# Metabolomics for Biomarkers of Type 2 Diabetes Mellitus: Advances and Nutritional Intervention Trends

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## Introduction

In addition to obesity, T2DM is a major risk factor for cardiovascular disease (CVD). Hyperglycemia and insulin resistance (IR) are powerful predictors of adverse cardiovascular events, and these two risk factors in combination exert a detrimental synergistic effect [1]. However, despite a number of recognized risk factors including family history of diabetes, age, sex, and numerous anthropometric, biochemical, socioeconomic, and lifestyle variables, the identification of individuals with increased risk of T2DM and/or CVD remains a laborious task [2]. Typical biomarkers in T2DM prediction models include elevated concentrations of fasting plasma glucose and insulin, glycated hemoglobin (HbA1c), serum ferritin, C-reactive protein (CRP), and interleukin-2 receptor alpha (IL2RA); these models also predict a decrease in effective serum insulin and adiponectin concentrations [3, 4]. Recent advances in "omics" such as genomics and metabolomic technologies are generating an increasing number of potential biomarkers to identify crucial stages in the pathogenesis and progression of a determined pathological state, including T2DM. In addition, they are also gaining insights in the underlying molecular disease-causing mechanisms through the discovery of associations between some genetic variants and key small molecules. These relationships highlight the power of integrating multiomic approaches (Systeomics) to better understand the causal mechanisms [5]. For instance, recently, Wang et al. [6] delineated the role of fatty acid desaturases (FADs) in regulating human liver lipid composition through a targeted lipidomic analysis and associated them with FADS single nucleotide polymorphisms (SNPs) from genome-wide association studies (GWASs). They suggested that FADS1 and its polymorphisms were related with long-chain fatty acid accumulation in human liver [6]. Metabolomics is defined as a comprehensive characterization of endogenous or exogenous metabolites representing the metabolome [7]; the use of this technology enables the detection of physiological or pathological changes in cells, tissues, or body fluids and represents a useful tool for biomarker detection [8, 9]. In diabetes research, metabolomics has been successfully applied to diagnostic and prognostic biomarker discovery, elucidation of disease pathways, identification of drug side effects, and discovery of functional biomarkers for drug activity [10]. The present review aims to summarize the distinct family of metabolites that have been proposed as potential biomarkers of different stages of T2DM by

metabolomic approaches. Additionally, the impact of diet, as an important lifestyle factor, on classical and metabolomic biomarkers will be reviewed for better understanding the pathophysiology of diabetes, aiming to implement healthcare strategies in the future.

## Metabolomic Biomarkers in Prediabetes

The term prediabetes refers to the impaired glucose tolerance (IGT) and/or impaired fasting glucose (IFG) of subjects with a relatively high risk of developing diabetes. IFG is characterized by fasting plasma glucose levels between 100 mg/dl (5.6 mmol/l) and 126 mg/dl (7.0 mmol/l). IGT is defined by 2-h plasma glucose values after an oral glucose tolerance test (OGTT); values between 140 mg/dl (7.8 mmol/l) and 200 mg/ dl (11.1 mmol/l) are considered indicative of IGT [11]. In addition to IFG and IGT, IR is also considered a crucial metabolic status because it can precede the dysglycemic states of prediabetes and T2DM [12]. Because prediabetic stages are asymptomatic, extended time periods may elapse before diagnosis of T2DM, hampering early detection. In addition to the most common test used to assess impaired insulin sensitivity (IS), homeostasis model assessment of insulin resistance (HOMA-IR), OGTTs are also indirectly used to assess insulin resistance.

Metabolomics may be extremely helpful in the identification of novel biomarkers of prediabetes and metabolic disturbances that precede the new onset of T2DM. In this regard, a targeted metabolomic approach has shown that IR emerges in insulin-dependent processes, such as proteolysis, lipolysis, ketogenesis, and glycolysis, in addition to the reduction in glucose uptake and suppression of gluconeogenesis, thus, reflecting a broad switch from catabolism to anabolism [13]. The understanding of the role of dyslipidemia in prediabetes has progressed significantly with the implementation of lipidomics, a new branch of metabolomics [14–16]. The current and advanced analytical techniques used in lipidomics such as chromatography coupled to mass spectrometry (MS) or nuclear magnetic resonance (NMR) as well as other spectroscopic approaches are powerful techniques used in lipidomics for lipid detection and characterization [17]. They allow detection and characterization up to several hundreds of lipids belonging to major lipid classes (i.e., fatty acyls, phospholipids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids) [17]. As an example of the high throughput obtained by these technologies, a recent lipidomic study reported over 500

