Systemic alterations in the metabolome of diabetic NOD mice delineate increased oxidative stress accompanied by reduced inflammation and hypertriglyceridemia

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Fahrmann J, Grapov D, Yang J, Hammock B, Fiehn O, Bell GI, Hara M. Systemic alterations in the metabolome of diabetic NOD mice delineate increased oxidative stress accompanied by reduced inflammation and hypertriglyceridemia. Am J Physiol Endocrinol Metab 308: E978–E989, 2015. First published April 8, 2015; doi:10.1152/ajpendo.00019.2015.—Nonobese diabetic (NOD) mice are a commonly used model of type 1 diabetes (T1D). However, not all animals will develop overt diabetes despite undergoing similar autoimmune insult. In this study, a comprehensive metabolomic approach, consisting of gas chromatography time-of-flight (GC-TOF) mass spectrometry (MS), ultra-high-performance liquid chromatography-accurate mass quadrupole time-of-flight (UHPLC-qTOF) MS and targeted UHPLC-tandem mass spectrometry-based methodologies, was used to capture metabolic alterations in the metabolome and lipidome of plasma from NOD mice progressing or not progressing to T1D. Using this multi-platform approach, we identified >1,000 circulating lipids and metabolites in male and female progressor and nonprogressor animals (n = 71). Statistical and multivariate analyses were used to identify age- and sex-independent metabolic markers, which best differentiated metabolic profiles of progressors and nonprogressors. Key T1D-associated perturbations were related with 1) increases in oxidation products glucono-δ-lactone and galactonic acid and reductions in cysteine, methionine and threonine acid, suggesting increased oxidative stress; 2) reductions in circulating polyunsaturated fatty acids and lipid signaling mediators, most notably arachidonic acid (AA) and AA-derived eicosanoids, implying impaired states of systemic inflammation; 3) elevations in circulating triacylglycerides reflective of hypertriglyceridemia; and 4) reductions in major structural lipids, most notably lysophosphatidylcholines and phosphatidylcholines. Taken together, our results highlight the systemic perturbations that accompany a loss of glycemic control and development of overt T1D.

Diabetic mice; metabolomics; inflammation; oxidative stress

Type 1 diabetes (T1D) is an autoimmune disease characterized by the selective destruction of pancreatic β-cells. Currently, it is considered that the autoimmune insult is the primary driver of β-cell destruction that leads to development of T1D. However, it is becoming increasingly evident that early metabolic perturbations are inherently involved in the development and progression of T1D (39, 40, 53). These metabolic alterations may potentiate or attenuate β-cell loss and dysfunction. Oresic et al. (40) reported that alterations in branched-chain amino acids (BCAA) and lipid metabolism preceded the appearance of autoantibodies in children who later progressed to T1D. Pflueger et al. (44) found that, independent of age-related differences, autoantibody-positive children had higher levels of odd-chain triglycerides and polyunsaturated fatty acid (PUFA)-containing phospholipids than autoantibody-negative children. Furthermore, it was found that children who developed autoantibodies by age 2 yr had a significantly lower concentration of circulating methionine than those who developed autoantibodies late in childhood or remained autoantibody negative (44). Collectively, these studies demonstrate an intrinsic relationship between metabolic perturbations, autoimmune insult, and β-cell destruction.

Metabolomics is the study of small molecules and biochemical intermediates (metabolites), which are highly relevant to other regulatory mechanisms (e.g., genomics, transcriptome, and proteome) and sensitive to environmental stimuli, forming detailed representations of organismal phenotypes. Over the past decade, the application of metabolomics has been used to gain new insights into the pathology of numerous diseases including type 2 diabetes, develop methods predictive of disease onset, and reveal new biomarkers associated with diagnosis and prognosis (20, 51). Therefore, the application of metabolomics to study T1D pathophysiology represents a promising avenue of research to identify candidate biomarkers related to disease development and progression.

Using a nonobese diabetic (NOD) mouse model, we recently demonstrated marked heterogeneity in pancreatic β-cell loss regardless of age or sex. Importantly, we found that chronic hyperglycemia (≥250 mg/dl) and overt T1D manifested only in mice that lost ~70% of their total β-cell mass (23). Using an untargeted metabolomics approach, we performed an initial evaluation of primary metabolites in plasma of young (<25 wk) and old (≥25 wk) nonprogressors (nondiabetic) vs. progressors (diabetic) to reveal distinct metabolomic pathways involved in disease progression (23). In the present study, we largely extended these investigations to capture alterations in primary metabolism, complex lipids, and lipid signaling mediators in the plasma of nonprogressors and progressors with the overall aim(s) of identifying circulating factors linked to disease progression and, potentially, β-cell destruction and dysfunction.

METHODS

Metabolomic, Lipidomic, and Oxylipin Analysis

NOD mice (n = 71) were assessed as diabetic or nondiabetic based on their fasting (4 h) blood glucose levels at death, which defined 31
hyperglycemic (glucose ≥ 250 mg/dl) and 40 normoglycemic animals. All procedures involving mice were approved by the University of Chicago Institutional Animal Care and Use Committee. Characteristics of our defined cohort are described in Table 1.

The MiniX database (47) was used as a laboratory information management system (LIMS) and for sample randomization prior to all analytical procedures. Detailed information on sample preparation and data acquisition for all analytical platforms are reported in the APPENDIX.

