Amino Acid Metabolism is Altered in Adolescents with Nonalcoholic Fatty Liver Disease—An Untargeted, High Resolution Metabolomics Study

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Objective To conduct an untargeted, high resolution exploration of metabolic pathways that was altered in association with hepatic steatosis in adolescents.

Study design This prospective, case-control study included 39 Hispanic-American, obese adolescents aged 11-17 years evaluated for hepatic steatosis using magnetic resonance spectroscopy. Of these 39 individuals, 30 had hepatic steatosis ≥5% and 9 were matched controls with hepatic steatosis <5%. Fasting plasma samples were analyzed in triplicate using ultra-high resolution metabolomics on a Thermo Fisher Q Exactive mass spectrometry system, coupled with C18 reverse phase liquid chromatography. Differences in plasma metabolites between adolescents with and without nonalcoholic fatty liver disease (NAFLD) were determined by independent *t* tests and visualized using Manhattan plots. Untargeted pathway analyses using Mummichog were performed among the significant metabolites to identify pathways that were most dysregulated in NAFLD.

Results The metabolomics analysis yielded 9583 metabolites, and 7711 with 80% presence across all samples remained for statistical testing. Of these, 478 metabolites were associated with the presence of NAFLD compared with the matched controls. Pathway analysis revealed that along with lipid metabolism, several major amino acid pathways were dysregulated in NAFLD, with tyrosine metabolism being the most affected.

Conclusions Metabolic pathways of several amino acids are significantly disturbed in adolescents with elevated hepatic steatosis. This is a novel finding and suggests that these pathways may be integral in the mechanisms of NAFLD. *(J Pediatr 2016;172:14-9)*.

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Nonalcoholic fatty liver disease (NAFLD) has increased in prevalence and now is the most common chronic liver disease in children.[1,2] Hispanic-Americans have the highest risk of NAFLD possibly because of genetic variations, predisposition to increased adiposity, and increased exposure to high consumption of sugar-sweetened beverages.[3-5] Much of our understanding of the pathogenesis of NAFLD is based upon evidence from animal models and studies in adults with NAFLD. Data in the pediatric population with NAFLD are still limited, and studies exploring potential mechanisms are needed.

High-resolution metabolomics is a powerful analytical tool that analyzes both individual metabolites and systemic alterations of signaling pathways for disease.[6,7] When applied as untargeted assays, high-resolution mass spectrometry (MS) can detect many endogenous metabolites, thus, allowing novel discovery that is not limited to narrowly focused hypotheses. Recent advances in data extraction for ultra-high resolution MS allow relative quantification of thousands of metabolites,[8] including metabolites in 146 out of 154 known human metabolic pathways.[9] A new pathway and network analysis tool used by our group and others, Mummichog, provides an approach for unbiased interrogation of high-resolution metabolomics data for all known metabolic pathways.[10] In the current pilot study, we used these approaches in an exploratory, unbiased, untargeted metabolomics analysis of plasma samples from a group of well-matched adolescent with NAFLD and control participants to identify metabolic pathways that are dysregulated in adolescents with NAFLD.

BCAA	Branched-chain amino acid
BMI	Body mass index
FDR	False discovery rate
LC	Liquid chromatography
m/z	Mass-to-charge ratio
MRS	Magnetic resonance spectroscopy
MS	Mass spectrometry
NAFLD	Nonalcoholic fatty liver disease

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Methods

The study protocol was approved by the Emory University Institutional Review Board and the Children's Healthcare of Atlanta Institutional Review Board; informed consent (parental consent for participants <18 years) and assent were obtained for each participant. Recruitment methods and inclusion/exclusion criteria have been described in detail elsewhere.[11] Briefly, we recruited adolescents aged 11-18 years, self-identified as Hispanics, body mass index (BMI) ≥85th percentile for age and sex, and daily consumption of sugar-sweetened beverages >2. Exclusion criteria included chronic alcohol consumption, previously known liver disease, any other chronic disease requiring daily medication, and any acute illness and anti-oxidation therapy/supplement prior to the enrollment. Cases and controls were recruited identically and assigned into the respective categories after evaluation and completion of the magnetic resonance spectroscopy (MRS) procedure. Presence of "presumed NAFLD" (cases) was defined as MRS for hepatic steatosis ≥5%[12,13] in combination with typical clinical history. Controls were defined as those with hepatic steatosis <5%. The MRS procedure is described in detail elsewhere.[14] Participants underwent a complete history, physical examination, and laboratory evaluation. Their blood samples were collected in ethylenediaminetetraacetic acid-coated tubes after an overnight fast (at least 12 hours), processed immediately, and stored at −80°C. All participants with baseline plasma samples available were included in this analysis.

