

Supplementary Text:

Sensitivity analysis with medication and clinical chemistry

We used sensitivity analysis to assess the effect of additional covariates on T2D metabolite associations. For sensitivity analysis, in addition to the covariates mentioned in the Materials and Methods, we added each of the following covariates separately and investigated their impact on the final result: HDL, LDL, TRI, creatinine, blood urea nitrogen (BUN), and medication. Medication consisted of statins; anti-hypertensives using a combination variable of use of angiotensin-converting enzyme [ACE], angiotensin II receptor blockers [ARBs], and calcium channel blockers [CC], referred to as ACE_ARB_CC; beta-blockers; thyroxine; and aspirin. **Supplementary Table 3** shows the results of sensitivity analysis, with metabolites that have been affected after adjusting for those covariates. After adjusting for statins, 18 metabolites associated with T2D became nonsignificant. Adjustment for ACE_ARB_CC, aspirin, BUN, and creatinine affected 18, 10, eight, and three metabolites, respectively. Statins were associated with the xenobiotics 2-hydroxyhippurate (salicylurate) and salicylate, along with seven other metabolites. Aspirin was associated with three xenobiotics – O-sulfo-L-tyrosine, 2-hydroxyhippurate (salicylurate), and salicylate – and two unknown metabolites. After creatinine and BUN adjustment, three and eight metabolites became non-significant, respectively, including O-sulfo-L-tyrosine and metabolites from leucine/isoleucine/valine metabolism, among others. Adjustment for TRI, HDL, and LDL revealed 34, 21, and 20 affected metabolites, respectively.

Shared and Unique metabolites:

We visualized significant metabolites of super-pathways in the eight clinical variables while highlighting the top most significant hits in either T2D or TRI (**Figure 2a**). We then identified 17 sub-pathways (≥ 6 metabolites) in amino acids (6 sub-pathways), lipids (10 sub-pathways) and peptides (one sub-pathway) to identify significant pathway associations shared between clinical variables (heatmap in **Figure 2b**). Based on the shared metabolic perturbations among the clinical variables, we computed correlations between the studied phenotypes (T2D, BMI, retinopathy, LDL, HDL, TRI, and LDL /HDL ratio) using regression statistics ($\text{sign}[\beta] * -\log_{10}[p\text{-value}]$) with the 373 metabolites (**Supplementary Figure 3**). Boxplots of selected metabolites associated with five or more variables are shown in **Supplementary Figure 4a-b**, and those associated only with T2D, retinopathy, or TRI are shown in **Supplementary Figures 4c-h**.

GGMs:

The second largest subnetwork consisted of sphingomyelins (20 metabolites, **Supplementary Figure 5b**), which were associated with two or more phenotypes, followed by two fatty acid subnetworks with 17 and 13 metabolites, respectively (**Figures 3a,3e**). The first fatty acid subnetwork comprised PUFAs, phospholipids, and monoacylglycerols and had seven metabolites associated only with T2D and seven associated only with TRI. The second subnetwork included eight metabolites associated with TRI, mainly long-chain fatty acids and PUFAs. Subnetworks which contained metabolites associated only with T2D are: dipeptides and unknowns (**Figure 3f**), monohydroxy fatty acid metabolism (**Figure 3b**), carbohydrates and sugars (**Figure 3c**), and a fungal/xenobiotic/unknowns subnetwork (**Figure 3d**). Finally,

BCAAs/phenylalanine and tyrosine subnetworks (**Supplementary Figures 5c,e**) and a glucoronides/unknowns subnetwork were identified (**Supplementary Figure 5d**).

Extended Discussion:

Among the most shared pathways (i.e. shared between four or more clinical outcomes) were the associations of T2D, BMI, LDL, retinopathy, and LDL/HDL with sphingolipids, and of T2D, BMI, LDL, and TRI with phospholipids, lysolipids, and fatty acids (monohydroxy, branched, acylcarnitine). Additionally, T2D, BMI, and TRI shared associations with BCAAs and carbohydrates (glycolysis, glycogen, fructose, mannose, pentose, amino sugar, and advanced glycation end-products [AGEs]), whereas T2D, BMI, and retinopathy shared associations with amino acids of the phenylalanine and tyrosine pathway, 12 purine/pyrimidines, and lysolipids. T2D, TRI, and retinopathy shared associations with 11 peptides.

In addition to those mentioned above, T2D and TRI also shared associations with xenobiotics, LCFAs, and PUFAs. Finally, T2D shared only a few associations (methionine, cysteine, SAM, and taurine pathways) with TRI, LDL/HDL, and retinopathy, and the urea cycle with BMI, whereas T2D was predominantly associated with those pathways. Of all clinical variables, TRI showed the strongest associations with metabolites from the phospholipids, diacylglycerol, and monoacylglycerol pathways, among others.

Sphingomyelins:

Our cohort uncovered several sphingomyelins with distinct chemical structures, correlated as shown in the GGM subnetwork (Figure 3 and Supplementary Figure 5). Sphingosine, the end product of sphingomyelin degradation [1], was elevated in our cohort, with a corresponding decrease in sphingomyelin levels in T2D. Sphingomyelins were previously associated with insulin resistance in clinical studies [2] and with diabetic nephropathy pathogenesis [3], and sphingomyelin levels were used to discriminate among healthy, T2D, and diabetic nephropathy patients [4]. Sphingomyelins are also involved in the pathogenesis of diabetic retinopathy [3], neuropathy [5] and cardiovascular disease [6].