different lipid molecular species among the main lipid classes in plasma of individuals [18]. The importance of lipoprotein fatty acid composition and its role in IS has been emphasized in a study using lipidomic techniques, including ultra-performance liquid chromatography coupled to mass spectrometry (UPLC/MS). In this study, the degree of fatty acid saturation in triacylglycerols (TAG) within the VLDLIDL- LDL axis and HDL were differentially related to IR [15]. Specifically, serum TAG molecules, such as 16:0/16:0/18:1 and 16:0/18:1/18:0, correlated positively with HOMA-IR; however, TAG containing essential fatty acids, such as 18:1/ 18:2/18:2, correlated negatively. The findings of this study reinforce the role that fatty acids may have in the pathogenesis of IR; therefore, the serum fatty acid composition may be considered a more precise marker of insulin resistance than total serum TAG concentrations [15]. Consistent with this hypothesis, in the Framingham Heart Study (FHS), serum TAG characterized by relatively low carbon number and double bond content (i.e., C46:1, 48:1) were positively associated with HOMA-IR; conversely, TAG with increased carbon number and double bond content (i.e., C56:9, C58:10) were not correlated with HOMA-IR [16]. These results were consistent even after the participants were grouped in quartiles according to HOMA-IR [16].

Zhao et al. [19] also observed a characteristic lipid profile for individuals with IGT as a prediabetic condition. Applying untargeted metabolomics using UPLC-qTOF-MS, the authors reported increased plasma levels of free fatty acids (FFA) (i.e., C16:0, C18:0, C18:1) and glycochenodeoxycholic acid as well as decreased concentrations of lysophosphatidylcholines (lysoPC) (i.e., C16:0, C18:0, C18:1 and C18:2) relative to subjects with normal glucose tolerance (NGT) [19]. In the same study, the NGT individuals trended towards lower plasma levels of saturated fatty acids (SFA), including palmitate and stearate, but not monounsaturated (MUFA) or polysaturated fatty acids (PUFA), such as oleate and arachidonic acid, respectively [19].

Beyond the isolated impact of dyslipidemia, amino acid signature has been also reported as a characteristic signature in obese prediabetic subjects. In a broad metabolic profiling study performed by Newgard et al., the PCA-component including certain amino acids, branched-chain amino acids (BCAA) (leucine/isoleucine and valine), methionine, glutamate/ glutamine, aromatic amino acids

(phenylalanine and tyrosine), as well as acylcarnitines (AcylCN) C3 and C5 was obesity associated and linearly related to IR assessed by the HOMA index [20]. These findings were supported by Huffman et al., who reported that a similar group of metabolites containing large neutral amino acids (proline, valine, leucine/ isoleucine, methionine, phenylalanine, tyrosine, histidine) and uric acid were related to IR in a mixed-sex population (n=73) at risk for T2DM [21]. Additionally, a group of metabolites including FFA and fatty acid oxidation byproducts was associated with an impaired pancreatic response. The authors suggested that a poor compensatory response to insulin production is associated with increased concentrations of circulating FFA, potentially having a toxic effect on β cells in prediabetic subjects [21]. Interestingly, the same authors also observed sex differences; men were more susceptible to amino acid-induced IR, whereas women were more vulnerable to lipid-mediated β cell toxicity [21]. More recently, using proton nuclear magnetic resonance (1HNMR)-based analysis, a set of 20 serum metabolites was also associated with IR in a cohort of 7098 young adults [22].

BCAAs, aromatic amino acids, glycolysis and gluconeogenesis intermediates, and fatty acid composition and saturation were positively correlated with HOMA-IR. Conversely, glutamine and ketone bodies (3-hydroxybutyrate and acetoacetate) exhibited an inverse correlation, as did the average number of double bonds per fatty acid chain. Furthermore, the authors observed interactions between four amino acids (leucine, isoleucine, valine, and tyrosine) and sex and obesity variables, with significant associations in women with central obesity [22]. Nevertheless, the ethnicity of populations has to take into account when interpreting results. Metabolomics [23], jointly with genomics [24], is a promising and needed tool for evaluating differences between different ethnical populations as previously some studies observed that different populations have different rates of T2DM [24]. A recent metabolomic study has shown that a pattern of reduced plasma glycine and increased aromatic and BCAA was related to individuals with high IS compared with low IS individuals in European-American subjects, while other ethnics (Hispanics or African Americans) did not show the same associations [23]. Further metabolomic studies are needed in ethnically and racially diverse populations. To segregate the effects of obesity and IR on the metabolic changes observed in prediabetic subjects, Tai et al. compared the metabolic profiling of two non-obese (BMI

