluting compounds for analysis by ultra-HPLC–tandem mass spectrometry (UPLC-MS/MS) by using positive ionization, *3*) for analysis by UPLC-MS/MS by using negative ionization, *4*) for analysis by using a UPLC-MS/MS polar platform with negative ionization, and *5*) a sample reserved for backup if needed.

Three types of controls were analyzed concomitantly with the experimental samples: *1*) samples generated from a pool of human plasma that has been extensively characterized by Metabolon, *2*) extracted water samples serving as process blanks, and *3*) a cocktail of standards spiked into every analyzed sample to allow monitoring of instrument performance. Instrument variability was determined by calculating the median relative SD (RSD) for the standards that were added to each sample before injection into the mass spectrometers (median RSD typically = 4–6%; $n \geq 30$ standards). Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., noninstrument standards) present in 100% of the pooled human plasma samples (median RSD = 10–14%; n = several hundred metabolites). Experimental samples and controls were randomized across the experimental runs.

Sample extracts were analyzed by using a standardized chromatographic UPLC-MS/MS method (20). All columns and solvents were obtained from a single manufacturer's lot for the sample analysis of this study. For each sample, vacuum-dried samples were dissolved in injection solvent containing ≥8 injection standards at fixed concentrations, depending on the platform. The internal standards were used to ensure consistency of injections and chromatography. Instruments were tuned and calibrated for mass resolution and mass accuracy daily.

The UPLC-MS/MS platform consisted of an Acquity UPLC (Waters Corp.) and a Q-Exactive (Thermo Scientific) mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and an Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried and then reconstituted in acidic or basic liquid chromatography–compatible solvents, each of which contained ≥8 injection standards at fixed concentrations. One aliquot was analyzed by using acidic, positive ion-optimized conditions. Another aliquot was analyzed by using basic, negative ion-optimized conditions. Two independent injections were done by using separate dedicated columns (Waters UPLC BEH C18–2.1 × 100 mm, 1.7 μm). Extracts reconstituted in acidic conditions were gradient-eluted by using water and methanol containing 0.1% formic acid, whereas the basic extracts, which also used water and methanol, contained 6.5 mmol ammonium bicarbonate/L. A third aliquot was analyzed via negative ionization after elution from an HILIC column (Waters UPLC BEH Amide 2.1 × 150 mm, 1.7 μm) by using a gradient consisting of water and acetonitrile with 10 mmol ammonium formate/L. The mass spectrometry (MS) analysis alternated between MS and data-dependent MS2 scans by using dynamic exclusion. The scan range was from 80 to 1000 *m/z*.

Metabolites were identified by an automated comparison of the ion features in the experimental samples against a reference library of chemical standard entries. The entries included molecular weight (*m/z*), retention time, preferred adducts, in-source fragment, and associated MS spectra. Entries have been curated by visual inspection for quality control by using software developed at Metabolon (21). The identification of known metabolites was based on comparison to metabolomic library entries of purified standards. Commercially available purified standard compounds were previously acquired and registered into the Laboratory Information Management System for determination of their detectable characteristics. Peaks were quantified by using AUC. Raw area counts for each metabolite

in each sample were normalized to correct for variation resulting from instrument interday tuning differences by the median value for each run day and therefore setting the medians to 1.0 for each run. This normalization preserved the variation between samples but allowed metabolites of widely different raw peak areas to be compared on a similar graphical scale. Missing values were imputed with the observed minimum after normalization.

Statistical analysis

The distributions of serum metabolites and HAZ values were examined in exploratory data analyses by using histograms and boxplots. The primary analysis of this study was based on linear growth as represented by HAZ as a continuous variable. There were no a priori power calculations for this discovery-phase metabolomics study. Partial Spearman correlations of each metabolite with the HAZ were estimated with adjustment for age, sex, and village. A false-discovery rate approach was used to correct for multiple testing (22–24). Q values were computed at the 0.05 false-discovery rate level. Wilcoxon's rank-sum test was used to compare serum metabolites between stunted and nonstunted children. This analysis was carried out in R software version 3.3.0. R package "ppcor" (25) and "corpcor" (26) were used for partial Spearman correlations. "Qvalue" was used for calculating the Q value (27).

