Comprehensive Metabolomics Study To Assess Longitudinal Biochemical Changes and Potential Early Biomarkers in Nonobese Diabetic Mice That Progress to Diabetes

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Supporting Information

ABSTRACT: A global nontargeted longitudinal metabolomics study was carried out in male and female NOD mice to characterize the time-profile of the changes in the metabolic signature caused by onset of type 1 diabetes (T1D) and identify possible early biomarkers in T1D progressors. Metabolomics profiling of samples collected at five different time-points identified 676 and 706 biochemicals in blood and feces, respectively. Several metabolites were expressed at significantly different levels in progressors at all time-points, and their proportion increased strongly following onset of hyperglycemia. At the last time-point, when all progressors were diabetic, a large percentage of metabolites had significantly different levels: 57.8% in blood and 27.8% in feces. Metabolic pathways most strongly affected included the carbohydrate, lipid, branched-chain amino acid, and oxidative ones. Several biochemicals showed considerable (>4×) change. Maltose, 3-hydroxybutyric acid, and kojibiose increased, while 1,5-anhydroglucitol decreased more than 10-fold. At the earliest time-point (6-week), differences between the metabolic signatures of progressors and nonprogressors were relatively modest. Nevertheless, several compounds had significantly different levels and show promise as possible early T1D biomarkers. They include fatty acid phosphocholine derivatives from the phosphatidylcholine subpathway (elevated in both blood and feces) as well as serotonin, ribose, and arabinose (increased) in blood plus 13-HODE, tocopherol (increased), diaminopimelate, valerate, hydroxymethylpyrimidine, and dulcitol (decreased) in feces. A combined metabolic signature based on these compounds might serve as an early predictor of T1D-progressors.

KEYWORDS: 1,5-anhydroglucitol, beta hydroxybutyric acid, branched-chain amino acids, diabetes mellitus, gender differences, hyperglycemia, kojibiose, metabolomics, nonobese diabetic (NOD) mice, sorbitol

INTRODUCTION

People affected by type 1 diabetes (T1D) lose their ability to produce insulin, as their β-cells are destroyed by an autoimmune process,1−4 and they require life-long administration of exogenous insulin. T1D affects about one in every 400–600 children and adolescents in the U.S., and its incidence rate ranges from 0.1 (China, Venezuela) to >40 (Sardinia, Finland) per 100 000/year.1−7 As with most other autoimmune diseases, the incidence of T1D has increased worldwide with an overall rate of about 3% per year, and the average age of onset has decreased by 3–5 years over the past decades for yet unknown reasons.5,8−10 Even a century after the introduction of insulin, T1D still represents a considerable therapeutic need, partly because of the inconvenience of the ongoing need for administration of exogenous insulin, and partly because chronic and degenerative complications still occur in a considerable fraction of T1D patients, despite considerable improvements in management and care.11,12

The etiology, pathogenesis, and onset mechanism of T1D are complex. It is now well-recognized that an interplay of genetic predisposition, yet unknown environmental factors, and probably other stochastic events is needed.13 It is also becoming increasingly clear that early metabolic perturbations are involved in T1D development and progression, and they might even serve as early disease indicators (refs 14−17 and references therein). To further elucidate the connections among metabolic perturbations, mounting of the autoimmune attack, and destruction of pancreatic β-cells, we carried out a comprehensive global nontargeted longitudinal metabolomics study in nonobese diabetic (NOD) mice, a common animal model of type 1 diabetes (T1D), to characterize the time-profile of the changes in the metabolic signature caused by...
onset of diabetes and identify possible early biomarkers separating T1D progressors from nonprogressors. The NOD model, an inbred strain developed from a line originally intended as a cataract-prone mouse strain,18 is the most widely used animal model of T1D, and it has contributed significantly to the study of this disease.19–23 These mice develop spontaneous diabetes in a manner that reproduces many crucial aspects of the human disease (e.g., the presence of islet-specific autoantibodies, inflammation of pancreatic islets, and dependence on MHC alleles), but there are also important differences (e.g., more severe insulitis, gender bias, etc.)21,23 In NODs, about 60–90% of females, but only about 20–50% of males, develop diabetes between the ages of 12 and 30 weeks. Diabetes development can be altered by many factors, and specific pathogen-free conditions are needed for such high incidence rates. Insulitis, the invasion of the pancreatic islets by a mixed population of leukocytes, starts around 4 weeks of age and amplifies progressively until the onset of overt diabetes involving various immune cells (T and B cells, macrophages and dendritic cells), which can take on the organization of typical tertiary lymphoid structures.24 Peri-islet infiltration is minimal in young NODs, but it becomes prominent around 9–10 weeks of age.24,25 By most estimates, at the time of hyperglycemia onset, NOD mice have already lost 80–90% of their insulin-producing β-cell mass26,27, a proportion similar to that in humans at the time of clinical manifestation of T1D.2,28 The progressive loss of β-cell function is a central aspect of T1D, and declining insulin responses have been observed in prediabetic NOD mice studied until the animals are 14 or 18 weeks of age,29,30 around the onset of hyperglycemia. Recently, in a longer study of up to 42 weeks of age, we have shown that β-cell function (insulin secretion) decreases with age in all NODs up to 28 weeks of age paralleling diabetes onset, and then it essentially disappears in diabetic animals, while it stabilizes at still adequate levels to maintain normoglycemia in nonprogressors.31

Some metabolomics studies on T1D onset31–33 and the metabolic perturbations it causes in children,34–37 as well as in animal models such as the NOD mice,38–41 have been performed in the past decade. Most, including studies on pancreatic islets, have been summarized relatively recently.14–17 An important limitation of these studies was the need for relatively large amounts of samples, for example, several million cells for liquid chromatography–mass spectrometry (LC–MS) type analyses (e.g., > 3 million),16, but with improved analytical methods this is no longer a serious issue. The method employed herein required 150 μL of blood and 200 mg (wet weight) of fecal samples making possible the longitudinal collection of samples from the same animals. This allowed more clear separation of the differences between the progressors and the nonprogressors as changes could be tracked within the same animals and also made it more likely to be able to identify early biomarkers than previous approaches. There are already more than 75 000 metabolites registered in the Human Metabolome Database (HMDB; http://www.hmdb.ca) of which more than 20 000 have been detected in the blood.42 Hence, with current methods, very detailed metabolomic signatures can be obtained.