~24 kg/m2) Asianethnic populations with IR. Using a combination of two metabolic platforms, tandem mass spectrometry (MS/MS), and gas chromatography coupled toMS (GC-MS), the authors identified significant changes in plasma and urine metabolites in individuals separated by tertiles of IR based on HOMA indices [25]. The results showed up to 26 clusters composed of amino acid and AcylCN, between other metabolites that contributed to the grade of IR. One of these clusters was composed of 10 amino acids that were significantly increased in individuals with a high HOMA index. The same trend was observed in another group composed of pyruvate, lactate, and arginine. Moreover, isobutyrylglycine and isovalerylglycine , included in another cluster, were significantly lower in the high-HOMA group than in the low-HOMA group but in only one population. Interestingly, no association between IR and traditional IR biomarkers such as inflammatory mediators and fatty acids was observed in this study [25].

Metabolic signatures of prediabetes composed of few metabolites have been proposed in several studies. Wang-Sattler et al. quantified 140 metabolites with an AbsoluteIDQTM p180 kit (BIOCRATES Life SciencesAG, Innsbruck, Austria) in fasting serum samples of subjects from the Cooperative Health Research in the KORA S4 study. The authors observed that glycine and lysoPC 18:2 were significantly decreased, whereas AcylCN C2 was increased in IGT individuals compared to the NGT group [26•]. Similar results were observed in the follow-up KORA F4 study. In the prospective KORA S4→F4 cohort, lower levels of glycine and lysoPC 18:2, but not C2 AcylCN, were found to be predictors of both IGT and T2DM. This was independently confirmed by the same authors in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam cohort [26•]. In other clinical studies, α-hydroxybutyrate (α-HB) has also been proposed as a strong prediabetic biomarker [27, 28•]. Based on a subset (n=399) of the EPIC cohort, Gall et al. identified α-HB as the most significant metabolite associated with IR and, interestingly, as an early marker for dysglycemia (IFG + IGT) independent of and in addition to IR [27]. Ferrannini et al. later confirmed the association of α-HB with IR, along with the novel metabolite linoleoylglycerophosphocholine (L-GPC) [28•]. Ferranini et al. observed a positive correlation between α-HB and IR in two prospective observational cohorts, the Relationship between Insulin Sensitivity and Cardiovascular Disease

(RISC) study (n=1261) and the Botnia Prospective Study (n=2580) with 3 and 9.5 years of follow-up, respectively. Additionally, L-GPC was negatively correlated with IR. α- HB was also reciprocally related to indices of β cell function derived from OGTT. In the follow-up of both studies, α-HB was a positive predictor and L-GPC a negative predictor of dysglycemia (RISC study) or T2DM(Botnia Study), independently of the family history of diabetes, sex, age, BMI, and fasting plasma glucose [27]. Recently, a novel branched-chain ketoacid derivative of isoleucine, called 3-methyl-2-oxovalerate, was found to be significantly associated to IFG, both in the plasma and urine of individuals with prediabetes relative to control individuals with diabetes [29]. The observation that 3-methyl-2-oxovalerate was the second strongest predictive biomarker for IFG, after glucose, was first reported in a cohort of 2204 females from Twins, UK, and was subsequently replicated in individuals with IFG (n=536) in the follow-up study KORA F4 [29]. Other metabolites that are not directly linked with major metabolic pathways have been significantly associated with prediabetes. For example, decreased urinary levels of gut microbiota-associated metabolite biomarkers, including hippuric acid, methylxanthine, methyluric acid, and 3- hydroxyhippuric acid, have been linked to IGT [19].

**Metabolomics Biomarkers and Pathways Altered in T2DM**

Because T2DM triggers multiple metabolic disorders, efforts have been made to elucidate the mechanisms causing these disorders and systemic complications. Metabolomics has been successfully applied in T2DM research to elucidate novel metabolic pathways as well as to define relationships between significant metabolites in these pathways. Nowadays, the knowledge of interactions between affected T2DM pathways is improving through building biological pathways and network analysis techniques which integrate data from the different Bomics^ [30, 31]. To obtain a more comprehensive analysis of the metabolic processes negatively regulated by T2DM, a summary of metabolomic studies and the resulting metabolites significantly altered in diabetics is shown in Table 1. Furthermore, Fig. 1 illustrates a summary of metabolic networks presumably affected by T2DM. To this end, we used the MetaCoreTM software (Thomson Reuters) fromGeneGo, uploading the complete list of T2DM biomarkers reported in literature (Table 1). Metabolomic studies