RESULTS

The demographic characteristics of the 400 children are shown in **Table 1**. Stunted children were older, less likely to have a father who was alive, and more likely to have a clean water source but less likely to have a pit latrine. There were differences in stunting between villages. There were 248 children (62%) who were stunted, as defined by HAZ <-2. The relation of HAZ with serum metabolites is summarized as a volcano plot in **Figure 1**. There were 136 metabolites that were significantly associated with HAZ. Sixty-two serum metabolites were negatively associated with HAZ, including 19 glycerophospholipids (notably 7 phosphatidylethanolamines) and 5 metabolites associated with cigarette smoking (**Table 2**). Seventy-four metabolites were positively associated with HAZ, including DHA, 5 glycerophospholipids with arachidonic acid (ARA) side chains [1-stearoyl-2-arachidonoyl-glycerophosphorylcholine (18:0/20:4), 1-stearoyl-2-arachidonoyl-glycerophosphorylcholine (18:0/20:4), 1-linoleoyl-2-arachidonoyl-glycerophosphorylcholine (18:2/20:4n–6), 1-(1-enyl-palmitoyl)-2-arachidonyl-glycerophosphorylcholine (P-16:0/20:4), and 1-arachindonyl-glycerophosphorylcholine (20:4n–6)], 12 sphingomyelins, 9 proteinogenic amino acids, 7 γ-glutamyl dipeptides, 6 glycerophosphocholines, and 4 neurosteroids (**Table 3**). Spearman correlations of serum metabolites, adjusted by age, sex, and village, with HAZ are shown in **Supplemental Table 1**. The medians and interquartile ranges of serum metabolites of children with and without stunting are shown in **Supplemental Table 2**.

DISCUSSION

Stunted children have lower serum phospholipid DHA and ARA, sulfated neurosteroids, carnitine, and γ-glutamyl amino acids and higher serum biomarkers related to cigarette smoke exposure. Stunting was associated with lower creatinine and components of collagen. Many of these metabolites are related to the development of the nervous and musculoskeletal systems. To our knowledge, this is the first study to show an association of linear growth of young children with abnormalities in specific metabolites such as DHA, ARA, sulfated neurosteroids, carnitine, γ-glutamyl amino acids, creatinine, and amino acids

TABLE 1
Characteristics of the study population[1]

	Not stunted[2] (n = 152)	Stunted[2] (n = 248)	P[3]
Age, mo	29.0 ± 12.5	36.9 ± 10.6	<0.001
Female	80 (53)	118 (48)	0.33
Weight-for-height z score	0.1 ± 0.9	0.2 ± 1.0	0.50
HAZ	−1.1 ± 0.9	−3.1 ± 0.8	<0.001
Primary caretaker is mother	148 (97)	233 (94)	0.12
Father is alive	152 (100)	232 (94)	0.001
Siblings, n	3.7 ± 1.8	3.8 ± 1.7	0.83
Individuals that sleep in same room as child, n	3.5 ± 1.2	3.2 ± 1.6	0.06
Home with a metal roof	28 (18)	47 (19)	0.90
Family owns bicycle	98 (64)	146 (59)	0.26
Animals sleep in house	46 (30)	98 (40)	0.06
Water from a clean source	89 (59)	183 (74)	0.002
Child uses pit latrine	127 (84)	184 (74)	0.03
Village			<0.001
Chamba	6 (4)	39 (16)	
Makwhira	7 (5)	20 (8)	
Masika	97 (64)	59 (24)	
Mayaka	25 (16)	71 (29)	
Mbiza	11 (7)	49 (20)	
Mitondo	6 (4)	10 (4)	

[1] Values are means ± SDs or n (%). HAZ, height-for-age z score.
[2] Stunted was defined as HAZ <-2.
[3] Determined by Student's t test for continuous variables or chi-square test for categorical variables.