As mentioned, NOD mice have been used in some previous metabolomic studies. One of the earliest studies had been reported by Sysi-Aho, Oresic, and co-workers.36 It involved a longitudinal lipodomics study using serum samples, which indicated that female NOD mice that later progress to autoimmune diabetes exhibit the same lipidomic pattern as prediabetic children. The study also involved a separate metabolomics analysis of isolated pancreatic islets and found several metabolites showing significant differences between animals judged as being at high versus low risk of developing diabetes. Madsen and co-workers compared blood samples from female NOD and C57BL/6 (wild type) mice and found that NOD mice had a distinct metabolic profile, which included increased glutamate and decreased TCA cycle metabolites and partly resembled what was previously observed in children progressing to T1D.39 More recently, the group of Hara and co-workers37,40,41 performed global metabolomics analyses in two consecutive studies, but they compared a group of diabetic versus nondiabetic NODs without doing longitudinal monitoring of the same mice. Hence, none of these was complete nontargeted metabolomics studies on longitudinal samples in NODs. In the present study, we collected detailed biochemical data from two matrices (blood and feces) in parallel and tracked changes with time in each animal for several hundred metabolites.

## MATERIALS AND METHODS

### Animal Care and Treatment

Female nonobese diabetic (NOD) mice (NOD/ShiLtJ; 4 weeks old; n = 30, 15 M + 15 F) were obtained from Jackson Laboratories (Bar Harbor, ME). Starting from week 10, glycosuria was monitored one or two times per week. In animals that turned positive, blood glucose levels (glycemia) were monitored two to three times per week. Animals with elevated glucose levels (nonfasting glycemia >300 mg/dL) on three consecutive days were identified as hyperglycemic and were designated as T1D progressors. Hyperglycemic animals were implanted with sustained release insulin pellets (LinBit, LinShin Canada, Inc., Toronto, Canada) to avoid severe hyperglycemia and minimize animal suffering. One or two pellets was used per animal and renewed as needed after 2–3 weeks as indicated by the glucose levels. All mice were sampled for whole blood and fecal pellets at 6, 11, 16, 21, and 26 weeks of age. Blood (150 μL blood, EDTA) and fecal material (200 mg wet weight) were collected and stored at −80 °C. At the end of the study, all samples from six (3 F + 3 M) nonprogressors, which remained nondiabetic, and eight (4 F + 4 M) progressors, which developed T1D, selected as representative were sent for analysis to Metabolon (Durham, NC) (total samples: 5 time-points × 2 samples × 14 animals = 140). At the end of the study, all animals were euthanized, and pancreas and other organ samples were collected and stored for analyses. All animal studies were reviewed and approved by the University of Miami Institutional Animal Care and Use Committee. All procedures were conducted according to the guidelines of the Committee on Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources (National Research Council, Washington, DC). Animals were housed in microisolated cages in Virus Antibody Free rooms with free access to autoclaved food and water at the Department of Veterinary Resources of the University of Miami.

### Immunofluorescence

Immunostaining was performed as described previously.25 Briefly, harvested tissues were embedded in Tissue-Tek O.C.T. (Sakura Finetek, Torrance, CA) and snap frozen by placing the tissue mold in dry ice. Tissue sections (5–10 μm) were collected on glass slides and kept at −80 °C until analysis.
Samples were fixed for 10 min in 4% paraformaldehyde, washed three times with PBS 1×, and permeabilized with PBS plus 0.1% Triton X100 for 1 h at room temperature. After blocking for at least 1 h in PBS with 10% Power Block (BioGenex, Fremont, CA) or normal goat serum as required by antibody detection, samples were incubated overnight at 4 °C with the primary antibodies following the manufacturer’s recommendations. Immunofluorescence staining was performed for insulin (1:100; Dako, Carpinteria, CA) and glucagon (1:100; Dako). Sections were washed five times with PBS plus 0.05 Tween20 and then incubated with the secondary antibodies, washed another five times with PBS plus 0.05 Tween20 and twice with PBS 1×, and mounted for analysis via confocal microscopy. Secondary antibodies were AlexaFluor-conjugated goat antirabbit 488, and goat antiguinea pig 647 (1:800; Life Technologies, Carlsbad, CA). Nuclei were detected by DAPI staining (Life Technologies). After final washes, slides were mounted using Vectashield (Vector Laboratories, Burlingame, CA). Immunofluorescence imaging was performed at the Diabetes Research Institute Analytical Imaging Core Facility. Images were captured using a Leica SP5 inverted confocal microscope with motorized stage as tiled single-layer (5× air) for whole pancreatic sections. Representative images were merged and edited for contrast in Adobe Photoshop (Adobe Systems, Inc., San Jose, CA).

Metabolomic Analysis

Metabolomics analysis was performed in a manner similar to that of our previous study with human pancreatic islets. In brief, individual blood and fecal samples were loaded in equivalent volumes and weights across the platform with no additional normalization performed prior to statistical analysis. Small molecules were extracted in a methanol solution containing process assessment standards as described previously. The resulting clarified supernatant extract was divided into five aliquots, one for each of the four individual LC–MS analyses and one spare, briefly evaporated to remove the organic solvent and stored overnight under nitrogen before preparation for analysis. The global biochemical profiling analysis was composed of four unique arms consisting of reverse phase chromatography positive ionization methods optimized for hydrophilic compounds (LC–MS Pos Polar) and hydrophobic compounds (LC–MS Pos Lipid), reverse-phase chromatography with negative ionization conditions (LC–MS Neg), as well as a HILIC chromatography method coupled to negative (LC–MS Polar). All of the methods alternated between full scan MS and data-dependent MSn scans. The scan range varied slightly between methods but generally covered 70–1000 m/z.

Experimentally detected metabolites were identified by matching the ion chromatographic retention index, accurate mass, and mass spectral fragmentation signatures with a reference library consisting of over 3200 entries created from authentic standard metabolites under the identical analytical procedure as the experimental samples. In-house peak detection and integration software was used. The data output from the software was a list of m/z ratios, fragmentation spectra, retention indices, and area under the curve (AUC) values. All proposed identifications were then manually reviewed and hand curated by an analyst who approved or rejected each identification based on the criteria above.