For analysis of primary metabolism, plasma aliquots (30 μl), stored at −80°C, were thawed, extracted, and derivatized, and metabolite levels were quantified by gas chromatography time-of-flight (GC-TOF) mass spectrometry as previously described (19). All samples were analyzed in one batch, throughout which data quality and instrument performance were monitored using quality control and reference plasma samples [National Institute of Standards and Technology (NIST)]. Quality controls (n = 8), comprising a mixture of standards and analyzed every 10 samples, were monitored for changes in the ratio of analyte peak heights and used to ensure equivalent instrumental conditions (P > 0.05; r-test comparing observed to expected ratios of analyte response factors) over the duration of the sample acquisition (19). Acquired spectra were further processed using the BinBase database (18, 47). Briefly, output results (30) were filtered based on multiple parameters to exclude noisy or inconsistent peaks. Detailed criteria for peak reporting, including mass spectral matching, spectral purity, signal to noise, and retention time, are discussed in detail elsewhere (17). Known artifact peaks such as polysiloxanes or phthalates were excluded from data export in BinBase. Missing values were replaced by investigating the extracted ion traces of the raw data subtracted by the local background noise. All entries in BinBase were matched against the Fiehn mass spectral library of 1,200 authentic metabolite spectra, using retention index and mass spectrum information or the NIST11 commercial library. Metabolites were reported if present in at least 50% of the diabetic or nondiabetic samples. Data reported as quantitative ion peak heights were normalized by the sum intensity of all annotated metabolites and used for further statistical analysis. For analysis of the complex lipids, plasma aliquots (20 μl), stored at −80°C, were thawed and extracted using a modified liquid-liquid phase extraction approach proposed by Matyash et al. (37) and analyzed on an Agilent 1290A Infinity Ultra High Performance Liquid Chromatography system with an Agilent Accurate Mass-6530-QTOF (UHPLC-QTOF). Data quality and instrument performance were monitored throughout the data acquisition using quality control (internal STDS) and reference pooled plasma samples. The data were processed using MZmine 2.10 software. Complex lipids were identified by searching against a precursor accurate mass and retention time library in conjunct with matching tandem mass spectra against the LipidBlast virtual MS/MS database (29). Metabolites were reported if positively detected at very high mass spectral confidence in at least 50% of the diabetic or nondiabetic samples. Data, reported as peak heights for the quantification ion (m/z) at the specific retention time for each annotated and unknown metabolite, was normalized to the class-specific internal standard (annotated) or to the internal standard that had the closest retention time (unknowns).

For analysis of oxylipins, samples were extracted in accordance with previously described protocols (60) and analyzed by Agilent 12000SL-AB Sciex 4000 Qtrap ultrahigh performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Optimized conditions and the MRMs transitions, as well as extraction efficiencies, were reported previously (60). Quality control samples were analyzed at minimum calibration throughout the analysis. Analyst software v. 1.4.2 was used to quantify peaks according to their corresponding standard curves. Absolute concentrations of oxylipins are presented as nanomoles per liter.

### Data Analysis

**Statistical analyses.** Statistical analyses were carried out on age and sex covariate adjusted metabolite values. A linear model was used to describe differences in metabolite abundances due to animal age and sex, the residuals from which were tested for differences between T1D and control animals by use of a nonparametric Mann-Whitney U-test. The significance levels (P values) were adjusted for multiple hypothesis testing according to Benjamini and Hochberg (4) at a false discovery rate (FDR) of 5% (abbreviated pFDR <0.05). Statistical tests were calculated separately for primary metabolite, lipidomic, oxylipin, and nonannotated measurements.

**Multivariate modeling.** Multivariate modeling was conducted using orthogonal signal correction partial least squares discriminant analysis (O-PLS-DA) (52) to identify robust predictors of metabolic differences between T1D and control animals. O-PLS-DA models were fitted to age and sex adjusted, natural logarithm transformed, and autoscaled data. The 71 animals were split between two-thirds training and one-third test data sets while the proportion of T1D and control animals in the full data was preserved. Training data were used for model optimization and feature selection. Final model performance was determined based on predictions for the held-out test set and Monte Carlo cross-validation results from the training data.

Model optimization and feature selection were carried out independently for primary metabolite, lipidomic, and oxylipin training data. Model latent variable (LV) number and orthogonal LV (OLV) number were selected using leave-one-out cross-validation. Feature selection was used to identify the top ~10% of all metabolic predictors for T1D from each biochemical domain. The full variable set was filtered to retain analytes that displayed 1) significant correlation with model scores (Spearman’s pFDR ≤ 0.05) (58) and 2) model loadings on LV1 in the top 90th quantile in magnitude (41) and Mann-Whitney U-test P values < 0.05.

The top 10% selected features (n = 44; 12 primary metabolites, 29 complex lipids, and 3 oxylipins) from each biochemical domain were combined and evaluated using Monte Carlo training and testing cross-validation and permutation testing. Internal training and testing were performed by further splitting the training set into two-thirds pseudo-training and one-third pseudo-test sets, while the proportion of T1D and control animals was preserved. This split was randomly repeated 1,000 times and used to estimate the distributions for the O-PLS-DA model performance statistics: the cross-validated fit to the training data (Q2), root mean squared error of prediction (RMSEP), area under the receiver operator characteristic curve (AUC), sensitivity (true positive rate), and specificity (true negative rate). The probability of achieving the models’ predictive performance was estimated through comparison of performance statistics to those of permuted models (random class labels) (45), which were calculated by replicating the internal training and testing procedures described above. Additionally, model performance was compared between the selected (n = 44; primary metabolites, 12; lipids, 29; oxylipins, 3) and excluded (bottom 90%, n = 351; primary metabolites, 168; lipids, 132; oxylipins, 51) feature sets.

Final model classification performance was validated through prediction of class labels for the originally held-out test set and are

### Table 1. Nonobese diabetic mice characteristics

<table>
<thead>
<tr>
<th></th>
<th>Nondiabetic</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>Male</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>Age, wk</td>
<td>36 (26,40)</td>
<td>38 (26,40)</td>
</tr>
<tr>
<td>Weigh, g</td>
<td>27.3 ± 4.8</td>
<td>19.5 ± 4.8*</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>94 ± 34</td>
<td>513 ± 101*</td>
</tr>
</tbody>
</table>

Values are reported as means ± SD (median, minimum, maximum). *Unpaired two-sample t-test, P ≤ 0.05.
RESULTS

Analysis of Plasma Metabolites in NOD Mice

Unbiased GC-TOF- and UHPLC-qTOF-based metabolomics coupled with targeted UPLC-MS/MS analysis of oxylipins was used to compare the metabolome of 41 nondiabetic and 30 diabetic animals (Table 1). A total of 1,041 metabolomic peaks were detected, of which 395 were structurally annotated metabolites. For GC-TOF- and UHPLC-qTOF-based metabolomics, measured peak heights are reported in normalized units using the diagnostic “unique ion” for each compound. Lipid signaling mediators (oxylipins) are reported as concentrations (nmol/l).