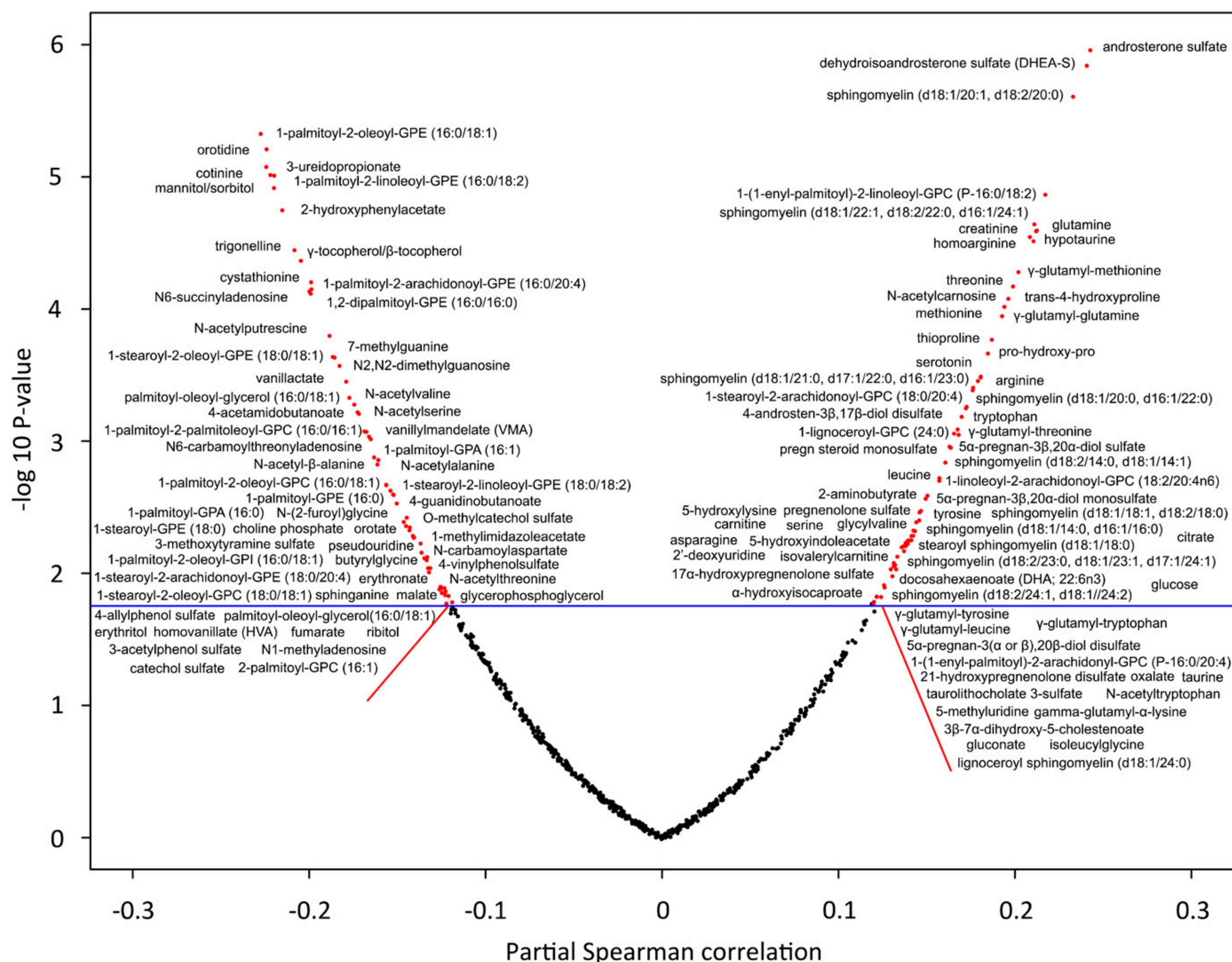

FIGURE 1 Volcano plot showing the relation of partial Spearman correlations, adjusted for age, sex, and village, between height-for-age z score and serum metabolites. The horizontal line indicates significance at $P < 0.0176$, which corresponds to a Q value <0.05. DHEA-S, dehyroepiandrosterone sulfate; GPA, glycerophosphate; GPC, glycerophosporylcholine; GPE, glycerophosphoethanolamine; GPI, glycerophosphatidylinositol.

involved in collagen assembly. The present study corroborates previous findings that child stunting is associated with lower serum proteinogenic amino acids and alterations in glycerophospholipids (16) and with tobacco use in poor families (28–30).