Statistical Analysis

Two types of statistical analyses were performed: (1) significance tests and (2) classification analysis. Standard statistical analyses were performed in ArrayStudio on log-transformed data. For analyses not standard in ArrayStudio, the programs R (http://cran.r-project.org/) and JMP were used. Following log transformation and imputation of missing values, if any, with the minimum observed value for each compound, ANOVA contrasts with a repeated measures component were used as significance test to identify biochemistry that differed significantly (p < 0.05) between experimental groups. An estimate of the false discovery rate (q-value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies. Classification analyses used included principal components analysis (PCA), hierarchical clustering, and random forest. For the scaled intensity graphics, each biochemical on the original scale (raw area count) was rescaled to set the median across all animals and time-points equal to 1. For the visualization of interaction networks and biological pathways of the significant metabolites, Metabolon’s MetaboLync plugin to Cytoscape (http://cytoscape.org) was used, an open-source software platform.

RESULTS AND DISCUSSION

Overview of Metabolomics Data

Blood and fecal pellet samples were collected at predefined time-points (6, 11, 16, 21, and 26 weeks of age) from NOD mice (15 males and 15 females) that were monitored and designated as progressors (type 1 diabetic, T1D) or non-progressors (control) depending on whether they became hyperglycemic or not by the end of the study (Supporting Information Figure S1). Diabetes onset in these mice followed a pattern typical for NOD mice, with diabetes starting to occur around week 12, and approximately 60% of female and 30% of male mice becoming diabetic by week 30. The corresponding Kaplan–Meier survival curves are shown in Figure 1 with a couple of onset rates from our previous studies included for comparison. At the end of the study, all samples from six (3 F + 3 M) male (M) and female (F) mice (n = 15 + 15) are shown separately in

![Figure 1](https://example.com/image.png)

**Figure 1.** Kaplan–Meier survival curves showing the overall rate of diabetes onset within the NOD mice of the present study. Data for male (M) and female (F) mice (n = 15 + 15) are shown separately in blue and red, respectively. Sample collections times are denoted with purple arrows at the bottom. For comparison, onset rates obtained by us in two previous studies with female NODs are also included (shown in lighter colors).
3 M) nonprogressors (control) and eight (4 F + 4 M) progressors (T1D) selected as representative were subjected to global metabolomics analysis, as described in the Materials and Methods section (n = 14 mice × 2 matrices × 5 time-points = 140 samples). Following data acquisition and curation, a total of 676 and 706 compounds of known identity (named biochemicals) were identified in blood and fecal samples, respectively. At each time-point, there were several metabolites that had significantly different concentrations in progressors as compared to nonprogressors (for detailed data, see Supporting Information Table S1). The number of metabolites expressed at significantly different levels increased strongly in the week 21 and 26 samples, when the progressors were diabetic, while the nonprogressor controls remained normoglycemic. Figure 2 provides a summary comparison of the metabolites (biochemicals) that exhibited statistically significant changes (p < 0.05) for these comparisons focusing on the difference between progressors versus nonprogressors at each of the five time-points. In blood, analysis by three-way ANOVA with repeated measures identified biochemicals exhibiting significant interaction and main effects for experimental parameters of gender, group, and time point. In feces, analysis by two-way ANOVA with repeated measures identified biochemicals exhibiting significant interaction and main effects for experimental parameters of group and time point.

Overall Data Segregation: Principal Component, Hierarchical Cluster, and Random Forest Analyses

Principal component analysis (PCA) projects differences across a large set of variables onto a smaller set of principal variables, and by reducing dimensionality, it allows the visualization of the clustering of individual samples. Transformations are done so that the first principal component (PC1) has the largest possible variance (meaning that it accounts for as much variability as possible along a single dimension), and then each additional component has the highest remaining variance possible, while also being orthogonal to all preceding components. Here, PCA was used to assess whether progressors could be segregated from nonprogressors at the various time-points based on the differences in their overall metabolic profiles.

Figure 2. Changes with age in the number of statistically significantly altered biochemicals in the blood and fecal samples of T1D progressor NOD mice. Data shown as stacked columns are from Table S1 with the number of compounds that are increased in progressors versus nonprogressors shown in red and those that decreased in blue.
metabolic signatures. The first two principal components account for a relatively modest portion of the variability in the data (18.1 + 14.5 = 32.6% and 23.5 + 10.7 = 34.2% in blood and feces, respectively); nevertheless, they show a significant clustering of both the blood and fecal data (Figure 3).

PCA showed clear segregation of the week 26 time-points from the rest of the time-points (weeks 6, 11, 16, and 21), especially in the blood samples, suggesting that by this time there is already a profound impact on global metabolism associated with diabetes progression despite the use of sustained release insulin implants (Figure 3). Note that there is little separation among the samples collected at different time points in NOD control mice suggesting stable metabolic phenotypes with aging in these normoglycemic controls. Interestingly, there is a clear separation by gender (male vs female) in the blood samples along principal component 2 (PC2, vertical axis) in both progressors and nonprogressors. Nevertheless, the delineation of the week 26 time-points from the rest is present in both females and males mainly along PC1 (horizontal axis) indicating that diabetes induces comparable changes in the global metabolism profile of male and female mice despite their gender-related metabolic differences.

PCA of the fecal samples also revealed good separation between the early (weeks 6 and 11) and late (weeks 21 and 26) time-points in both progressors and nonprogressors reflecting age- and T1D-related differences in global fecal metabolites. A good segregation of the week 21 and 26 fecal samples of the progressor NODs from those at the other time points is clearly present suggesting profound diabetes-mediated differences in global metabolism at these time points (Figure 3B).

Hierarchical cluster analysis (HCA), which groups metabolically similar samples close to one another, was also performed on these data to obtain a different perspective into potential group segregation. Overall, HCA showed similar trends as PCA with a clear segregation of the later time-point samples (i.e., week 26) of the progressors (Figure 4).