Ultra-High Resolution Metabolomics Analysis and Data Processing

Frozen plasma samples were transported on dry ice to the Emory Department of Medicine Clinical Biomarkers Laboratory and maintained at −80°C until analysis. Thawed samples were processed and analyzed using liquid chromatography (LC) with ultra-high resolution MS as previously described.[15] Briefly, 20 samples, along with pooled reference sample, were prepared and analyzed on a daily basis to prevent freeze/thaw cycles. For each sample, 65 μL of plasma was used and acetonitrile containing a mixture of 14 stable isotope internal standards was added to the aliquot at 2:1 in order to precipitate proteins.[15] The samples were kept on ice for 30 minutes and then centrifuged for 10 minutes at 13 400 × rpm at 4°C. The supernatant was then removed and placed into autosampler vials. Mass spectral data were collected with a 10-minute gradient on a Dionex UltiMate 3000 rapid separation LC system coupled with a Thermo Q Exactive MS system (Thermo Fisher Scientific, San Diego, California). Ions were scanned in the mass-to-charge ratio (m/z) range from 85-1275 in the positive ionization mode with a resolution of 70 000. Three technical replicates were run for each sample using dual column chromatography procedure[15] with C18 chromatography (Higgins analytical, 100 × 2.1 mm columns). Data were stored as raw files and converted to computable document format using Xcalibur file converter software (Thermo Fisher Scientific) for further processing. Following LC with ultra-high resolution MS, the data were processed using apLCMS[16] and xMSanalyzer[8] to perform peak detection, noise filtering, m/z, and retention time alignment, and feature quantification. The metabolite values were averaged for triplicates; and data were log2 transformed and subjected to quality assessment including exclusion of data for technical replicates with overall Pearson correlation (r) <0.70. Extraction of mass spectral data initially yielded 9583 metabolites. Of these, 7711 metabolites were present in >80% of samples and were used for subsequent analysis.

Statistical Analyses

Descriptive statistics for demographic and clinical data were performed using independent *t* tests or Mann-Whitney U tests (for variables without normal distribution). Sex was compared by Fisher exact tests. The differential expression of plasma metabolites between NAFLD and controls was determined using *t* tests and visualized using Manhattan plots. False discovery rate (FDR) was computed using Benjamini-Hochberg method,[17] which is important in biomarker discovery where adjustments for multiple comparisons are needed to protect against FDR. For our pathway discovery analysis, we used a more conservative approach that avoided FDR error, by including all metabolites that were significant (raw *P* value <.05) and then performing statistical testing of these metabolites for pathway enrichment. The 478 significant metabolites were depicted by a heat map and subjected to pathway analysis using Mummichog,[10] a set of algorithms specifically designed for high-throughput metabolomics. To complement univariate statistics, we also performed linear regression model, adjusted for age and sex, to test the significance of metabolite association with steatosis.

Results

The demographics and clinical data of the study population are summarized in **Table I**. All 39 participants were obese (BMI >95th percentile for age and sex) and self-identified as Hispanic (16 boys and 23 girls). The average age and body weight of participants was 13.8 ± 2.43 years and 80.8 ± 18.2 kg (mean ± SD), respectively; and hepatic steatosis ranged from 2.66%-27.0%. Compared with controls, adolescents with ≥5% hepatic steatosis had increased liver enzymes, plasma triglycerides, insulin, as well as insulin resistance ($P < .05$ for all). No significant differences were observed between the 2 groups in terms of age, sex, body weight, BMI z-score, plasma glucose, or other lipid measurements.

Significant Metabolites Distinguish NAFLD from Controls

To determine the metabolic differences between controls and adolescents with NAFLD, the 7711 metabolites were analyzed by independent *t* tests. Manhattan plots depict each as a