Stunted children had lower serum phospholipid DHA (22:6n–3), an ω-3 long-chain PUFA, and lower serum phospholipid arachidonic acid (20:4n–6), an ω-6 long-chain PUFA. DHA is essential for normal growth and development and is the most abundant ω-3 fatty acid in the brain (31). ARA content of phosphatidylcholine has been related to infant growth (32) and child stunting (33). Moreover, cognitive development is enhanced when infants receive formula containing ARA and DHA (34). DHA may be formed by biosynthesis from the precursor α-linoleic acid (18:3n–3). ARA is critical for infant growth, brain development, and health (35, 36). ARA may be formed by biosynthesis from linoleic acid (18:2n–6). Breast milk is a rich source of DHA and ARA, and the infant receives most of its supply of these long-chain PUFAs preformed because human metabolism from the 18-C precursors is very limited (37). During weaning and after cessation of breastfeeding, infants and young children in developing countries are at a high risk of insufficient DHA and ARA, because complementary foods contain inadequate amounts or are even devoid of DHA and ARA (31). Rich dietary sources of DHA and ARA include fish and eggs and meats and eggs, respectively.

Child stunting was associated with lower sulfated neurosteroids, pregnenolone sulfate, and dehydroepiandrosterone sulfate, which play an important role in brain development and are involved in cognition, learning, memory, neuronal development, and neuroprotection (38–40). Dehydroepiandrosterone sulfate is mainly produced by the adrenal glands (41) and exerts diverse effects in the brain through the modulation of various receptors. Stunting was also associated with lower serum 17α-hydroxypregnenolone sulfate, 21-hydroxypregnenolone disulfate, a metabolite formed from pregnenolone, and metabolites of progesterone (5α-pregnan-3β,20α-diol monosulfate, 5α-pregnan-3β,20α-diol disulfate), and metabolites of dihydrotestosterone (androsterone sulfate, 4-androsten-3β-diol disulfate). The sulfated steroids can act as circulating reservoirs for the peripheral formation of bioactive hormones (42). Lower serum sulfated steroids could potentially reflect lower availability of these steroids for brain development.

TABLE 2
Serum metabolites negatively associated with height-for-age *z* score[1]