Comparison of NOD Mice’s Physical and Biochemical Characteristics

Statistical analyses were used to compare physical characteristics and metabolomic measurements between diabetic and nondiabetic NOD mice. Diabetic mice displayed significant elevations in fasting glucose but reduced body weight compared with nondiabetic mice (Table 1).

A nonparametric Mann-Whitney U-test with FDR correction was used to identify 493 (47%) significantly altered age- and sex-adjusted metabolic features ($P_{adj} \leq 0.05$) between diabetic and nondiabetic mice (see Supplemental Table S1 in supplemental materials, linked to the online version of this article). Statistical comparisons of major classes of molecules and biochemical subdomains identified general T1D-dependent increases in the majority of carbohydrates, aromatics, amino acids or amides, nucleotides, and triacylglycerides (TGs) whereas prostacyclins, C18-diols, C18- and C20-ketones, C20-hydroxy acids, triols, sterols, acylcarnitines (ACs), phosphatidylethanolamines (PEs), phosphatidylcholines (PCs), phosphatidylinositols (PIs), sphingomyelins (SMs), and lysophosphatidylcholines (LPCs) showed a general decrease (Table 2).

Table 2. Significantly altered biochemical domains in T1D vs. control animals

<table>
<thead>
<tr>
<th>Biochemical Class</th>
<th>Control</th>
<th>Diabetic</th>
<th>FC</th>
<th>$P$ Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid or amide</td>
<td>1440000 ± 570000</td>
<td>1730000 ± 5e+05</td>
<td>1.2</td>
<td>0.02</td>
</tr>
<tr>
<td>Aromatic (6)</td>
<td>3070 ± 1800</td>
<td>4200 ± 2300</td>
<td>1.37</td>
<td>0.01</td>
</tr>
<tr>
<td>Carbohydrate (55)</td>
<td>2690000 ± 9e+05</td>
<td>4560000 ± 640000</td>
<td>1.69</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Nucleotide (14)</td>
<td>29100 ± 11000</td>
<td>37700 ± 17000</td>
<td>1.3</td>
<td>0.03</td>
</tr>
<tr>
<td>TG (50)</td>
<td>21500000 ± 8900000</td>
<td>32600000 ± 2.3e+07</td>
<td>1.52</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Based on Mann-Whitney U-test. † Number of measured molecules. ‡ Oxylipins are reported in nM; all others in peak area heights.
Multivariate classification modeling using O-PLS-DA coupled with feature selection was used to identify the top metabolic determinants of the T1D plasma phenotype. A subset of 44 metabolites (10%; Supplemental Table S1) was selected as part of the validated model (see Appendix Table A1) for discrimination between diabetic and nondiabetic NOD mice.

For primary metabolites and oxylipins, biochemical and chemical similarity network analysis was conducted to calculate and visualize relationships between precursor and product metabolite reactant pairs and molecules sharing a high degree of structural similarity (Fig. 1). A heatmap based on hierarchical cluster analysis was used to summarize the relationships between classes of measured molecules, fasting blood glucose, and body weight (Fig. 2).

A Gaussian graphic model network was calculated to identify conditionally independent relationships (partial correlation, $P_{adj} \leq 0.05$) between all significant ($P_{adj} \leq 0.05$) T1D-associated metabolic perturbations (Fig. 3).

**Diabetic NOD Mice Display Altered Carbohydrate Profiles**

Total plasma carbohydrate levels were increased by 169% in diabetic mice (Table 2) and were generally positively correlated with measured fasting blood glucose and negatively correlated with body weight (Fig. 2). Moreover, circulating levels of glucose, gluconic acid lactone, glucuronic acid, idonic acid, ribose, cellobiose, and 2-deoxytetronic acid, all of which were elevated in diabetic mice compared with nondiabetic mice, were selected as being within the top 10% discriminants of the diabetic phenotype (Supplemental Table S1). Elevations in ribose, 2-deoxytetronic acid and cellobiose were shown to be both biochemically (Fig. 1) and empirically (Fig. 3) related to general alterations in most carbohydrates. Similarly, we observed T1D-associated increases in organic acids and metabolites related to energetics including a 240% increase in 2-hydroxy-2-methylbutanoic acid, which was found to be the single most discriminatory metabolite of the T1D phenotype (Supplemental Table S1). 2-Hydroxy-2-methylbutanoic acid was positively related to the observed increase in indole-3-lactate and ribose and negatively correlated to PC (38:2), whereas indole-3-lactate was also positively correlated to the T1D-associated increase in isocitric acid (Fig. 3). Whereas most carbohydrates were found to be elevated in diabetic mice, we found significant T1D-dependent decreases in circulating 1,5-anhydroglucitol, glycerol-3-galactoside, and threonic acid. Threonic acid was similarly identified as a top descriptor of T1D and paralleled an observed decrease in methionine (Fig. 3).

**Diabetic NOD Mice Display Alterations in Amino Acid Profiles**

Overall, 10 of the 47 measured amino acid or amine metabolites (21%) were significantly altered in diabetic compared with nondiabetic animals (Fig. 1). Of these changes, six were significantly decreased in diabetic mice, including creatinine.

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**Fig. 1.** Biochemical network displaying metabolic differences between diabetic and nondiabetic NOD mice. Metabolites are connected based on biochemical relationships (orange, KEGG RPAIRS) or structural similarity (blue, Tanimoto coefficient $\geq 0.7$). Metabolite size and color represent the importance (O-PLS-DA model loadings, LV1) and relative change (gray, $P_{adj} > 0.05$; green, decrease; red, increase) in diabetic vs. nondiabetic NOD mice. Shapes display metabolites’ molecular classes or biochemical subdomains, and top descriptors of type 1 diabetes (T1D)-associated metabolic perturbations (Supplemental Table S1) are highlighted with thick black borders.
5-methoxytryptamine, methionine, cysteine, tyrosine, and tryptophan (Fig. 1). Inversely, four metabolites showed T1D-associated elevations, including a 180% increase in panthenolic acid and 140% increases in valine, isoleucine, and 5-hydroxyindole-3-acetic acid (Supplemental Table S1). Of the noted shifts, only methionine was found to be included as a top predictor of the T1D phenotype. Overall, amino acids were negatively correlated with body weight but not fasting blood glucose (Fig. 2).