Figure 3. Principal component analysis (PCA) of the present metabolomics data in blood (left) and fecal samples (right) obtained longitudinally (weeks 6–26) from NOD mice that progressed (Type 1) or not (control) to diabetes.
To identify biomarkers that allow separation among groups, random forest analysis (RFA) was also performed. This is a statistical tool that utilizes a supervised classification technique based on an ensemble of decision trees. Using primary grouping of progressors (type 1) and nonprogressors (control) at the five different time-points, RFA of the blood and fecal metabolic profiles resulted in 47% accuracy in differentiating the groups in both matrices (Supporting Information Figure S2). These are much better than random chance alone (10% accuracy for \(2 \times 5 = 10\) groups) indicating that the differences in the biochemical profiles between the two groups were pronounced. However, the predictive accuracy for the blood samples was mainly driven by the low class error at the week 26 time-point between progressors and nonprogressors, and the one for the fecal samples was mainly mediated by the low class errors at the week 26 and 26 time-points in progressor mice (Figure S2). RFA of the week 26 blood samples was able to classify T1D from control group with a predictive accuracy of 100% (data not shown) implying profound differences in the metabolic phenotype at this time point when all progressors were diabetic. RFA also ranks biochemicals by their importance to the classification scheme, and the 30 top-ranking metabolites in each matrix are summarized in Figure S2. As indicated, they span several different metabolic pathways. In blood samples, glucose and other biochemicals related to carbohydrate metabolism as well as established markers of glycemic control (1,5-anhydroglucitol) and lipid metabolites were consistently identified as contributing the most to the separation of groups.

Changes in Diabetic NOD Mice

At the last sampling (week 26), all progressors were already hyperglycemic for at least 4 weeks (Figure 1) so this comparison can be considered as reflecting differences between diabetic and nondiabetic NOD mice. A large proportion of the metabolites detected had significantly different levels in the T1D group versus the nondiabetic controls (Figure 2): 57.8% in blood (266 \(\uparrow\) + 125 \(\downarrow\) = 391 of the 676 detected) and 27.8% in feces (82 \(\uparrow\) + 114 \(\downarrow\) = 196 of the 706 detected). This indicates that the overall metabolic landscape was profoundly altered by diabetes onset, which is even more remarkable as hyperglycemia in these animals was at least partially controlled by use of sustained release insulin implants. Many of these compounds showed considerable changes. Compounds that showed statistically significant (\(p < 0.05\)) and at least four-fold changes either upward or downward versus the nondiabetic controls at the last sampling are summarized in Table S2. The longitudinal time-profiles of their average fold changes are shown in Figure 5 as an indication of their changes as T1D starts to develop. Most of these changes are likely due to the onset of hyperglycemia and not of the autoimmune reaction itself, but clear delineation is difficult with the present data. As a possible indication, we looked at the correlation of their concentrations with that of glucose across all \(5 \times 14 = 70\) individual data-points; the corresponding \(r^2\) values are included in Table S2. For the majority of these strongly changed metabolites, their blood concentrations indeed correlate closely with that of glucose (\(r^2 > 0.3\)); those that do not and, hence, are less likely to be hyperglycemia related changes include, for example, reduced glutathione (GSH), 4-hydroxy-nonenal-glutathione, xanthosine, taurodeoxycholate, \(\beta\)-muricholate, and nicotinamide N-oxide (Table S2).

As a further verification, we also calculated the fold changes in T1D NODs versus their corresponding values at week 6 in
the same mice for both blood and fecal samples. Metabolites showing again at least four-fold change in T1D that change no more than 1.33-fold in control animals are summarized in Supporting Information Figure S3. In agreement with the trends shown in Figure S5, compounds showing the largest fold change in blood include, for example, maltose, kojibiose, and 3-hydroxybutyrate (↑) as well as 1,5-anhydroglucitol and β-muricholate (↓). Biochemicals with the largest change in feces
include, among others, hippurate, cinnamoylglycine, allantoin, 1-methylnicotinamide, and phenylacetylglycine (↑) as well as ursodeoxycholate sulfate, valylglutamine, and tauro-β-muricholate (↓).

Line plots illustrating the average time-profiles from week 6 to 26 for some of the compounds that showed the largest fold-change in diabetic animals (either increase or decrease) are shown in Figure 6. Corresponding spaghetti plots with all individual changes are included in Supporting Information Figure S4. Note, for example, that maltose in blood showed one of the largest increases in diabetes progressors: it was consistently increased more than two-fold at all time-points starting from week 11, and it reached more than 20-fold difference by week 26 (22×, ↑) (Figures 5A and 6). In the meantime, glucose increase was essentially parallel, but it reached just a 3.1-fold difference at week 26, when all progressors were already diabetic. Note, however, that sustained release insulin pellets were used in diabetic mice to avoid excessive hyperglycemia and minimize animal suffering, and this somewhat limited the differences in glucose.

Fewer compounds showed relative decrease in T1D animals with β-muricholate being the only one showing more than four-fold change in both matrices (B, 4.9×; F, 4.5×, ↓) and 1,5-anhydroglucitol showing one of the largest fold-changes observed in blood (50×, ↓) as it drops to very low levels in diabetic animals. Overall, diabetes onset significantly affected
several metabolic pathways in diabetic NOD mice compared with nondiabetic controls from the same batch; main metabolic pathways affected are illustrated using the Metabolic Pathway Classification Network view of the metabolomic data for both blood and fecal samples in Figures 7 and 8, respectively. These graphs clearly highlight some of the metabolic subpathways that were strongly and consistently affected by T1D onset such as "fructose, mannose, and galactose," "leucine, isoleucine, and valine," "diacelyglycerol," "polyunsaturated fatty acid," "fatty acid monohydroxy," and others.