Metabolite	Description
1-Palmitoyl-2-oleoyl-GPE (16:0/18:1)	Phosphatidylethanolamine, monoene
Orotidine	Intermediate in pyrimidine nucleotide biosynthesis
3-Ureidopropionate	Intermediate in the metabolism of uracil
Cotinine	Major alkaloid in tobacco and marker of exposure to tobacco smoke
1-Palmitoyl-2-linoleoyl-GPE (16:0/18:2)	Phosphatidylethanolamine, diene
Mannitol/sorbitol	Isomers, alcohol sugars
2-Hydroxyphenylacetate	Substrate of the enzyme oxidoreductases in styrene degradation pathway
Trigonelline (N'-methylnicotinate)	Metabolite associated with cigarette smoking
γ-Tocopherol/β-tocopherol	Form of vitamin E
Cystathionine	Formed from transsulfuration of homocysteine
1-Palmitoyl-2-arachidonoyl-GPE (16:0/20:4)	Phosphatidylethanolamine, polyene
N6-Succinyladenosine	Aspartic acid derivative
1,2-Dipalmitoyl-GPE (16:0/16:0)	Phosphatidylethanolamine, saturate
N-Acetylputrescine	*N*-Acetylated form of putrescine, a common polyamine
7-Methylguanine	Metabolite of DNA methylation and depurination
1-stearoyl-2-oleoyl-GPE (18:0/18:1)	Phosphatidylethanolamine, monoene
N2,N2-Dimethylguanosine	Primary degradation product of transfer RNA
Vanillactate	Acidic catecholamine metabolite
N-Acetylvaline	*N*-Acetylated form of valine
Palmitoyl-oleoyl-glycerol (16:0/18:1)	Diglyceride, monoene
N-Acetylserine	*N*-Acetylated form of serine
4-Acetamidobutanoate	In family of γ amino acids and derivatives, the conjugate base of 4-acetamidobenzoic acid
1-Palmitoyl-2-palmitoleoyl-glycerophosphorylcholine (16:0/16:1)	Phosphatidylcholine, monoene
Vanillylmandelate	Product of catecholamine catabolism
N6-Carbamoylthreonyladenosine	Minor constituent of milk
1-Palmitoleoyl-glycerophosphorylcholine (16:1)	Phosphatidic acid, monoene
N-Acetyl-β-alanine	β-Amino acid
N-Acetylalanine	*N*-Acyl-aliphatic-α amino acid
1-Palmitoyl-2-oleoyl-glycerophosphorylcholine (16:0/18:1)	Phosphatidylcholine, monoene
1-Stearoyl-2-linoleoyl-GPE (18:0/18:2)	Phosphatidylethanolamine, diene
1-Palmitoyl-GPE (16:0)	Lyso-phosphatidylethanolamine, saturate
1-Stearoyl-GPE (18:0)	Lyso-phosphatidylethanolamine, saturate
N-(2-Furoyl)glycine	Metabolite associated with cigarette smoking
O-Methylcatechol sulfate	Polyphenol metabolite
1-Methylimidazoleacetate	Main metabolite of histamine
1-Palmitoyl-GPA (16:0)	Lyso-phosphatidic acid, saturate
Orotate	Minor dietary constituent of dairy products, root vegetables
4-Guanidinobutanoate	γ-Amino acid
Choline phosphate	Intermediate in synthesis of phosphatidylcholine
3-Methoxytyramine sulfate	Dopamine metabolite
Pseudouridine	C-Glycoside isomer of the nucleoside uridine
N-Carbamoylaspartate	Intermediate product in pyrimidine biosynthesis
4-Vinylphenol sulfate	Metabolite associated with cigarette smoking
1-Palmitoyl-2-oleoyl-GPI (16:0/18:1)	Phosphatidylinositol, monoene
Butyrylglycine	Acyl glycine, minor metabolite of fatty acids
N-Acetylthreonine	*N*-Acetylated form of threonine
Erythronate	Oxidation product of *N*-acetyl-D-glucosamine
1-Stearoyl-2-arachidonoyl-GPE (18:0/20:4)	Phosphatidylethanolamine, polyene
Malate	Intermediate in tricarboxylic acid cycle
Sphinganine	Blocker of postlysosomal cholesterol transport
1-Stearoyl-2-oleoyl-glycerophosphorylcholine (18:0/18:1)	Phosphatidylcholine, monoene
Glycerophosphoglycerol	Diglyceride, monoene
Palmitoyl-oleoyl-glycerol (16:0/18:1)	Diglyceride, monoene
4-Allylphenol sulfate	Phenolic found in fruit
Homovanillate	Dopamine metabolite
3-Acetylphenol sulfate	Sulfated hydroxy-substituted alkyl phenyl ketone
Ribitol	Metabolic end product formed by the reduction of ribose
Fumarate	Dicarboxylic acid precursor to malate in tricarboxylic acid cycle
Erythritol	Small molecule found in foods
N1-Methyladenosine	Purine nucleoside
2-Palmitoleoyl-glycerophosphorylcholine (16:1)	Lyso-phosphatidylcholine, monoene
Catechol sulfate	Metabolite associated with cigarette smoking

[1] GPA, glycerophosphate; GPE, glycerophosphoethanolamine; GPI, glycerophosphatidylinositol.