evaluating individuals with T2DM confirm that metabolic networks of primary biomolecules, such as carbohydrates, lipids, and amino acids, are altered as a consequence of the diabetic stage (Fig. 1). Hyperglycemia and glycosuria are the major biomarkers of uncontrolled T2DM [29, 54]. However, abnormal levels of other metabolites reflect dysregulation of carbohydrate metabolism (i.e., fructose, mannose) (Fig. 1). Impairment of glycolysis and gluconeogenesis has been demonstrated by metabolomic approaches through the identification of metabolites included in the sepathways: glycerol-3-phosphate, phosphoenolpyruvate, pyruvate, and lactate. Additionally, downstream tricarboxylic acid cycle (TCA) metabolites, such as citrate, 2-oxoglutarate, succinate, fumarate, and malate, are deregulated in diabetes (Table 1). Controversial results suggest that levels of circulating and urinary glucogenic amino acids [29, 33, 36, 39, 41, 45, 46, 52, 55] in diabetic subjects indicate deregulation of glucose biosynthesis (Table 1). Furthermore, significant increases in three ketone bodies, acetone, acetoacetate, and β-hydroxybutyrate, in plasma [32, 41] and urine [34], reflect a reduction in glucose uptake and the onset of ketosis in T2DM [56]. A considerable number ofmetabolomic studies have reported a positive association between abnormal circulating concentrations of lipid derivatives and T2DM progression. Although not necessarily consistent with prediabetes with respect to saturation, higher concentrations of long-chain (i.e., oleic, palmitic) and lower concentrations of medium-chain FFAs (i.e., caproate, pelargonate, 10-undecenoate) are a characteristic lipid signature among individuals with T2DM [41, 43, 45, 57]. Using GC-MS analysis, Han et al. demonstrated that primarily esterified fatty acids (EFA) are decreased in patients with T2DM, while non-esterified fatty acids (NEFA) are increased. This occurs even when including the variability of groups with different stages of diabetic nephropathy, suggesting a combination of lipotoxicity and toxicology repair mechanism [47]. Applying a lipidomic approach, Ståhlman et al. characterized the lipid composition of ApoB-containing lipoproteins isolated from control, normolipidemic, and dyslipidemic individuals with T2DM [58]. Significant increases in PC 16:0-20:3 (in VLDL and LDL) and PC 18:0-20:3 (in LDL) were detected in normolipidemic T2DM compared with control individuals. These alterationsweremore pronounced in the dyslipidemic T2DM group, which also had a relatively increased amount of PC 16:0-16:1. Similarly, significant increases in CE 16:1 (in VLDL and LDL) and CE 20:3 (in LDL) were detected in lipoproteins from

dyslipidemic T2DM participants. Furthermore, levels of palmitic acid (C16:0) in VLDL and LDL TAG correlated positively with IR [58]. Abnormal circulating levels of distinct subclasses of phospholipids, including PC, lysoPC, phosphatidylinositol (PI), PE, lysoPE, SM, PG, and sphingosine-1-phosphate (Table 1), have also been identified bymetabolomic approaches and were considered potential biomarkers of the diabetic dyslipidemia [48, 49, 53]. Ceramides, another important class of bioactive lipids, have recently garnered attention due their pathophysiological relevance in the development of IR and impaired glycemic control [59]; therefore, ceramide concentrations are significantly deregulated in T2DM [37]. A research focused on acylcarnitines (AcylCN) and their byproducts has generated insights into the dysregulation of fatty acid oxidation associated with T2DM. Variations in the levels of AcylCN, mostly from short to medium chains, have been detected by applying targeted metabolomic analyses to T2DM before its onset, there is great demand for reliable, predictive biomarkers. Targeted metabolomic studies have increasingly aided in the development of novel biomarkers in large prospective studies [16, 60••, 61, 62]. Consistent with previous observations in individuals with IR, Rhee et al. observed a characteristic association between carbon number and bond content that was predictive of developing T2DM. Specifically, TAG with a lower carbon number and double bond content were associated with an increased odds ratio (OR) for diabetic subjects, while TAG with a higher carbon number and double bond content were associated with an OR of less than one. Moreover, the inverse relationship between diabetes risk, carbon number, and double bond content persisted after multivariable adjustment for lysoPC, PC, and possibly lysophosphatidylethanolamines (lysoPE), but not for cholesterol esters (CE) [16]. In another but complementary study, Wang et al. carried out two parallel and independent studies based on the same sample population. These authors discovered two novel metabolic signatures for the prediction of T2DM [60••, 62]. In the first study, higher levels in a panel of five amino acids (isoleucine, leucine, phenylalanine, tyrosine, and valine) showed a strong association with future development of diabetes. Moreover, a combination of three amino acids (isoleucine, tyrosine, and phenylalanine) was shown to be a better predictor of future diabetes than all five amino acids; individuals in the top quartile of this 3-amino acid score had a five- to sevenfold higher risk of developing new-onset diabetes compared with individuals in the lowest quartile [60••]. These results,