These changes are in general agreement with previous observations in diabetic NODs (e.g., by Hara and co-workers\textsuperscript{40,41}), and some of the main ones are discussed below. So far, there are only a limited number of studies comparing metabolic fingerprints between T1D and corresponding normal controls in humans (children) using plasma or urine samples.\textsuperscript{34–37} A recent detailed metabolomics study at Mayo Clinics, which followed up on a smaller earlier study,\textsuperscript{34} found 154 known metabolites significantly altered even in well-controlled T1D children (glycated hemoglobin <6.5%), belonging to 26 pathways including glycolysis, gluconeogenesis, bile acids, tRNA biosynthesis, amino acids, branch chain amino acids, retinol, and vitamin D metabolism.\textsuperscript{37} An earlier study by Balderas and co-workers found only relatively moderate changes in the plasma and urine samples of T1D children that were under insulin treatment and good glycemic control.\textsuperscript{35} A recent study by Galderisi and co-workers found 59 urinary metabolites having a higher level in children with T1D including gluco- and mineralocorticoids, phenylalanine and tryptophan catabolites (kynurenine), small peptides, glycerophospholipids, fatty acids, and gut bacterial products.\textsuperscript{36}

Figure 7. Effect of diabetes onset on NOD mouse metabolism (i.e., T1D vs control at week 26) shown using MetaboLync Pathway Classification Network for blood samples for selected superfamilies. Within each pathway, the size of the circle correlates with the magnitude of change, and the color indicates significant change versus control \((p < 0.05; \text{red increased, blue decreased})\).
Gender Differences in the Metabolome

Since PCA indicated a clear separation by gender in the blood samples along PC2 in both progressor and nonprogressor NOD mice (Figure 3, vertical axis), we took a more detailed look to identify the main metabolites responsible for this separation. This is of particular interest as there is gender bias in many autoimmune diseases with females being, in general, more susceptible.51,52 While there is no significant gender bias for T1D in humans, there is a strong one in NOD mice, where females are much more likely to develop diabetes than males (typically, 60−90% vs 20−50%),21,23 probably due to roles played by sex hormones in the autoimmune process.51 Some of the biochemicals quantified here showed considerable gender differences having up to 2−3-fold different blood concentrations (irrespective of the T1D progressor status); illustrative line plots are shown in Figure 9. Compounds with the largest and most consistent differences in blood include, for example, β-alanine, carboxyethyl-GABA, imidazole lactate, hexanoylglycerine, 4-cholesten-3-one, several sphingosine derivatives, and tryptophan. There were fewer differences in the fecal samples with 4-cholesten-3-one and 3-hydroxyhexanotae showing differences in both blood and fecal samples, and some cholate sulfates being most different in feces. There seemed to be no relationship between these differences and the progressor status: one would expect female nonprogressors to have values closer to those of the generally more protected males, but this was not the case for any of these compounds (Figure 9).

Changes in Carbohydrate Metabolism and in Biochemical Markers of Glycemic Control and Insulin Deficiency

In addition to significant elevations in glucose (at week 21 and 26; Figure 6), significantly elevated levels of sorbitol, fructose, mannose, and maltose have also been found both in blood and feces. Part of these may be generated upon shuttling of excess...
glucose into the sorbitol pathway (Figure 10). Some of these compounds such as maltose and 3-hydroxybutyrate in blood (also kojibiose in feces) increased more dramatically in T1D than glucose itself showing more than 10−20-fold increases. Nevertheless, the blood concentrations of these compounds correlate closely with that of glucose, the corresponding $r^2$ values being 0.86, 0.75, 0.71, and 0.70 for maltose, fructose, sorbitol, and mannose, respectively, which are some of the highest such $r^2$ values (Table S2). The sorbitol pathway is important for handling excess glucose, but increased activity can deplete the reserves of the anabolic cofactor NADPH resulting in increased oxidative stress, reduced nitric oxide production, and increased protein glycation to form advanced glycation end products (AGEs).53 Together with the inability of sorbitol to cross cell membranes and the resulting induction of osmotic stress, these negative consequences of the activation of the sorbitol pathway may lead to microvascular diabetic complications. Compared with normoglycemic controls, T1D mice at week 26 also showed somewhat elevated levels of pyruvate (1.4×) and the glycolytic end product lactate (1.3×) (Figure 10) suggesting possible differences in glucose utilization.

Significant reductions in 1,5-anhydroglucitol (1,5-AG), a marker of glycemic control,54 together with significant elevations in 3-hydroxybutyric acid (3-hydroxybutyrate, beta-hydroxybutyric acid, BHBA), a ketone body and an indicator of diabetic ketoacidosis due to insulin deficiency in T1D,55,56 which are both used clinically, corroborate the findings of elevated circulating glucose and infer a signature of insulin deficiency in diabetic animals. 1,5-AG is a stable, long-term marker of glycemic control known to exhibit serum levels that are inversely proportional to circulating glucose because it competes with glucose for reabsorption in the kidney. Consequently, urinary excretion of 1,5-AG is increased under hyperglycemic conditions and the serum levels fall. Therefore, the fact that our nontargeted metabolomics approach identified 1,5-AG (50×, ↓) and BHBA (12×, ↑) among the most strongly and significantly changed blood biochemicals in T1D animals (Figure 6) as indicative of higher circulating glucose concentrations nicely confirms their clinical use in T1D. Their blood concentrations also correlate closely with that of glucose giving $r^2$ values of 0.61 and 0.62 for 1,5-AG and BHBA, respectively (Table S2). Interestingly, we also found 2-hydroxybutyric acid (alpha-hydroxybutyrate, AHB) as elevated (3×) in diabetic NODs (week 26). Metabolomics studies have indicated AHB as a marker of insulin resistance in type 2 diabetes (T2D) progressors.57,58 AHB can arise from states of lactic and ketoacidosis or disrupted energy metabolism. It is thought to be a byproduct during the formation of alpha-ketobutyrate (AKB) via a reaction catalyzed by lactate dehydrogenase or alpha-hydroxybutyrate dehydrogenase. This increased conversion of AKB to AHB is thought to occur when either the rate of AKB formation exceeds the rate of catabolism to propionyl CoA (propionylcarnitine) for anaplerotic incorporation into the TCA cycle or when there is product inhibition of the dehydrogenase responsible for AKB conversion.57

**Changes in TCA Cycle Metabolism**

The dysregulation of glucose metabolism in diabetic NOD mice relative to control mice suggested that oxidative metabolism may also be altered. T1D NODs exhibited an accumulation of TCA cycle intermediates including citrate, α-ketoglutarate, succinate, fumarate, and malate (Figure 7, bottom). The levels of these biochemicals were most strongly affected in T1D blood at week 26 and may be indicative of a disruption in the TCA cycle or increased anaplerotic contributions to attempt to replenish TCA cycle intermediates. Hence, these findings suggest the TCA cycle is altered in T1D NOD mice and may be indicative of mitochondrial dysfunction.