Diabetic NOD Mice Display Perturbed Lipid Profiles

Cumulatively, alterations in lipid metabolism best delineated diabetic from nondiabetic mice, encompassing 28 of the top 44 most discriminatory metabolites (64%) between diabetic and nondiabetic animals (Supplemental Table S1). Jointly, diabetic animals displayed significant elevations in virtually all measured triacylglycerides with TG (49:1), TG (50:1), TG (12:0/18:2/20:5), TG (51:1), TG (54:1), TG (54:2), TG (54:4), TG (58:3), and TG (58:10) all being ranked in the top 10% of discriminatory metabolites (Supplemental Table S1) and being empirically related to each other (Fig. 3). Only TG (50:3) and TG (56:4) were found to be significantly lower in diabetic than in nondiabetic mice (Supplemental Table S1). The increase in TG (54:1) was negatively correlated to LPC (16:1), whereas TG (58:3) and TG (16:0/18:2/20:5) were both negatively related to CE (20:4) (Fig. 3). Notably, all measured LPCs were significantly reduced in diabetic compared with nondiabetic animals (Supplemental Table S1 and Table 2). The T1D-associated reduction in LPC was positively related to similar reductions in several other lipid species, including SM (d40:2), A, SM (d18:2/23:0), and PI (40:6) (Fig. 3). Intriguingly, LPC (16:1) was positively associated with methionine, which was reduced in diabetic mice and positively correlated to the T1D-associated reduction in LPC (20:4) (Fig. 3). LPC (20:4) coordinately correlated with LPC (16:0), which served as a central hub and was positively related to numerous PC and SM lipid species, including PC (36:4), PC (p-36:3) or PC (o-36:3), PC (38:5), SM (d18:1), and SM (d18:2/23:0) (Fig. 3). In accord with the observed T1D-associated decrease in all measured LPCs, nearly all measured PCs and SMs were similarly reduced in diabetic compared with nondiabetic mice (Table 2). Only SM (d16:1/20:1), PC (35:2), PC (35:2) B, PC (p-36:2) or PC (o-36:3), and PC (36:5) were found to be elevated in diabetic mice (Supplemental Table S1). The T1D-associated reduction in PC (38:5) was also positively associated with similar reductions in PC (38:2), PC (37:2), and PI (38:4).
Likewise, the T1D-dependent decrease in PE (38:6) B was positively related to PE (38:6), which positively correlated with PI (38:4). Comparable to PCs and SMs, many of the measured PEs and PIs were decreased in diabetic animals (Table 2). On a global scale, PCs, SMs, PIs, LPCs, and PEs were all positively correlated with body weight and negatively correlated with fasting blood glucose, whereas TGs were negatively correlated with body weight and positively correlated with fasting blood glucose (Fig. 2).

Plasma free fatty acids were generally lower in diabetic mice, led by a 60% reduction in palmitoleic acid and 40% reductions in palmitic acid and arachidonic acid (Supplemental Table S1). The reductions in circulating free fatty acids were negatively correlated with fasting blood glucose (Fig. 2).

**Diabetic NOD Mice Display Perturbations in Lipid Signaling Mediators**

Overall, 24 of the 54 measured oxylipins (43%) were found to be significantly altered between diabetic and nondiabetic animals. Furthermore, all 24 oxylipins were found to be significantly decreased in the plasma of diabetic compared with nondiabetic mice (Fig. 1). Particularly interesting was that many oxylipins derived from arachidonate metabolism were significantly reduced in diabetic mice, including LTB₄, PGE₂, PGD₂, TXB₂, 8-HETE, 12-HETE, 15-HETE, 12-OxoETE, and 15-OxoETE (Fig. 4). Moreover, global concentrations of circulating 20C-ketones, 20C-hydroxy acids, and prostacyclins were all positively correlated with body weight and negatively correlated with fasting blood glucose (Fig. 2 and Supplemental Table S1). Only 12-oxo-ETE, 9-oxo-ODE, and PGF₂α were identified as being within the top 10% of discriminant metabolites (Supplemental Table S1). The T1D-associated decrease in 12-oxo-ETE paralleled the observed reductions in PC (38:2) and threonic acid (Fig. 3).

**DISCUSSION**

In the previous study, using NOD mice, we characterized progressors (diabetic) and nonprogressors (nondiabetic) to
T1D and performed an initial screening of primary metabolites in the plasma of these mice with the aims of identifying circulating factors linked with β-cell viability and/or T1D progression (23). In the current study, we aimed to expand upon our initial observations by using a multiplatform approach to recapitulate our initial metabolomic findings in addition to capturing perturbations in the lipidome and lipid signaling mediators. Using this unique multiplatform approach, we detected more than 1,000 lipids and metabolites (396 annotated) in the plasma of progressors and nonprogressors.

Our analysis of the primary metabolites (carbohydrates, amino acids, organic acids, nucleotides, free fatty acids) revealed numerous age- and sex-adjusted T1D-dependent alterations that extended to multiple biochemical domains. Importantly, 29 of the 60 annotated metabolites distinguishing progressors from nonprogressors were also found to be significantly different in our initial study (23) (see Appendix Table A2). Furthermore, all 29 metabolites maintained the same direction in change providing validation for both our initial and current study (see Appendix Table A2).

In comparison with nonprogressors, diabetic mice indicated a 69% increase in global circulating carbohydrates. Most notably, diabetic mice displayed significant elevations in circulating glucose and significant reductions in 1,5-anhydroglucitol (1,5-AG), a marker of glycemic control (28). The elevation in glucose and reduction in 1,5-AG is consistent with the diabetic model and provides assurance in our analytical analysis.