TABLE 3
Serum metabolites positively associated with height-for-age *z* score

Metabolite	Description
Androsterone sulfate	Sulfated neurosteroid
Dehydroisoandrosterone sulfate	Sulfated neurosteroid with memory enhancing, neuroprotective effects
Sphingomyelin (d18:1/20:1, d18:2/20:0)	Sphingomyelin
1-(1-Enyl-palmitoyl)-2-linoleoyl-glycerophosphorylcholine (P-16:0/18:2)	Phosphatidylcholine plasmalogen, diene
Sphingomyelin (d18:1/22:1, d18:2/22:0, d16:1/24:1)	Sphingomyelin
Glutamine	Conditionally essential amino acid
Hypotaurine	Intermediate in biosynthesis of taurine
Creatinine	Breakdown product of creatine phosphate in muscle
Homoarginine	Nonproteinogenic amino acid formed from lysine, involved in vascular homeostasis
γ-Glutamyl-methionine	Dipeptide composed of γ-glutamate and methionine
Threonine	Essential amino acid
Trans-4-hydroxyproline	Major component of protein collagen (role in collagen stability)
N-acetylcarnosine	Related to carnosine but with extra acetyl group, found especially in muscle tissue
γ-Glutamyl-glutamine	Dipeptide composed of γ-glutamate and glutamine
Methionine	Essential amino acid
Thioproline	Metabolite of 5-hydroxytryptamine catabolism
Pro-hydroxy-pro	Major component of collagen
Serotonin (5-hydroxytryptamine)	Monoamine neurotransmitter, derived from tryptophan
Sphingomyelin (d18:1/21:0, d17:1/22:0, d16:1/23:0)	Sphingomyelin
Arginine	Conditionally essential amino acid
1-Stearoyl-2-arachidonoyl-glycerophosphorylcholine (18:0/20:4)	Phosphatidylcholine, polyene
Sphingomyelin (d18:1/20:0, d16:1/22:0)	Sphingomyelin
Tryptophan	Essential amino acid
4-Androsten-3β,17β-diol disulfate	Metabolite of dihydrotestosterone
1-Lignoceroyl-glycerophosphorylcholine (24:0)	Lyso-phosphatidylcholine, long-chain saturate
γ-Glutamyl-threonine	Dipeptide composed of γ-glutamate and threonine
Pregn steroid monosulfate	Endogenous excitatory neurosteroid, with cognitive and memory-enhancing effects
5α-Pregnan-3β,20α-diol sulfate	Sulfate of pregnanediol isomer, metabolite of plasma progesterone
Sphingomyelin (d18:2/14:0, d18:1/14:1)	Sphingomyelin
Leucine	Essential amino acid
2-Aminobutyrate	Intermediate in biosynthesis of ophthalmic acid
1-Linoleoyl-2-arachidonoyl-glycerophosphorylcholine (18:2/20:4n–6)	Phosphatidylcholine, polyene
5α-Pregnan-3β, 20α-diol monosulfate	Sulfate of pregnanediol isomer, metabolite of plasma progesterone
1-(1-Enyl-palmitoyl)-2-arachidonyl-glycerophosphorylcholine (P-16:0/20:4)	Phosphatidylcholine plasmalogen, polyene
Tyrosine	Nonessential amino acid
Sphingomyelin (d18:1/18:1, d18:2/18:0)	Sphingomyelin
Pregnenolone sulfate	Endogenous excitatory neurosteroid, with cognitive and memory-enhancing effects
Sphingomyelin (d18:1/14:0, d16:1/16:0)	Sphingomyelin
Glycylvaline	Dipeptide of glycine and valine
Serine	Nonessential amino acid
Carnitine	Conditionally essential nutrient, nonproteinogenic amino acid
Stearoyl sphingomyelin (d18:1/18:0)	Sphingomyelin
Sphingomyelin (d18:2/23:0, d18:1/23:1, d17:1/24:1)	Sphingomyelin long chain
5-Hydroxyindoleacetate	Breakdown product of serotonin
Asparagine	Nonessential amino acid
Isovalerylcarnitine	C5 acylcarnitine
Citrate	Acid in the tricarboxylic acid cycle
DHA (22:6n–3)	Very-long-chain PUFA with a 22-carbon backbone and 6 double bonds, polyene
5-Hydroxylysine	Hydroxylated derivative of lysine present in certain collagens
γ-Glutamyl-leucine	Dipeptide composed of γ-glutamate and leucine
Sphingomyelin (d18:2/16:0, d18:1/16:1)	Sphingomyelin
1-(1-Enyl-stearoyl)-2-archidonoyl-glycerophosphoethanolamine (P-18:0/20:4)	Phosphatidylethanolamine, plasmalogen polyene
γ-Glutamyl-tryptophan	Dipeptide composed of γ-glutamate and tryptophan