with the exception of a nonsignificance of isoleucine, were replicated by the same authors in the Malmö Diet and Cancer (MDC) study [60••]. Later, Wang et al. reported that the odds of developing T2DM were increased fourfold for individuals in the higher quartile of plasma 2-aminoadipic acid (2-AAA) concentrations over the 12-year follow-up period, relative to those in the lowest quartile. These results were replicated in the MDC study and were confirmed in a heterogeneous cohort from the FHS-Offspring study (n=1561) [62]. Additionally, fasting concentrations of 2- AAAwere moderately correlated with fasting insulin, HOMAIR, HOMA of β cell function, and OGTT. However, concentrations of 2-AAA were poorly correlated with the previous set of five amino acids associated with future diabetes risk, suggesting that these biomarkers are regulated by distinct pathophysiological pathways [62]. More recently, Floegel et al. confirmed that dysfunctional levels of lipid-related metabolites and amino acids are potent biomarkers for future T2DM prediction [61]. In the EPIC-Potsdam study, researchers identified 14 metabolites that were independently and significantly associated with T2DM risk. They used a PCA to identify 2 factors which included different metabolites. Metabolite factor 1, consisting of primarily acyl-alkyl-PCs, sphingomyelins (SM), and lysoPC, was associated with a significant 69 % reduced risk of T2DM when comparing extreme quintiles of metabolite factors. Conversely, metabolite factor 2, consisting of diacyl- PCs, BCAA and aromatic amino acids, propionylcarnitine, and hexose, was associated with a significantly greater risk of T2DM. Remarkably, when these metabolites were added to classical models using recognized risk factors of T2DM, discrimination was slightly but significantly improved [61].

**Nutritional Interventions in Metabolomics Biomarkers of T2DM** Pharmacological and lifestyle interventions have a significant impact on T2DMpatients [63]. Among lifestyle factors, diet is a strong modulator of health status [64]. Diets rich in whole grains, fruits, vegetables, legumes, and nuts combined with moderate consumption of alcohol and lower in refined grains, red or processed meats, and sugar-sweetened beverages have been shown to reduce the risk of diabetes and also improve glycemic control and blood lipids in patients with diabetes [65••, 66, 67]. A recent systematic review and meta-analysis of dietary T2DM management approaches has highlighted that low-carbohydrate, low-glycemic index (GI), Mediterranean (MedDiet), and high-protein diets effectively improve various markers of cardiovascular risk [68]. These diets successfully reduced HbA1c,

stimulated weight loss, and increased HDL concentrations in people with diabetes. These studies suggest that dietary patterns should be considered in the overall strategy of diabetes management [68]. The MedDiet has received particular attention due to its demonstrated efficacy in preventing CVD [69••], in addition to its association with reduced incidence of metabolic syndrome, prediabetes [70], and T2DM [71]. Salas-Salvadó et al. recently showed a positive effect of MedDiet on T2DM prevention, comparing two types of MedDiet and using a low-fat diet as a control in subjects with high CVD risk [72, 73]. In an interventional study, the authors discovered that without energy restrictions, MedDiet enriched with either extra-virgin olive oil (EVOO) or mixed nuts reduced the risk of T2DM [72]. By assessing the efficacy of long-term adherence to MedDiet (median follow-up, 4.1 years), the authors found that the EVOO MedDiet group exhibited a lower incidence of T2DM compared to the nut-enriched MedDiet or control diet [73]. In 2011, several diet-quality scores (Healthy Eating Index [HEI], the alternative HEI [aHEI], the alternative Mediterranean Diet (aMED), and the Dietary Approaches to Stop Hypertension [DASH]) were studied to be associated with incident T2DM in the Health Professionals Follow-Up Study [66]. They observed that three scores (aHEI, aMED, and DASH) were significantly related with a decreased risk of T2DM[66]. Recently, the BInterAct Consortium studied the association between aHEI and DASH scores and three reduced rank regression (RRR)-derived dietary pattern scores from different studies (the American Nurses' Health Study, German EPICPotsdam study, and the British Whitehall II study, respectively) with T2DM^ [67]. Only adherence to these RRR-derived dietary pattern scores decreased type 2 diabetes risk in the EPIC-InterAct Study [67]. It is noteworthy to comment that adherence to these scores were represented by high intake of plant-derived foods and low intake of red and processed meat and sugar-sweetened beverages [66, 67]. In this line, alkylresorcinol has been described as a valid biomarker for a Nordic diet (ND) which is rich in whole-grain cereals [74]. The alkylresorcinol C17:0/C21:0 ratio has been inversely related with increased IS [75]. There has been moderate-grade evidence that the intake of whole-grains protected against T2DM [76]. Otherwise, further studies are required for examining associations between ND and its characteristic foods and T2DM [76, 77]. In recent years, metabolomics has been widely applied to interventional studies to identify variations in human metabolic profiling in response to food [78–83]; however, this approach has rarely been applied to