In concurrence with the increases in circulating carbohydrates, carbohydrate oxidation products glucono-δ-lactone (GDL), an oxidation product of glucose, were elevated in diabetic compared with nondiabetic mice (Fig. 1). The elevation in circulating GDL and galactonic acid may reflect elevated states of oxidative stress, a well-known hallmark of T1D (36). Consistent with this notion, we observed significant T1D-dependent reductions in circulating levels of methionine and cysteine (Fig. 1). Methionine and cysteine are both components of the transsulfuration pathway, which leads to the synthesis of methionine (not measured), an important intracellular antioxidant. Previous studies have demonstrated that insulin-deprived T1D subjects have reduced rates of homocysteine-methionine remethylation and increased rates of transsulfuration compared with control subjects (1). Accordingly, patients with poor glycemic control have been shown to have depleted glutathione pools and reduced erythrocyte free cysteine concentrations (11). The reduction in circulating methionine and cysteine may, therefore, reflect an increased flux into the transsulfuration pathway and glutathione synthesis. In the current investigation, the reduction in circulating methionine paralleled the observed reduction in threonic acid, a breakdown product of the antioxidant ascorbic acid (vitamin C; Fig. 4) (54). The T1D-associated reduction in circulating threonic acid suggests impaired ascorbic acid metabolism. This is a noteworthy observation given that ascorbic acid uptake into the tissue of diabetic animals is often depressed during states of hyperglycemia (10), rendering cells more susceptible to oxidative stress. Additionally, diabetic mice indicated a significant (raw \( P \text{ value} \leq 0.017 \)) 33% reduction in α-tocopherol, a lipophilic antioxidant (see Supplemental Table S2). Collectively, the above-mentioned metabolic aberrations point toward a heightened state of oxidative stress in mice that progress to T1D. In this sense, it is
interesting to note that cysteine, threonic acid, and methionine were negatively correlated with fasting blood glucose and positively correlated with 1,5-AG (see Appendix Table A3). We therefore postulate that reductions in circulating cysteine, threonic acid, and methionine serve as candidate biomarkers for monitoring oxidative stress and predicting T1D development and progression.

Although it was evident that there were many T1D-associated alterations in primary metabolism, one of the more pronounced differences reflective of the progression to T1D was observed in lipid species and lipid signaling mediators. Consistent with our initial finding, diabetic NOD mice displayed significant reductions in selected free fatty acids, most notably arachidonic acid (AA), palmitoleic acid (PA) and oleic acid (OA) compared with nonprogressors (Fig. 1). Free fatty acids exhibit complex interactions with β-cells, acting as both secretagogues (14) and agents of apoptotic signaling (9, 26, 42, 55). Moreover, the development of T1D is known to have a strong inflammatory component (3, 34, 43). Cyclooxygenase (COX)-2 and 12-lipoxygenase (LOX) derived products of AA, such as prostaglandin E2 (PGE2) and 12-hydroxyecosatetraenoic acid (12-HETE), have been shown to play critical roles in cytokine-induced human β-cell destruction (9, 26, 42, 55). Thus, one may anticipate that the reduction in circulating AA represents a shift in equilibrium toward eicosanoid production. On the contrary, we found significant T1D-associated reductions in several AA-derived eicosanoids, including thromboxane A2 (TXA2), leukotriene B4 (LTB4), PGD2, PGE2, 11-,12- and 15-HETE, and 12-oxo-ETE. This suggests a reduced bioavailability of AA rather than an increased flux into these lipid signaling pathways (Fig. 4). The reduction in circulating oxylipins, particularly those derived from AA, also suggests that mice that have progressed to T1D are under a reduced state of systemic inflammation. Kriegel et al. (32) previously reported marked infiltration of CD11c+CD11b+dendritic cells (DC) into the pancreatic islets of NOD mice. Interestingly, these DCs exhibited a hypoactive phenotype, failed to induce proliferation of diabetogenic CD4+ T-cells in vitro, and were potent suppressors of diabetes development (32). Additionally, Bouma et al. (6) demonstrated that NOD mice exhibit impaired recruitment of leukocytes into sites of inflammation in the peritoneum and subcutaneously elicited air pouches. It should be noted that in both studies the cohort of NOD mice were under 8 wk of age and, as such, cannot be exclusively compared with our diabetic cohort. Despite this limitation, both studies demonstrate marked modulation of overall immune and inflammatory status in NOD mice and T1D development. Moreover, both studies hint toward the fact that reduced recruitment of inflammatory mediators accompanies T1D development. However, it is important to note that we evaluated systemic alterations of inflammatory mediators as opposed to pancreas-specific changes; thus, changes in circulation reflect the contributions all organ types. Despite these findings, AA, in addition to PA and OA, has been also been shown to attenuate the deleterious effects of high glucose on human pancreatic β-cell turnover and function (12, 13, 35). Similarly, reductions in 9,10-DiHODE and 11- and 12-HETE have been observed in men with hyperlipidemia, a frequent T1D-associated complication (15, 48). Future studies will be required to confirm our initial observations and fully elucidate how the observed reductions in circulating lipid mediators and free fatty acids relate to T1D development and progression.

The coexistence of lipid dysfunction and hyperlipidemia is a known to be intertwined with T1D pathophysiology. Hypertriglyceridemia is strongly linked to poor glycemic control (21, 46). Our analysis of the lipidome revealed significant T1D-associated increases in virtually all circulating triglycerides (Supplemental Table S1). Moreover, global abundances of triglycerides were positively correlated with fasting blood glucose (Fig. 3), indicative of hypertriglyceridemia. Inverse to the observed increases in circulating triglycerides, diabetic NOD mice displayed striking reductions in several lysophospholipids, phospholipids, and sphingolipids (Supplemental Table S1). It is important to note that these major lipid classes were highly correlated with body weight, which was found to be significantly lower in our diabetic cohort, and negatively correlated with fasting blood glucose (Fig. 3). T1D is characterized by increases in amino acid catabolism (25) and fatty acid oxidation (24, 27), a consequence of the inability to utilize glucose as a source of energy. Weight loss is frequently observed in T1D, which is attributed to several T1D-associated complications such as muscle atrophy and dehydration (57, 59).

