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Research Paper

Child Stunting is Associated with Low Circulating Essential Amino Acids

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ABSTRACT

Background: Stunting affects about one-quarter of children under five worldwide. The pathogenesis of stunting is poorly understood. Nutritional interventions have had only modest effects in reducing stunting. We hypothesized that insufficiency in essential amino acids may be limiting the linear growth of children.
Methods: We used a targeted metabolomics approach to measure serum amino acids, glycerophospholipids, sphingolipids, and other metabolites using liquid chromatography-tandem mass spectrometry in 313 children, aged 12–59 months, from rural Malawi. Children underwent anthropometry.
Findings: Sixty-two percent of the children were stunted. Children with stunting had lower serum concentrations of all nine essential amino acids (tryptophan, isoleucine, leucine, valine, methionine, threonine, histidine, phenylalanine, lysine) compared with nonstunted children ($p < 0.01$). In addition, stunted children had significantly lower serum concentrations of conditionally essential amino acids (arginine, glycine, glutamine), non-essential amino acids (asparagine, glutamate, serine), and six different sphingolipids compared with nonstunted children. Stunting was also associated with alterations in serum glycerophospholipid concentrations.
Interpretation: Our findings support the idea that children with a high risk of stunting may not be receiving an adequate dietary intake of essential amino acids and choline, an essential nutrient for the synthesis of sphingolipids and glycerophospholipids.

1. Introduction

Stunting affects about one-quarter of children under five years of age worldwide (Black et al., 2013; UNICEF/World Health Organization/World Bank Group, 2015). Stunting is considered the best available summary measure of chronic malnutrition. Child stunting may develop during the first two years of life and is largely attributed to inadequate nutrition and infectious diseases (Black et al., 2013). In 2014, there were an estimated 159 million children who were stunted, with nearly all stunted children living in low-income countries (UNICEF/World Health Organization/World Bank Group, 2015). Stunting is associated with decreased survival, impaired cognitive and motor development, reduced economic productivity, and higher chance of living in poverty in adulthood (Black et al., 2013; Grantham-McGregor et al., 2007). The World Health Assembly has set a global target of a 40% reduction in the number of stunted under-five children by 2025 (de Onis et al., 2013). This targeted reduction in child stunting is included in the United Nations Sustainable Development Goal #2 (Murray, 2015). Nutritional interventions only have a modest impact upon stunting and will be insufficient to meet this goal alone. Even if ten evidence-based nutritional interventions were all applied at 90% coverage, stunting would be reduced by only ~20% (Bhutta et al., 2013), which falls short of international goals to reduce stunting. The pathogenesis of stunting remains poorly understood. There may be as yet unknown or limiting nutritional factors that contribute to child stunting.

In the 1950s and 1960s, international organizations were focused on protein malnutrition in children in developing countries (Semba, 2008). The emphasis shifted away from proteins to micronutrient malnutrition in the 1970s (Semba, 2008), under the assumption that most children received adequate protein. This assumption needs to be re-examined for several reasons. New evidence shows human growth is controlled by the master growth regulation pathway, the mechanistic target of

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rapamycin complex C1 (mTORC1) (Laplante and Sabatini, 2012). When specific amino acids are deficient in the diet, mTORC1 senses amino acid deficiency and represses protein and lipid synthesis and cellular growth (Laplante and Sabatini, 2012). The linear growth of children is dependent upon the chondral growth plate (Baron et al., 2015). Bone growth by the chondral plate is regulated by mTORC1 and the availability of amino acids, such as the essential amino acid leucine (Kim et al., 2009). Children at high risk of stunting may have limitations of essential amino acids in their diet such as tryptophan and lysine (Nuss and Tanumihardjo, 2011). The amino acid requirements of young children were not directly established and are currently derived based on a factorial computation (Pillai and Kurpad, 2012). Whether current dietary recommendations of essential amino acids are sufficient for children in low-income settings – where infectious diseases are common and catch-up growth is important – is unclear. The burden of infectious disease and metabolic needs for immune system activation may partition limited essential amino acids to support immune function at the expense of growth (Kampman-van de Hoek et al., 2016).

Recent advances in metabolomics and mass spectrometry now facilitate rapid and absolute quantification of serum amino acids and other metabolites in large epidemiological studies. We hypothesized that stunted children have decreased concentrations of circulating essential amino acids and other metabolites. To address this hypothesis, we used a targeted metabolomics approach to investigate serum amino acids and other metabolites in a cohort of young children in rural Malawi.