**Changes in Lipid Metabolism**

Substantial changes in several aspects of lipid metabolism were observed when comparing diabetic and nondiabetic animals at the 26 week time-point. In particular, accumulation of several
free fatty acids, including medium-chain, long-chain, saturated, unsaturated, polyunsaturated, branched-chain, and dicarboxylic fatty acids, was observed in diabetic NOD mice compared with nondiabetic control NOD mice. These changes are clearly highlighted by the accumulation of red markers in the corresponding "fatty acid" subpathway branches on the lipid superpathway in Figure 7. Concomitant with the elevations in free fatty acids, increased blood levels of mono- and diacylglycerols along with glycerol (nonsignificant increase), a marker of lipolysis, were also elevated. Taken together, these findings are suggestive of insulin deficiency at the level of adipose tissue resulting in increased release of free fatty acids and glycerol. Alternatively, this could also be due to an increase in fatty acid synthesis as evidenced by increases in malonylcarnitine, citrate, and glucose.

Moreover, elevations in circulating acylcarnitines and many short-, medium-, and long-chain acylcarnitines in diabetic NOD animals may be associated with increased β-oxidation of fatty acids or mitochondrial dysfunction in liver and muscle tissue as conjugation of fatty acids with carnitine is required for transport across mitochondrial membranes and subsequent β-oxidation. Accumulation of acylcarnitines was accompanied by considerable (12X) elevations in the ketone body BHBA, an indicator of diabetic ketoacidosis due to insulin deficiency in T1D, which is in use as a clinical test as discussed earlier.55,56 Ketones are produced from excess acetyl-CoA in the liver, generally from β-oxidation of fatty acids, and are utilized by peripheral tissues, such as brain and cardiac muscle, to meet energy demands when glucose is (or is perceived to be) limiting. Of note, circulating ketones are typically elevated with greater fatty acid utilization. Therefore, the observed changes are indicative of a signature of altered lipid metabolism in diabetic animals consisting of altered lipolysis, increased β-oxidation of fatty acids despite some degree of mitochondrial dysfunction, and increased production of ketones: all findings that may be related to the underlying issue of severe insulin deficiency. These changes were largely absent in the progressor animals at week 16, when they were still in prediabetic stage. These findings suggest that the observed perturbations in lipid metabolism may also be associated with the conversion to

Figure 10. Glycolytic and sorbitol pathways of glucose utilization with line plots included showing metabolites detected here and significantly altered in diabetic NOD mice versus corresponding controls. All metabolites shown are significantly different (p < 0.05) at the week 26 time-point, when all progressors were diabetic (lactate is also shown; for it, the difference is only close to being significant, p = 0.077). Numbers in the upper left corner of the corresponding insets indicate the average fold-change (T1D vs normoglycemic controls) at the last time-point. Additional related metabolites that were found to be highly (>10X) altered in T1D animals are also shown (left); they include maltose as well as 1,5-anhydroglucitol and 3-hydroxybutyric acid, which are clinically used as biomarkers (see text).
the diabetic phenotype and that the development of dyslipidemia is accelerated with the severity of diabetes. Conversely, a significant decrease in fatty acids and carnitine-conjugated fatty acids was observed in the feces of T1D mice at week 26 compared with controls. This suggests concomitant decrease in excretion leading to relative accumulation of fatty acids and their conjugates in the blood (note the accumulation of blue markers in the corresponding “fatty acid” subpathway on the lipid superpathway in Figure 8).

Changes in Branched-Chain Amino Acid Metabolism

One of the most strongly and thoroughly affected pathways by diabetes onset was the branched-chain amino acid (BCAA) subpathway (Figure 11). The BCAAs valine, isoleucine, and leucine constitute a large portion of amino acids incorporated into proteins and also serve as carbon sources to meet energy demands in times of need. BCAAs are important nutrient signals, play a role in modulating insulin secretion at the level of the β-cell, and altered BCAA metabolism has been implicated in the pathogenesis of T2D. In particular, a signature of elevated circulating BCAAs has been reported to be associated with insulin resistance and T2D. As with increased acylcarnitines, accumulation of BCAAs and their degradation products may be reflective of mitochondrial dysfunction in the liver, muscle, and adipose tissue. However, in parallel with these elevations in blood, a significant decrease in BCAAs and their metabolites was observed in the feces of diabetic NOD mice (week 26; compared with control), suggesting simultaneous decrease in excretion of BCAAs and their metabolites leading to relative accumulation in the blood. Finally, it should also be noted that the BCAAs isoleucine, leucine, and valine together with the aromatic amino acids tyrosine and phenylalanine were the five amino acids found in human studies to have highly significant associations with future T1D in a metabolite profiling study by Wang, Gerszten, and co-workers. This clinical study suggested that fasting concentrations of these amino acids are higher in progressors than in nonprogressors. This was not the case here as none of these amino acids was found to have elevated levels in progressor NOD mice at the earlier time-points (Figure 11).

Inflammation and Oxidative Stress

Consistent elevations in several biochemical markers of inflammation and oxidative stress were observed in T1D NOD mice. For example, polyunsaturated fatty acids (PUFAs), such as linoleate (3.7×, †) and arachidonate (1.3×, †) (Figure 7), and fatty acid-derived inflammatory mediators, such as 12-HHTrE (2.5×, †) and 12-HEPE (1.6×, †), were increased in T1D NOD mice suggestive of an inflammatory milieu in these animals. It should be noted that these are contrary to the observations of Hara and co-workers in NODs indicating reductions in circulating PUFAs and lipid signaling mediators (arachidonate and derived eicosanoids) implying impaired states of systemic inflammation. Kynurenate, which we have recently found to be highly induced by inflammatory cytokines in isolated human islets, was also slightly, but significantly elevated at week 26 (1.6×, †). It is derived from tryptophan by indoleamine 2,3-dioxygenase (IDO), an enzyme that is known