(Continued)

TABLE 3 (*Continued*)

Metabolite	Description
5-Bromotryptophan	Nonproteinogenic α-amino acid
3-Hydroxy-5-cholestenoic acid	Found in the primary bile acid biosynthesis pathway
1-Arachindonoyl-glycerophosphorylcholine (20:4n–6)	Lyso-phosphatidylcholine, polyene
17α-Hydroxypregnenolone sulfate	Prohormone in the formation of dehydroepiandrosterone, itself a prohormone of the sex steroids
2′-Deoxyuridine	Naturally occurring nucleoside
γ-Glutamyl-tyrosine	Dipeptide composed of γ-glutamate and tyrosine
Sphingomyelin (d18:2/24:1, d18:1//24:2)	Sphingomyelin, long-chain unsaturate
Glucose	Monosaccharide, primary source of energy
α-Hydroxyisocaproate	End product of leucine metabolism
21-Hydroxypregnenolone disulfate	Essential intermediate in corticosterone synthesis
5α-Pregnan-3(α or β),20β-diol disulfate	Sulfate of pregnanediol isomer, metabolite of plasma progesterone
N-Acetyltryptophan	Stabilizer
Oxalate (ethanedioate)	Dicarboxylic acid found in vegetables and produced endogenously by metabolism of glyoxylic acid or ascorbic acid
Taurine	Sulfonic acid with wide distribution in tissues
Taurolithocholate 3-sulfate	Sulfated bile acid
5-Methyluridine (ribothymidine)	Endogenous methylated nucleoside
γ-Glutamyl-α-lysine	Dipeptide composed of γ-glutamate and lysine
3β-7α-Dihydroxy-5-cholestenoate	Found in the primary bile acid biosynthesis pathway
Gluconate	Mild organic acid, abundant in plant foods, fruit, rice
Isoleucylglycine	Dipeptide composed of isoleucine and glycine
Lignoceroyl sphingomyelin (d18:1/24:0)	Sphingomyelin long chain saturate

Stunted children had lower serum carnitine, a conditionally essential nutrient that plays a critical role in β-oxidation of fatty acids and energy production. Carnitine is found mainly in animal-source foods, such as red meat, chicken, fish, and dairy products. Plant foods contain negligible amounts of carnitine (43). Carnitine is also synthesized in the body from lysine and methionine (**Figure 2**). We recently showed that environmental enteric dysfunction is associated with secondary carnitine deficiency (44).

Child stunting was associated with major abnormalities in the γ-glutamyl cycle of glutathione metabolism. Glutathione, a tripeptide consisting of γ-glutamine, cysteine, and glycine, is the major intracellular thiol that participates in cellular redox reactions and thioester formation. Glutathione protects cells against oxidative damage generated during energy production and immune response. Glutathione also plays a role in signal transduction, gene expression, and apoptosis (45). Stunting was associated with lower serum concentrations of 7 γ-glutamyl amino acids. Six of the dipeptides included essential amino acids whereas the seventh included glutamine, a conditionally essential amino acid. Glutathione in extracellular fluids is broken down at the cell membrane into cysteinyl glycine and γ-glutamyl amino acids (Figure 2). The amino acids can be used for synthesis of glutathione by the cells (46). It was previously believed that the γ-glutamyl cycle is used for transport of γ-glutamyl amino acids into cells; however, such dipeptide transport would require much higher energy compared with other known amino acid transporters (47).