2. Methods

2.1. Study Design and Participants

The study design was cross-sectional. The study subjects consisted of a community-based sample of 313 children, aged 12–59 months, seen in six villages (Masika, Makhwira, Mitondo, Mbiza, Chamba, and Mayaka) in rural southern Malawi in 2011. Children were eligible for the study if they had no evidence of severe acute malnutrition, congenital or chronic disease, or caretaker-reported diarrhea. The study enrolled 540 children, of whom venous blood samples were obtained from 483 children. A simple random sample of 313 children was used for the analysis of serum metabolites. Children underwent anthropometry conducted by trained, experienced field workers. Weight was measured to the nearest 5 g using a digital scale (Seca 334, Chino, CA). Recumbent length (for children <24 months) or standing height was measured to the nearest 0.1 cm using a rigid height board (Seca 417). Height-for-age Z-scores (HAZ) and weight-for-height Z-scores were calculated using World Health Organization growth curves (de Onis et al., 2012). Stunting was defined as HAZ < −2 (de Onis et al., 2012). Chichewa-speaking Malawian research nurses obtained written and oral informed consent from each child's caretaker before enrollment in the study. Community consent for the study also was obtained from the village chief and local health officials. The protocol for this study was approved by the College of Medicine Research 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.

2.2. Measurement of Serum Metabolites

Venous blood was drawn by study nurses and doctors. Serum samples were processed and snap frozen in liquid nitrogen within 4 h of blood drawing. Cryovials were stored at −80 °C. Sera were not thawed prior to metabolite measurements. Samples with no hemolysis were used for the analyses. Serum metabolites were measured in a masked fashion using liquid chromatography tandem mass spectrometry (LC-MS/MS). Metabolites were extracted and concentrations measured using the AbsoluteIDQ p180 kit (Biocrates Life Sciences AG, Innsbruck, Austria) following the manufacturers protocol for a 5500 QTrap (Sciex, Framingham, MA) mass spectrometer equipped with an electrospray ionization source, a CBM-20A command module, LC-20AB pump, and a SIL-20AC-HT autosampler, a CTO-10Ac column oven heater (all Shimadzu, Tokyo, Japan), and running with Analyst 1.5.2 software (Biocrates), as described in detail elsewhere (Schmerler et al., 2012). The method measured 139 metabolites: 22 amino acids, 3 biogenic amines, 6 amino acid metabolites, 15 sphingolipids, 8 acylcarnitines, and 85 glycerophospholipids (lyso-, diacyl-, and acyl-alkyl phosphatidylcholines). Glycerophospholipids are differentiated on the basis of ester and ether bonds in the glycerol moiety. Diacyl or "aa" indicates that fatty acids are bound with ester bonds at the sn-1 and sn-2 positions on the glycerol backbone. Acyl-alkyl or "ae" indicates that the fatty acid at the sn-1 position is bound with an ether bond. The total number of carbon atoms and double bonds in fatty acid chains is represented by "C x:y", where x denotes the number of carbons and y denotes the number of double bonds. Phosphatidylcholine (PC), lysophosphatidylcholine (lysoPC), and sphingomyelin (SM), and hydroxysphingomyelin (SM [OH]) are used in the abbreviations. The MS spectra were evaluated using Analyst/MetIDQ software (Biocrates). Human serum samples spiked with standard metabolites were used to monitor the reproducibility of the assay. Metabolites that were below the limit of quantification were excluded. The inter-assay and intra-assay coefficients of variation ranged from 5% to 15% for nearly all analytes.

2.3. Statistical Analysis

The sample size of 313 children was based upon >90% power to detect at least a 10% difference in serum leucine between stunted and non-stunted children, given a 60% prevalence of stunting, $\sigma = 47$ μmol/L (Reinehr et al., 2015), no matching, $\alpha = 0.05$, and a two-sided test. Of all the essential amino acids, we chose leucine for power calculations, since it is the most well-characterized amino acid sensed by the mTORC1 pathway (Laplante and Sabatini, 2012; Saxton et al., 2016). Univariate exploratory data analyses using histograms and boxplots were used to examine the distribution of serum metabolites. Linear regression was performed of HAZ on serum metabolites in separate models using one model per metabolite and a combined model with all metabolites. Bivariate exploratory analyses were used to relate each metabolite to HAZ and to each other to ensure linear relations between metabolites and HAZ. Spearman correlations were used to examine correlations between HAZ and serum metabolites. Wilcoxon rank-sum test, adjusted by age and gender, was used to compare serum metabolites between stunted and non-stunted children. A Bonferroni adjustment to type I error was made to account for the multiple metabolites, many which are closely correlated, into five general classes (amino acids, biogenic amines/amino acid metabolites, sphingolipids, acylcarnitines, glycerophospholipids) so that p-value < 0.01 (=0.05/5) was considered statistically significant. Statistical analyses were conducted using R version 3.1.

2.4. Role of the Funding Source

The study sponsors had no role in the study, writing of the report, or decision to submit the paper.

3. Results

The characteristics of the 313 children in the study are shown in Table 1. There were nearly equal numbers of girls and boys. Over 60% (194/313) of the children were stunted. A summary heatmap of the top fifty serum metabolites by HAZ is shown in Fig. 1. Serum amino acid and biogenic amines in children with and without stunting, adjusted by age and gender, are shown in Supplementary Table 1. All nine essential amino acids (tryptophan, isoleucine, leucine, valine, methionine, threonine, histidine, phenylalanine, lysine), three conditionally essential amino acids (arginine, glycine, glutamine), three non-essential