the assessment of the effect of foods on particular pathologic states [84–86]. For instance, regular consumption of cocoa powder decreased the levels of endogenous metabolites related tometabolic disorders, such as carnitine metabolites and tyrosine sulfate [86]. However, the impact of dietary interventions such as MedDiet on the metabolome of T2DM subjects has not been well characterized. Future studies are warranted to develop novel biomarkers in response to diet challenges. This could establish appropriate dietary therapeutic strategies to improve the life course of T2DM patients.

## Conclusions

Metabolomics is a rapidly growing field to identify novel biomarkers for different stages of T2DM. In the face of the current diabetes epidemic, future research should consider the relevance of novel biomarkers for the prediction and diagnosis of T2DM and the elucidation of disease pathways implicated in this disease. Metabolomic approaches have identified distinct classes of metabolites as potential biomarkers for different stages of T2DM. Several studies have demonstrated that the metabolism of carbohydrates, lipids, and amino acids are considerably altered in the prediabetic state and at different stages of T2DM progression. The identification of intermediate metabolites included in glycolysis, gluconeogenesis, the tricarboxylic acid cycle, lipolysis, and proteolysis have provided evidence for this metabolic dysfunction. Due to the scarcity of information on the effects of lifestyle changes on metabolomic biomarkers, more effort should be directed in expanding our knowledge to the metabolic modulations caused by dietary patterns in T2DM patients. Lifestyle interventions have a significant impact on diabetes prevention and control through modeling peripheral classical biomarkers of T2DM; therefore, future studies should aim to develop novel biomarkers that are sensitive to food challenge. This could establish appropriate dietary strategies to help improve the life course of T2DM patients.

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## Compliance with Ethics Guidelines

Conflicts of Interest Cristina Andres-Lacueva, Francisco J Tinahones, Jordi Salas-Salvadó, Mireia Urpi-Sarda, Sara Tulipani, and Enrique Almanza-Aguilera have no conflicts of interest. Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal performed by any of the authors.

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583

584 **FIGURES**


585

586 Fig. 1 Summary of metabolic networks affected in T2DM according to the presence of metabolites
587 in urine (top orange bar) and serum/plasma (lower blue bar)*. *Networks listed were obtained by an
588 Enrichment Analysis inMetaCoreTM (Genego, St. Joseph, MI) and ordered according to the major
589 metabolic pathways involved. Figure includes metabolites listed in Table 1 separated by urine and
590 serum/plasma and their direction. A figure with the full list of metabolic networks resulting from
591 MetaCoreTM is available in the supplementary material