Consequently, the observed reduction in major structural lipids may be a consequence of increased fatty acid β-oxidation and reduced bioavailability of fatty acids for phospholipid/structural lipid synthesis, a notion that is partially supported by the observed decrease in circulating free fatty acids and weight loss. Despite the strong association between body weight and major structural lipids, alterations in lipid metabolism are involved in the etiology of T1D. Transiently elevated LPC serum levels have been positively associated with seroconversion to islet autoantibody positivity in children who subsequently progress to T1D (40). Furthermore, LPCs have been shown to improve blood glucose levels in mouse models of T1D and T2D (61) and act as insulin secretagogues (50). Sysi-Aho et al. (53) found that female NOD mice that did not progress to T1D after 8 wk of age despite being insulin autoantibody (IAA) positive had elevated levels of selected serum LPC species compared with progressors. Additionally, it was demonstrated that IAA positivity and LPC concentrations were able to reasonably discriminate between progressors and nonprogressors (53). Collectively, the findings by Sysi-Aho et al. and others suggest a protective effect by LPCs. It is important to note that the studies by Sysi-Aho and colleagues focused primarily on metabolic alterations that occur prior and during seroconversion in young (<25 wk) NOD mice. The cohort of NOD mice used in the current investigation extended beyond 25 wk and included NOD mice that escaped chronic hyperglycemia, a group that has been largely excluded in previous studies. The inclusion of mice that avoided developing overt T1D was particularly important, since it allowed us to directly compare between progressors and nonprogressors both undergoing similar autoimmune insults. In our model system, LPCs were negatively correlated with fasting blood glucose (Fig. 3) corroborating the notion that LPCs are associated with glycemic control and β-cell dysfunction/destruction. Interestingly, selected LPCs were positively related to the T1D-associated reduction in methionine (Fig. 4). Pflueger et al. (44) previously found that concentrations of methionine were lower in children who developed autoantibodies by age 2 yr compared with those who developed autoantibodies later in child-
hood or remained autoantibody negative. Methionine is a vital component in one-carbon metabolism and serves as the precursor for s-adenosylmethionine synthesis. s-Adenosyl methionine in turn acts as a methyl donor for the synthesis of PCs from PEs (38). One-carbon metabolism has been shown to be perturbed in animal models of TID (38). The observed reduction in methionine in diabetic mice may therefore not only be an indicator of increased flux into the transsulfuration pathway but may also partially account for the TID-dependent reductions in PCs and related LPCs. This is a notable observation as Oresic et al. (40) demonstrated that children who developed TID had reduced serum levels of PCs at birth and were consistently low in progressively compared with nonprogressors. Similarly, analysis of umbilical cord serum lipids in infants who later developed TID revealed distinct reductions in major choline-containing phospholipids, including sphingomyelins and PCs (39). Our results suggest that the reduction in circulating LPC and PC species can be used as potential biomarkers for T1D onset and progression and β-cell loss.

In conclusion, a comprehensive metabolomics approach was used to identify 493 (192 annotated) significant age- and sex-independent metabolic alterations associated with TID development and progression. Our findings suggest that progression to TID is characterized by increased oxidative stress, perturbed states of inflammation, and altered lipid metabolism. More importantly, these findings serve as a basis for the identification of metabolic states (i.e., altered amino acid profiles in conjunction with altered lipid profiles) that can be used as diagnostic and prognostic indicators of TID pathophysiology and consequently β-cell death. In particular, we propose that reductions in circulating threonic acid, methionine, and cysteine may serve as stable markers of oxidative stress and TID progression, whereas reductions in phosphophatidylycholines and related lysophosphatidylycholines may serve as candidate biomarkers of glycemic control, T1D onset, and β-cell destruction.

APPENDIX

Analysis of the Metabolome, Lipidome, and Lipid Signaling Mediators

For analysis of primary metabolites, 30-µl plasma aliquots, which were extracted with 1 ml of degassed acetonitrile-isopropanol-water (3:3:2) at −20°C and centrifuged, the supernatant was removed, and solvents were evaporated to dryness under reduced pressure. To remove membrane lipids and triglycerides, dried samples were reconstituted with acetonitrile-water (1:1), decanted, and taken to dryness under reduced pressure.

Internal standards, C8–C30 fatty acid methyl esters (FAMEs), were added to samples and derivatized with methoxymethylene hydrochloride in pyridine and subsequently by MSTFA (Sigma-Aldrich) for trimethylsilylation of acidic protons and analyzed by GC-TOF-MS. An Agilent 7890A gas chromatograph (Santa Clara, CA) was used with a 30 m long, 0.25 mm ID Rtx5Sil-MS column with 0.25-µm 5% diphenyl film; an additional 10-m integrated guard column was used (Restek, Bellefonte, PA) (16, 30, 56). A Gerstel MPS2 automatic liner exchange system (ALEX) was used to eliminate sample cross-contamination during the GC-TOF analysis. A 0.5-µl of sample was injected at 50°C (ramped to 250°C) in splitless mode with a 25-s splitless time. The chromatographic gradient consisted of a constant flow of 1 ml/min, ramping the oven temperature from 50°C to 330°C over 22 min. Mass spectrometry was done using a Leco Pegasus IV TOF mass spectrometer, 280°C transfer line temperature, electron ionization at −70 V, and an ion source temperature of 250°C. Mass spectra were acquired at 1,525 V detector voltage at m/z 85–500 with 17 spectra/s. Acquired spectra were further processed using the BinBase database (18, 47). Briefly, output results (30) were filtered based on multiple parameters to exclude noisy or inconsistent peaks. All entries in BinBase were matched against the Fiehn mass spectral library of 1,200 authentic metabolite spectra, using retention index and mass spectrum information or the NIST11 commercial library.