Figure 11. “Leucine, isoleucine, and valine metabolism” subpathway with line plots included showing metabolites detected here and significantly altered in diabetic NOD mice versus corresponding controls (week 26). All metabolites shown are significantly different (p < 0.05) at the week 26 time-point, when all progressors are diabetic. Figure and table included at right illustrate the individual fold-change values.
to be stimulated by pro-inflammatory cytokines, such as interferon-γ and tumor necrosis factor-α. Several markers of oxidative stress including reduced glutathione (GSH; 6.7×, ↑), methionine sulfone (3.6×, ↑), and sulfoxide (1.5×, ↑), 13-HODE + 9-HODE (hydroxyoctadecadienoic acid; 6.1×, ↑), 12,13-DHOME (3.2×, ↑), and 9,10-DiHOME (5.2×, ↑) were altered in diabetic animals as well. These changes in biochemical indicators of oxidative stress were accompanied by elevations in antioxidants, including the dietary-derived compound ergothioneine (1.2×, ↑) and the vitamin E isoforms β-tocopherol (3.3×, ↑). These findings are indicative of increased oxidative stress and inflammation in diabetic NOD mice. With a few exceptions related to inflammation and antioxidant capacity, the above-mentioned biochemical markers did not exhibit substantial changes in feces.

Changes in Gut Microbiome Derived Metabolites

Many environmental factors have been implicated in altering diabetes susceptibility in NOD mice including exposure to

Figure 12. Longitudinal time-profile of the compounds showing the largest and statistically significant (p < 0.05) differences between progressors (red) and nonprogressors (green) at the early 6 week sample in (A) blood and (B) feces. Data (scaled intensity) shown as average ± SD (n = 8 and 6); numbers in the upper left corner of the corresponding insets indicate average fold-change and p-values at week 6 (gray ovals), when all animals are nondiabetic. Chemical structures are also included for illustration. Corresponding spaghetti plots with individual lines for each animal are included in Supporting Information Figure S7.
dietary factors and changes to gut microbiota. While the exact triggers for autoimmune diabetes are still unknown, the role of the gut microbiota has been of increasing interest. Recent studies have suggested that microbiota regulates T1D through the toll-like receptor. Commensal microbes can trigger both pro- and antiadipogenic signals; the former signals control the microbiota, while the latter induce tolerance to self-antigen. Consistent with the literature, difference in microbiome-derived metabolites was observed in diabetic versus nondiabetic NOD mice. In particular, many of the changes observed in fecal samples could be tightly connected to changes in gut microbiome. The observed large change caused by T1D onset in kojibiose (blood, 4.7×; feces, 37×, ↑) is most likely due to altered microbiome metabolism. Kojibiose is a naturally occurring carbohydrate that is a promising prebiotic (i.e., nondigestible food component selectively fermented in the colon and beneficially affecting the growth or activity of bacteria there). It was found to have the highest prebiotic index among disaccharides, and it had beneficial effects when fed to hyperglycemic rats.

The metabolism of aromatic amino acids, phenylalanine, tyrosine, and tryptophan occurs in part through the involvement of enzymes encoded within the microbiome. Significant fluctuations in microbial flora-derived tryptophan, 3-(4-hydroxyphenyl)propionate, 3-indoxyl sulfate, phenyllactyl-glycine, and indolepropionate in diabetic NODs compared with controls at the later time-points could indicate changes in microbial composition or activity with progression of diabetes in these mice. Trimethylamine N-oxide (TMAO), a gut flora-dependent metabolite derived from choline and L-carnitine, was significantly (1.7×, ↑) elevated in diabetic NOD mice at week 26. Elevated blood levels of TMAO are linked to increased risk of cardiovascular disease, and the increased levels of TMAO in diabetic NOD mice might be contributed by the altered gut microbiome. Consistent with an altered gut-microbiome, we also observed changes in the benzoate pathway, which is derived from microbiome metabolism of phenolic compounds in the diet. Certain metabolites are the products of gut microbial or mammalian metabolism; others, such as hippurate, are mammalian-microbial “cometabolites”. Altered blood levels of hippurate (2.5X, ↑), catechol sulfate (2.2X, ↑), p-cresol sulfate (2.6X, ↑), hydroxyacinnamate (2.1X, ↑), 4-hydroxyacinnamate (1.9X, ↑), and caffeic acid sulfate (4.7X, ↑) may reflect either reduced intestinal permeability or altered gut microbial metabolism with progression of diabetes in NOD mice. Coupled with changes in gut microbiome-derived aromatic amino acids and TMAO, we also observed perturbed levels of some secondary bile acids in diabetic NODs, which may also indicate alterations in microbiome composition. These T1D mice showed an increase in circulating deoxycholate (2.3X, ↑) and taurodeoxycholate (4.5X, ↑) relative to the control group.

Possible Early Biomarkers in T1D Progressors

An important goal of the present longitudinal sampling-based work was to identify possible early biomarkers that might distinguish T1D progressors from nonprogressors. For this, we compared the metabolic signatures of progressors and nonprogressors at 6 weeks of age. This was the earliest time-point sampled here, and one when all animals were still nondiabetic, and the inflammatory and autoimmune attack should be in its earliest phase (first diabetes onset occurred much later at week 13). Islet morphology is known to change with age in NOD mice. Insulitis might start as early as 4 weeks of age, but peri-islet infiltration is typically still lacking at 6 week becoming apparent around 9−10 weeks of age. Our earlier studies also confirmed that pancreatic islets begin to show peri-islet mononuclear infiltrates around 9−10 weeks of age, just before the onset of the first cases of hyperglycemia, and that these peri-islet infiltrates are consistently present around islets in all older animals, even in those that do not progress to diabetes. A set of confocal images further illustrating this is included in Supporting Information Figure S5. The role of these peri-islet leukocytes is not entirely clear; nevertheless, they also tend to be present in humans with T1D, but much less prominently and homogeneously than in NOD mice. They probably contribute to the destruction of β-cells (i.e., via soluble factors) and, often, but not always, they lead to islet infiltration (insulitis). Insulitis in humans is often patchy, and it is less severe and invasive than in NOD mice. Interestingly, we have also shown that β-cell proliferation increases dramatically in NOD islets with the start of this infiltration in agreement with observations suggesting that soluble factors secreted by T-cells promote β-cell proliferation.