592

593

## TABLES

**Table 1** Metabolomic studies showing metabolites significantly associated to T2DM

| Study design | Analytic technique | Biofluid | Metabolites significantly affected by the disease* | Reference |
|---|---|---|---|---|
| T2DM subjects ($n$=33)<br>Healthy subjects ($n$=20) | $^1$H NMR | Urine | (↑): Lactate, alanine, citrate, DMA, TMAO, hippurate, glycine, creatine, acetate, betaine, acetone, acetoacetate, β-hydroxybutyrate | [32] |
| T2DM subjects ($n$=11)<br>Healthy subjects ($n$=16) | $^1$H NMR | Plasma | (↑): Lactate<br>(↓): Leucine, isoleucine, valine, β-hydroxybutyrate, alanine, glutamine, citrate, tyrosine, formate | [33] |
| | | Urine | (↑): Alanine, citrate, phenylalanine, tyrosine, hippurate, phospho(enol)pyruvate<br>(↓): Glutamate, glutamine, *N*-methylnicotinamide, uridine | [33] |
| T2DM subjects ($n$=30)<br>Healthy subjects ($n$=12) | $^1$H NMR | Urine | (↑): TMA, DMA, DMG, betaine, TMAO, citrate, acetate, acetoacetate, butyrate, 2-hydroxybutyrate, β-hydroxybutyrate, alanine, glutamine, ornithine, taurine, *N*-methylnicotinamide, *N*-methyl-2-pyridone-5-carboxamide<br>(↓): Creatine, creatinine, malate, fumarate, succinate, 2-oxoglutarate, leucine, isoleucine, histidine, tryptophan, allantoin, *N*-methylnicotinate | [34] |
| T2DM subjects ($n$=28)<br>Healthy subjects ($n$=26) | GC-MS | Urine | (↑): 4-Aminobenzoic acid<br>(↓): Maleic acid dimethyl ester, oxyl acetic acid, 2,5-bisoxy-benzeneacetic acid | [35] |
| T2DM subjects ($n$=82)<br>Healthy subjects ($n$=36) | GC-MS | Serum | (↑): Butyrate, valine, glutamate, palmitate (C16:0), urate, oleate (C18:1), stearate (C18:0), arachidonate, maltose, octadecanoate<br>(↓): Lactate, lysine, glucuronolactone | [36] |
| Obese subjects with T2DM ($n$=13)<br>Healthy subjects ($n$=14) | GC-MS | Plasma | (↑): Ceramides: C18 (*N*-stearoylsphingosine), C20 (*N*-eicosanoylsphingosine), C24:1 Ceramide | [37] |
| T2DM subjects ($n$=48)<br>Healthy subjects ($n$=31) | GC×GC-TOF-MS | Plasma | (↑): Glucose, 2-hydroxyisobutyric acid | [38] |
| T2DM subjects ($n$=74)<br>NGT subjects ($n$=80) | $^1$H NMR | Serum | (↑): Glucose<br>(↓): Isoleucine, leucine, valine, alanine, methionine, glutamine, citrate, lysine, choline, lactate, tyrosine, phenylalanine, histidine | [39] |
| T2DM subjects ($n$=33)<br>Healthy subjects ($n$=25) | UPLC-oaTOF-MS | Serum | (↓): Phytosphingosine, dihydrosphingosine, leucine | [40] |
| T2DM subjects ($n$=26)<br>Healthy subjects ($n$=26) | GC-MS | Plasma | (↑): Lactate, alanine, 2-hydroxyisobutyric acid, β-hydroxybutyric acid, phosphate, leucine, isoleucine, serine, pyroglutamic acid, palmitic acid, oleic acid, stearic acid, arachidonic acid, 1-monopalmitin, 1-monostearin<br>(↓): 2-ketoisocaproic acid | [41] |
| Obese subjects with T2DM ($n$=44)<br>Obese subjects without T2DM ($n$=12) | HPLC-MS | Plasma | (↑): AcylCN: total-free, C2 (acetylcarnitine), C6 (hexanoyl carnitine), C8 (octanoyl carnitine), *cis*-3, 4-methylene-nonanoyl carnitine, C14 (myristoyl carnitine), C18:1 (oleoyl carnitine), C8-dicarb (suberoyl carnitine), summed C10-C14 AcylCN, total AcylCN<br>(↓): AcylCN: C3 (propionyl carnitine) | [42] |
| Obese subjects with T2DM ($n$=44)<br>Obese subjects without T2DM ($n$=12) | GC-TOF-MS | Plasma | (↑): β-hydroxybutyrate, oleic acid, gluconic acid, fructose, palmitoleic acid, 3,6-anhydrogalactose, glucuronic acid, glucose, heptadecanoic acid, inulobiose, leucine, 2-hydroxybutyrate, 2-deoxyerythritol, palmitic acid, 2-ketoisocaproic acid, uridine, cysteine, xylose, histidine, stearic acid<br>(↓): Benzylalcohol, benzoic acid, lysine, ethanolamine, arachidonic acid, glycine, glycerol-3-phosphate | [43] |
| T2DM subjects ($n$=10)<br>Lean subjects without T2DM ($n$=12)<br>Obese subjects without T2DM ($n$=14) | HPLC-ESI-MS/MS | Plasma | (↑): AcylCN: C3 (propionyl carnitine), C5 (isovaleryl carnitine), C8 (octanoyl carnitine), C4-OH (3-hydroxy-butyryl carnitine), C5-OH (3-hydroxy-isovaleryl carnitine), C6-OH (hydroxyhexanoyl carnitine) | [44] |
| T2DM subjects ($n$=40)<br>Healthy subjects ($n$=60) | UHPLC-MS/MS2<br>GC-MS<br>NMR | Serum and plasma | (↑): Desoxyhexose, glucose, glycolipids (HB-HNAc2-NANA, HNAC, HNAc-H2-dH), uronic acid, dihexose, mannose, creatinine, glutamylvaline, gamma-glutamylisoleucine, β-hydroxybutyrate, PAGN, phenylalanine, | [45] |