For analysis of the lipidome, plasma aliquots (20 µl), stored at −80°C, were thawed and extracted using a modified liquid-liquid phase extraction approach purported by Matyash et al. (37). Briefly, 225 µl of chilled methanol containing an internal standard mixture [PE(17:0/17:0); PG(17:0/17:0); PC(17:0/0:0); C17 sphingosine; C17 ceramide; SM (d18:0/17:0); palmitic acid-d3; PC (12:0/13:0); cholesterol-d7; TG (17:0/17:0/17:0)-d5; DG (12:0/12:0/0:0); DG (18:1/12:0/0:0); MG (17:0/0:0/0:0); PE (17:1/0:0); LPC (17:0); LPE (17:1)], and 750 µl of chilled MTBE containing the internal standard 22:1 cholesterol ester was added to 10-µl aliquots of sample. Samples were shaken for 6 min at 4°C using an Orbital Mixing Chilling/Heating Plate (Torrey Pines Scientific Instruments) followed by the addition of 188 µl of room temperature distilled water. Samples were vortexed and centrifuged, and the upper layer was transferred to a new 1.5-ml Eppendorf tube. An aliquot was dried to completeness using a Labconco Centrivap. Upon complete dryness, samples were resuspended in methanol-toluene (90:10) with 50 ng/ml CUDA ([(12-cyclohexylamino)carbonyl]amino]-dodecanoic acid, Cayman Chemical). Samples were vortexed, sonicated for 5 min, and centrifuged, whereafter 100 µl of the sample was transferred to an amber glass vial (National Scientific C4000-2W) with a microinsert (Supelco 27400-U). Lipid extracts were subsequently analyzed on an Agilent 1290A Infinity Ultra High Performance Liquid Chromatography system with an Agilent Accurate Mass Mass-6530-QTOF in both positive and negative modes. The column (65°C) was a Waters Acquity UPLC CSH C18 (100 mm length × 2.1 mm ID; 1.7 µm particles) coupled with a Waters Acquity VanGuard CSH C18 1.7 mM µm precolumn. The solvent system included (A) 60:40 vol/vol acetonitrile-water (LCMS grade) containing 10 mM ammonium formate and 0.1% formic acid and (B) 90:10 vol/vol isopropanol-acetonitrile containing 10 mM ammonium formate and 0.1% formic acid. The gradient started from 0 min 15% (B), 0–2 min 50% (B), 2.5–5 min 48% (B), 2.5–11 min 82% (B), 11–11.5 min 99% (B), 11.5–12 min 99% (B), 12–12.1 min 15% (B), and 12.1–15 min 15% (B). The flow rate was 0.6 ml/min and with an injection volume of 1.67 µl for ESI (+) and 5 µl for ESI (−) mode acquisition. ESI capillary voltage was +3.5 kV and −3.5 kV with collision energies of 25 and 40 eV for MS/MS collection in positive and negative acquisition modes, respectively. Data were collected at a mass range of m/z 60–1700 Da with a spectral acquisition speed of 2 spectra/s. Method blanks and pooled sterile human plasma samples were included to serve as additional quality controls. CUDA was used to monitor instrument performance. Data were processed using MZmine 2.10. All peak intensities are representative of peak heights. Annotations were completed by matching experimental accurate mass MS/MS spectra to MS/MS libraries, including Metlin-MSMS, NIST12, and LipidBlast (29). Spectral matching was automated using the MSPePSearch tool and manually curated using The NIST Mass Spectral Search Program v. 2.0g. Metabolite libraries were created, in positive and negative ionization modes, containing all confirmed identified compounds. MZmine’s Custom Database Search tool was used to assign annotations based on accurate mass and retention time matching.

For analysis of lipid signaling mediators (oxylipins), samples were extracted in accordance with previously described protocols (60). Briefly, plasma samples underwent solid-phase extraction (SPE) on 60-µg Waters Oasis–HLB cartridges (Milford, MA). The elutions from the SPE cartridges were evaporated to dryness using a Speedvac (Jouan, St-Herblain, France) and reconstituted in 200 nM CUDA in methanol and analyzed by UPLC-MS/MS. The LC system used for
analysis was an Agilent 1200 SL (Palo Alto, CA) equipped with a 2.1 x 150 mm Eclipse Plus C18 column with a 1.8 µm particle size (Agilent). The autosampler was kept at 4°C. Mobile phase A consisted of water with 0.1% glacial acetic acid. Mobile phase B consisted of LCMS-grade acetonitrile-methanol (84:16 vol/vol) with 0.1% glacial acetic acid. Gradient elution was performed at a flow rate of 250 µl/min. Chromatography was optimized to separate all analytes in 21.5 min according to their polarity, with the most polar analytes, prostaglandins, and leukotrienes eluting first, followed by the hydroxyl and epoxy fatty acids. The column was connected to a 4000 QTrap tandem mass spectrometer (Applied Biosystems Instrument, Foster City, CA) equipped with an electrospray source (Turbo V). The instrument was operated in negative multiple reaction monitoring (MRM) mode. The optimized conditions and the MRM transitions, as well as extraction efficiencies, were reported previously (60). Quality control samples were analyzed at minimum calibration throughout the analysis. Analyst 1.4.2 software was used to quantify peaks according to their standard curve.

Table A1. O-PLS-DA model performance and validation statistics

<table>
<thead>
<tr>
<th>Model</th>
<th>Q²</th>
<th>RMSEP</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Training data</td>
<td>0.691 ± 0.05*</td>
<td>0.341 ± 0.06*</td>
<td>0.844 ± 0.09*</td>
<td>0.902 ± 0.08**</td>
<td>0.786 ± 0.17*</td>
</tr>
<tr>
<td>Test data</td>
<td>0.652</td>
<td>0.277</td>
<td>0.944</td>
<td>1</td>
<td>0.889</td>
</tr>
</tbody>
</table>

Q², cross-validated fit to the training data; RMSEP, root mean square error of prediction; AUC, area under the receiver operator characteristic curve.

*Performance worse than only 5% of all permuted models. **Performance worse than only 10% of all permuted models.

Table A2. List of compounds significantly different in both the initial and follow-up studies