Among all biochemicals detected, a few were found to have significantly different levels at this earliest preinflammatory time-point sampled here (6 week); hence, they are possible early biomarkers identifying T1D progressors. Because there is only a limited number of such compounds among the large number of total detected compounds (e.g., ~2% in blood), there is much too much noise, and RFA cannot efficiently separate progressors and nonprogressors based on these data (week 6) (Supporting Information Figure S6). A list of all significantly different compounds at this earliest time-point is compiled in Supporting Information Table S3 ranked by decreasing order of significance (ANOVA contrast), and average concentration time-profiles for selected compounds of potential interest are shown in Figure 12. Corresponding individual profiles are also included as spaghetti plots in Supporting Information Figure S7. The metabolic subfamilies that were most different between progressors and nonprogressors at this earliest sampling time-point are summarized in Supporting Information Figure S8 using metabolic pathway enrichment (PE) values.

Notably, several fatty acid phosphocholine derivatives in the phosphatidylcholine subpathway showed significant difference between progressors and nonprogressors at this early time-point. While the corresponding fold-changes are relatively modest in the blood (1.06−1.16X, ↑), they are somewhat larger in feces (1.44−1.75X, ↑). More importantly, they are among the most significant ones detected and are consistently present in blood and fecal samples for several compounds, such as 1-palmitoyl-2-oleoyl-GPC (glycero-3-phosphocholine), 1-palmitoyl-2-arachidonoyl-GPC, and 1-stearoyl-2-arachidonoyl-GPC. While this is contrary to the observation of Oresic and co-workers of lower phosphatidylchines in children who later progress to diabetes in the DIPP study of 129 genetically high-risk children who progressed to T1D in early childhood, it agrees with the observation of Pfueger and co-workers of significantly higher levels of cluster of lipids dominated by polyunsaturated fatty acid (PUFA)-containing phosphatidylcholines (and triglycerides) in the larger BABYDIAB study of 1650 children of mothers or fathers with T1D. Our observation also seems to be somewhat contrary to the finding of Sysi-Aho, Oresic, and co-workers that NOD mice that did not progress to diabetes despite being insulin autoantibody positive had elevated levels of selected serum lysophosphatidylcholines species compared with progressors. Further, we
found no confirmation here of the lower methionine or hydroxyproline levels observed in that study. The progressors to nonprogressors ratios for these compounds were all very close to unity and far from showing statistical significance either in blood or in feces. It should be noted that while these studies all used serum samples, our study used whole blood. As already mentioned, another human study, which looked at metabolite profiling in the Framingham Offspring Study, suggested that five branched-chain and aromatic amino acids (isoleucine, leucine, valine, tyrosine, and phenylalanine) are associated with future diabetes with fasting concentrations being higher. None of these was found to be increased in early blood or fecal samples here; however, as discussed earlier, they were all highly increased in blood at later time-points when animals were already diabetic (week 21 and 26; Figure 11).

In addition to these phosphocholine derivatives, other potential early biomarker candidates for T1D progressors include serotonin, ribose, and arabino (elevated) in blood as well as tocopherol (elevated) plus diaminopimelate, valerate, hydroxymethylpyrimidine, and dulcitol (decreased) in feces (Figure 12, Figure S6). Although none of these compounds showed highly significant differences in blood samples (for all compounds, 0.01 < p < 0.05), eight compounds showed more significant differences in feces (p < 0.01; Table S3). While these compounds by themselves may not be different enough to serve as early disease indicators (biomarkers), a combination of them could provide a sufficiently reliable early biomarker signature to distinguish T1D progressors from nonprogressors.

Serotonin might be of particular interest as it was significantly elevated in T1D progressors at week 6 in blood (1.5×, ↑) and it stayed elevated (even if not significantly) at all time-points (1.1–1.3×, ↑). It was also elevated at week 6 in fecal samples (1.2×, ↑). In pancreatic islet, serotonin is an autocrine signal coreleased with insulin, and it has been recently shown that during heightened metabolic demands, such as pregnancy or high-fat diet feeding, serotonin is produced in the islet and can stimulate β-cell proliferation via 5-HT2B and 5-HT3 receptors. Finally, xanthine, hypoxanthine, and cysteine S-sulfate could be of potential interest as they show a clear peak at week 11 in T1D progressors, a time when the autoimmune inflammatory attack begins in progressors (Supporting Information Figure S9).

**CONCLUSIONS**

In summary, our comprehensive nontargeted metabolomics study quantified the longitudinal change of several hundred biochemicals in the blood and feces of NOD mice as they progressed to hyperglycemia. Diabetes onset was found to profoundly alter the overall metabolic landscape: in diabetic animals, a large proportion of the detected metabolome was significantly altered not only in blood (57.8%), but also in feces (27.8%), despite the presence of sustained-release insulin. Several biochemicals showed considerable, more than four-fold change, and the metabolic pathways most strongly affected included carbohydrate, lipid, branched-chain amino acid, and oxidative metabolisms. In T1D animals, some compounds, such as 3-hydroxybutyrate, maltose, and kojibiose, increased very dramatically showing more than 10–20-fold increases, while 1,5-anhydroglucitol decreased even more. Differences between the metabolic signatures of progressors and nonprogressors at the earliest time-point (6 week) were relatively modest. Nevertheless, several compounds had significantly different levels and showed promise as possible early disease indicators, even if not alone by themselves, but conceivably as part of a combined metabolic signature.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00512.

Summary of statistically significantly altered biochemicals; metabolites that show the largest fold-change in diabetic versus nondiabetic controls; metabolites with significantly different levels at week 6 in NOD mice that later progressed to diabetes; schematic design of present study; random forest analysis of all present metabolomics data; compounds with largest fold-change in T1D mice; spaghetti plots for compounds with largest fold-change in T1D progressors; representative pancreatic sections immunostained for insulin in different mice; random forest analysis of week 6 metabolomics data; spaghetti plots for compounds with largest and statistically significant differences between progressors and non-progressors at early 6 week sample; pathways most different between diabetes progressor and nonprogressor NOD mice at earliest time-point analyzed; line plots for compounds showing large and statistically significant differences between progressors and nonprogressors at week 11 sample (PDF)

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**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

Financial support from the Diabetes Research Institute Foundation is gratefully acknowledged.

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