Lysolipids, Phospholipids & Plasmalogens:

The anti-inflammatory and glucose-lowering effects of lysolipids make them good indicators of metabolic disorder, obesity, and cardiovascular disease risk for T2D patients [7]. Lysolipids were decreased with T2D in our cohort but increased with high TRI levels. However, phospholipids that significantly increased with TRI also increased with T2D. Altered phospholipid metabolism is central to the pathogenesis of metabolic diseases [2] and is associated with insulin resistance and sensitivity [8]. We found that 17 ethanolamine- and choline-containing phospholipid metabolites—the most abundant phospholipids in human metabolism [9]—were altered in T2D (5 increased and 12 decreased).

Consistent with a decrease in PUFAs, plasmalogen and lysoplasmalogen, which are enriched in PUFA (arachidonate), were decreased in T2D. Plasmalogens, the primary constituents of phospholipids [10], are inversely associated with T2D and cardiovascular disease [11] [12]. They may be used as an oxidative stress marker for diabetic patients [13], as they are thought to act as endogenous antioxidants; thus, decreased concentrations indicate higher oxidative stress [14].

Oxidative Stress:

Excess free fatty acids (FFA) can lead to insulin resistance and elevated tissue lipolysis. This then leads to additional elevation of plasma FFA, as observed in obese individuals, and increases biosynthesis of signaling molecules such as DAG and sphingolipids. This can also be explained by the production of adipokines, which often results in the release of fatty acids from adipose tissue and their deposition in distal tissues; this promotes the development of lipotoxicity. The resulting oxidative stress has been shown to trigger ceramide accumulation. Moreover, elevated sphingolipid synthesis contributes to cell death through endoplasmic reticulum stress, which has been shown to contribute to the insulin resistance that leads to the development of T2D. Excess fatty acids have been found to mediate lipotoxic cellular dysfunction through such mitochondrial dysfunction, and mitochondrial overload with substrates for β -oxidation can increase cell oxidative stress.

Amino Acids and Oxidative Stress:

3-HIB is known to be secreted from muscle cells and to activate endothelial fatty acid transport. It also stimulates muscle fatty acid uptake *in vivo*; promotes lipid accumulation in muscle, leading to insulin resistance in mice; regulates the trans-endothelial flux of fatty acids; and links the regulation of fatty acid flux to BCAA catabolism [15].

Glutathione, which is a major intracellular antioxidant and plays a key role in reducing the effects of oxidative stress, was found to be decreased in T2D patients in our cohort (oxidized cys-gly, cysteine-glutathione disulfide) compared to controls, in agreement with what was previously found in diabetic patients with known microvascular complications [16].

Taurine is another important amino acid biomarker for T2D because of its antioxidant effect, known to be related to obesity and non-alcoholic fatty liver disease (NAFLD) [17]. All metabolites involved in the urea cycle were decreased, and amino acids involved in the urea cycle are known to be associated with inflammatory markers and oxidative stress. Arginine, threonine, and metabolites in the alanine and aspartate metabolism pathway are associated chronic heart disease in diabetic patients [18].

T2D metabolites and link to oxidative stress

Metabolites associated only with T2D are involved in kidney function or chronic kidney disease. These include 1,5-anhydroglucitol, creatinine and guanidinoacetate (creatine metabolism) [19], N-acetylglutamine and pyroglutamine (glutamate metabolism), pseudouridine, tartronate (novel to T2D) and 4-hydroxychlorothalonil [20] [21]. Uric acid and creatinine are positively correlated with β -cell function in T2D patients [19]. BCAA metabolites associated only with T2D include 3-methylglutaconate (novel metabolite) of the leucine pathway [22] and isobutyrylglycine and isovalerylglycine, which are both related to insulin resistance [23]. Proline is known to protect mammalian cells against oxidative stress [24], and the novel associations of thioproline and N-methylproline are associated only with T2D; thioproline, which is an antioxidant that protects the body from oxidative stress [25], was decreased. Also novel and associated only with T2D were N-acetylarginine, which affects oxidative stress in rats [26], and dopamine sulfate. Dopamine is of special importance in neuronal redox-homeostasis and viability and is

linked to oxidative stress; its degradation results in the production of ROS, and its oxidation can lead to endogenous neurotoxins, while some dopamine derivatives also show antioxidative effects [27].

Metabolic Signatures:

Figure 4e shows a signature that combines an LCFA (palmitate), phenylalanine and a BCAA (3-methyl-2-oxovalerate) among other lipids that are at their lowest levels in the largest portion of retinopathy patients having the lowest levels of TRI. Palmitate, a biomarker for T2D [28], is increased in T2D compared to controls, yet shows the highest levels in the patients with the highest TRI levels. It has been reported to induce toxicity via oxidative stress, leading to reactive oxygen species (ROS) generation and Ca^{2+} -mediated pathogenic changes [28]. It is also involved in the *de novo* biosynthesis of sphingolipids [29] and is known to be perturbed in T2D complications such as retinopathy and nephropathy; its accumulation in T2D patients with persistent hyperglycemia accelerates retinal mitochondrial DNA damage via glucolipotoxicity, and it is involved in the progression of diabetic nephropathy [30]. The low levels of phenylalanine and 3-methyl-2-oxovalerate in the highest portion of retinopathy patients requires investigation of the importance of administering those amino acids in retinopathy patients.

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