Children with stunting had higher serum cystathionine, which suggests that homocysteine is diverted from the sulfur amino acid cycle by increased transsulfuration to cystathionine (Figure 2). The sulfur amino acid cycle plays a role in the biosynthesis of carnitine from lysine and methionine. The cysteine formed from cystathionine can be metabolized to cysteine sulfinate, hypotaurine, and taurine or can enter the γ-glutamyl cycle to produce γ-glutamyl cysteine through γ-glutamylcysteine ligase. Decreased γ-glutamyl amino acids may reflect less cysteine entering the γ-glutamyl cycle. Other metabolites in the sulfur amino acid pathway and taurine pathway that were significantly lower included methionine, serine, glutamine, hypotaurine, and taurine.

Serum creatinine is generated from creatine and creatinine phosphate, 95% of which is found in skeletal muscle (48). Stunted children had lower serum creatinine, a surrogate for skeletal muscle mass (49).

Stunting was associated with metabolic abnormalities suggestive of abnormal collagen prolyl and lysyl hydroxylation, 2 processes essential to the assembly of collagen, the main structural protein of the extracellular space of connective tissue. Stunted children had lower serum 5-hydroxylysine and trans-4-hydroxyproline, 2 posttranslationally modified amino acids that are found in collagen. Lysine in the α-chains of procollagen is converted to 5-hydroxylysine by lysyl hydroxylases. Proline is converted to 4-hydroxyproline on individual unfolded procollagen α-chains by prolyl 4-hydroxylases. 4-Hydroxylation of collagen proline residues is essential for the proper assembly and stabilization of collagen triple helices through intramolecular hydrogen bonding (50).

In the present study, stunted children had higher serum concentrations of cotinine, a well-known marker of exposure to tobacco smoke; catechol sulfate; 4-vinylphenol sulfate; trigonelline (*N*′-methylnicotinate); 3 serum metabolites of tobacco associated with smoking; and *N*-(2-furoyl)glycine, a metabolite found in tobacco smoke (51–53). Cotinine is the predominant metabolite of nicotine, which is found in tobacco leaves but not in firewood used for cooking fires. Paternal smoking was associated with child stunting among poor families in rural Indonesia and Bangladesh (28, 29). The diversion of family income from

FIGURE 2 Relation of the sulfur amino acid cycle, carnitine biosynthesis pathway, γ-glutamyl cycle and glutathione metabolism, and taurine pathway. The thick up and down arrows indicate higher or lower serum metabolite concentrations in children with stunting, respectively. THF, tetrahydrofolate.

food to tobacco increases the risk of household food insecurity (30). The prevalence of smoking in Malawi is 25.9% among men and 2.9% among women (54). Although data on tobacco use was not collected in the present study, the elevated serum concentrations of 5 markers of exposure to tobacco smoke in stunted children raises concerns that scarce household resources are being diverted from food to tobacco, thus increasing the risk for children. Exposure to secondhand smoke also has detrimental effects on lung development and cardiovascular health in young children (55, 56).

The strengths of this study are the large, community-based sample size of children from an environment where stunting is highly prevalent and the use of an advanced metabolic platform. Findings from Malawi cannot necessarily be generalized to other settings because of environmental, genetic, and cultural differences. This cross-sectional study can show epidemiologic associations. Inferences of causality would be strengthened by longitudinal analyses and controlled trials.

Two potential interventions are identified for the reduction of child stunting: increasing the intake of animal-source foods (the main source of carnitine, essential amino acids, choline, and ω-3 and ω-6 long-chain PUFAs) and tobacco control. A recent trial shows increased egg consumption can reduce child stunting (57). Further efforts are underway to corroborate these encouraging findings.

The authors' responsibilities were as follows—RDS, RM, IT, KMM, NS, and MJM: designed the research; IT, KMM, and MJM: conducted the research; RDS, KK, and MJM: provided essential reagents or materials; XL and MIO: analyzed the data; RDS, IT, NS, RM, and MJM: wrote the manuscript; RDS: had primary responsibility for the final content; and all authors: read and approved the final manuscript. None of the authors reported a conflict of interest related to the study.

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