<table>
<thead>
<tr>
<th>Metabolite†</th>
<th>FC‡</th>
<th>Direction§</th>
<th>Padj**</th>
<th>FC‡</th>
<th>Direction§</th>
<th>Padj**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,5-Anhydroglucitol</td>
<td>0.4</td>
<td>Down</td>
<td>&lt;0.00000</td>
<td>0.13</td>
<td>Down</td>
<td>&lt;0.00000</td>
</tr>
<tr>
<td>2-Deoxytetronic acid</td>
<td>1.4</td>
<td>Up</td>
<td>0.04390</td>
<td>1.12</td>
<td>Up</td>
<td>0.00384</td>
</tr>
<tr>
<td>2-Hydroxy-2-methylbutanoic acid</td>
<td>2.2</td>
<td>Up</td>
<td>0.00061</td>
<td>2.44</td>
<td>Up</td>
<td>&lt;0.00000</td>
</tr>
<tr>
<td>3,6-Anhydrogalactose</td>
<td>4.3</td>
<td>Up</td>
<td>&lt;0.00000</td>
<td>1.82</td>
<td>Up</td>
<td>0.00002</td>
</tr>
<tr>
<td>4-Hydroxybutyric acid</td>
<td>2.5</td>
<td>Up</td>
<td>&lt;0.00000</td>
<td>1.23</td>
<td>Up</td>
<td>0.02300</td>
</tr>
<tr>
<td>5-Hydroxylindole-3-acetic acid</td>
<td>2</td>
<td>Up</td>
<td>0.00002</td>
<td>1.41</td>
<td>Up</td>
<td>0.03900</td>
</tr>
<tr>
<td>Adenosine-5-phosphate</td>
<td>0.5</td>
<td>Down</td>
<td>0.00113</td>
<td>0.52</td>
<td>Down</td>
<td>0.00021</td>
</tr>
<tr>
<td>Arabinose</td>
<td>1.6</td>
<td>Up</td>
<td>0.01850</td>
<td>1.59</td>
<td>Up</td>
<td>0.00755</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>0.7</td>
<td>Down</td>
<td>0.00136</td>
<td>0.57</td>
<td>Down</td>
<td>0.00065</td>
</tr>
<tr>
<td>Creatine</td>
<td>0.6</td>
<td>Down</td>
<td>0.01130</td>
<td>0.36</td>
<td>Down</td>
<td>0.00374</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.7</td>
<td>Down</td>
<td>0.03990</td>
<td>0.67</td>
<td>Down</td>
<td>0.03400</td>
</tr>
<tr>
<td>Erythritol</td>
<td>1.6</td>
<td>Up</td>
<td>0.01140</td>
<td>1.85</td>
<td>Up</td>
<td>0.00391</td>
</tr>
<tr>
<td>Galactitol</td>
<td>2.6</td>
<td>Up</td>
<td>0.00964</td>
<td>1.15</td>
<td>Up</td>
<td>0.04980</td>
</tr>
<tr>
<td>Galactonic acid</td>
<td>2.2</td>
<td>Up</td>
<td>&lt;0.00000</td>
<td>1.61</td>
<td>Up</td>
<td>0.00531</td>
</tr>
<tr>
<td>Glucose</td>
<td>3</td>
<td>Up</td>
<td>&lt;0.00000</td>
<td>2.08</td>
<td>Up</td>
<td>&lt;0.00000</td>
</tr>
<tr>
<td>Glycerol-α-phosphate</td>
<td>0.6</td>
<td>Down</td>
<td>0.00003</td>
<td>0.52</td>
<td>Down</td>
<td>0.00091</td>
</tr>
<tr>
<td>Indole-3-lactate</td>
<td>2.2</td>
<td>Up</td>
<td>0.00114</td>
<td>2.08</td>
<td>Up</td>
<td>0.00379</td>
</tr>
<tr>
<td>Isocitric acid</td>
<td>1.4</td>
<td>Up</td>
<td>0.02390</td>
<td>1.56</td>
<td>Up</td>
<td>0.00564</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.9</td>
<td>Up</td>
<td>0.01870</td>
<td>1.43</td>
<td>Up</td>
<td>0.01250</td>
</tr>
<tr>
<td>Isoteric acid</td>
<td>1.5</td>
<td>Up</td>
<td>0.00584</td>
<td>1.69</td>
<td>Up</td>
<td>0.00415</td>
</tr>
<tr>
<td>Lactobionic acid</td>
<td>3.2</td>
<td>Up</td>
<td>0.00044</td>
<td>2.86</td>
<td>Up</td>
<td>&lt;0.00000</td>
</tr>
<tr>
<td>Maltose</td>
<td>3.1</td>
<td>Up</td>
<td>&lt;0.00000</td>
<td>2.50</td>
<td>Up</td>
<td>&lt;0.00000</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>0.7</td>
<td>Down</td>
<td>0.03470</td>
<td>0.68</td>
<td>Down</td>
<td>0.04830</td>
</tr>
<tr>
<td>Piperocetic acid</td>
<td>2</td>
<td>Up</td>
<td>0.00901</td>
<td>1.85</td>
<td>Up</td>
<td>0.03890</td>
</tr>
<tr>
<td>Saccharic acid</td>
<td>3.3</td>
<td>Up</td>
<td>&lt;0.00000</td>
<td>1.64</td>
<td>Up</td>
<td>0.01020</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>2.4</td>
<td>Up</td>
<td>0.00048</td>
<td>2.27</td>
<td>Up</td>
<td>0.00003</td>
</tr>
<tr>
<td>Threonic acid</td>
<td>0.7</td>
<td>Down</td>
<td>0.00209</td>
<td>0.36</td>
<td>Down</td>
<td>&lt;0.00000</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.9</td>
<td>Down</td>
<td>0.03990</td>
<td>0.65</td>
<td>Down</td>
<td>0.03700</td>
</tr>
<tr>
<td>Valine</td>
<td>2</td>
<td>Up</td>
<td>0.00785</td>
<td>1.43</td>
<td>Up</td>
<td>0.04440</td>
</tr>
</tbody>
</table>

*GC-TOF. †Metabolite name reported as a BinBase identifier. ‡Fold change of means in diabetic vs. nondiabetic animals. §Direction in change relative to nondiabetic animals. **False discovery rate adjusted P value.

Supplemental Table S3. List of all identified peaks which were not significantly different in T1D compared to control animals.

Table A3. Spearman rank correlations between fasting blood glucose, threonine, methionine, cysteine and 1,5-anhydroglucitol.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.F. and J.Y. performed experiments; J.F., D.G., and J.Y. analyzed data; J.F., D.G., J.Y., and M.H. interpreted results of experiments; J.F. and D.G. prepared figures; J.F., D.G., and M.H. drafted manuscript; J.F., D.G., J.Y., B.H., O.F., G.I.B., and M.H. approved final version of manuscript; J.F., D.G., B.H., O.F., G.I.B., and M.H. edited and revised manuscript; O.F., G.I.B., and M.H. conception and design of research.

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Table A3. Spearman rank correlations between fasting blood glucose, threonic acid, methionine, cysteine and 1,5-anhydroglucitol

<table>
<thead>
<tr>
<th>Correlation Matrix (Spearman)</th>
<th>Blood Glucose</th>
<th>Threonic Acid</th>
<th>Methionine</th>
<th>Cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose</td>
<td></td>
<td>-0.696*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threonic acid</td>
<td>0.732*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>-0.643*</td>
<td>0.636*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td>-0.537*</td>
<td>-0.553*</td>
<td>0.573*</td>
<td>-0.839*</td>
</tr>
</tbody>
</table>

1Values represent Spearman rank correlation coefficients (R). *P value < 0.001.

REFERENCES