**Table 1** (continued)

| Study design | Analytic technique | Biofluid | Metabolites significantly affected by the disease* | Reference |
|---|---|---|---|---|
| | | | 3-indoxyl sulfate, kynurenine, homocitrulline, myristate, palmitate, 2-hydroxypalmitate, margarate, 10-heptadecenoate, stearate, 2-hydroxystearate, oleate, linoleate, linoleamide, linolenate, eicosenoate, dihomo-alpha-linolenate, adrenate, isoleucine, leucine, gamma-glutamylleucine, valine<br>(↓): 1,5-anhydroglucitol, caprate, heptanoate, pelargonate, glycerophosphorylcholine, PC a C20:4, PC aa (OH, COOH) C28:4, PC aa C34:4, SM C14:0, SM C22:2, 10-undecenoate, arachidonate | |
| T2DM subjects ($n$=18)<br>Healthy subjects ($n$=19) | $^1$H NMR | Serum | (↑): Fumarate, methylguanidine, pyruvate, glucose, DMA, methylamine, mannose, TMAO, uridine<br>(↓): Taurine, pyroglutamate, threonine, phenylalanine, serine, glycine | [46] |
| T2DM subjects ($n$=30)<br>Healthy subjects ($n$=30) | GC-MS | Plasma | (↑): NEFA (C16:0, C18:2, C18:1, C18:0, C20:4, C20:3, C20:2, C20:0, C22:6)<br>(↓): NEFA (C10:0) EFA (C10:0, C14:0, C16:0, C16:1n-9, C18:2, C18:1, C18:0, C20:4, C20:5, C20:3, C20:2, C22:6) | [47] |
| T2DM subjects ($n$=30)<br>Healthy subjects ($n$=30) | LC-TOF-MS | Plasma | (↑): LysoPC (C18:2)<br>(↓): PC (C18:0/20:4), PI (C18:0/20:4) | [48] |
| T2DM subjects ($n$=26)<br>Healthy subjects ($n$=27) | UPLCQ-TOF-MS | Plasma | (↑): Dodecanoic acid, myristic acid, leucine, lysine, phenylalanine, propionyl carnitine, octanoyl carnitine, decanoyl carnitine, dodecanoyl carnitine, palmityl carnitine, heptadecanoyl carnitine, linoleyl carnitine, vaccenyl carnitine, lysoPC (14:0, 16:1, 18:1, 18:3, 20:5, 22:6), lysoPE (18:2, 22:6)<br>(↓): serine, lysoPE (18:1) | [49] |
| T2DM subjects ($n$=9)<br>Lean subjects without T2DM ($n$=39)<br>Obese subjects without T2DM ($n$=64) | HPLC-ESI-MS/MS | Plasma | T2DM vs other groups:<br>(↑): 3-hydroxyoleoylcarnitine<br>(↓): AcylCN (C2, C6) leucine, isoleucine, valine, phenylalanine, methionine, alanine | [50] |
| T2DM subjects ($n$=60)<br>Healthy subjects ($n$=25) | HPLC | Plasma | (↑): Myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, γ-linolenic acid, eicosadienoic acid, arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid | [51] |
| T2DM subjects (obese $n$=31, lean=95)<br>Healthy subjects (obese $n$=80, lean $n$=20) | LC-MS/MS | Plasma | (↑): Hydroxyproline, glutamine, ethanolamine, citrulline, sarcosine, β-alanine, glutamate, 3-methyl histidine, γ-aminobutyric acid, β-aminoisobutyric acid, proline<br>(↓): Phosphoserine, phosphoric acid ethanolamine, taurine, serine, aspartate, histidine, 1-methylhistidine, arginosuccinic acid, carnosine, anserine, α-aminoadipic acid, δ-hydroxylysine, lysine, homocysteine, leucine, tryptophan | [52] |
| T2DM subjects ($n$=105)<br>Healthy subjects ($n$=77) | UPLC-ESI-QTOF-MS | Plasma | (↑): Itaconic acid, leucine, PC (18:0/0:0), sphingosine-1-phosphate, PG (18:0/18:1)<br>(↓): Inosine, uric acid, 3-hydroxymethylglutaric acid, succinic acid, taurine, PE (P-16:0/22:6) | [53] |
| | | Urine | (↑): *N*-acetyl-D-phenylalanine,serotonin<br>(↓): 2-ketoglutaric acid, 2-ketobutyric acid, 1-methylhistidine, kynurenic acid, xanthurenic acid, pyruvic acid | |
| T2DM subjects ($n$=115)<br>Healthy subjects ($n$=1897) | UPLC-MS/MS | Plasma | (↑): 2-hydroxybutyrate, proline, 3-methyl-2-oxobutyrate, 3-methyl-2-oxovalerate, 4-methyl-2-oxopentanoate, isoleucine, leucine, valine, fructose, mannose, glucose, lactate, arabinose, malate, erythrytol<br>(↓): *N*-acetylglycine, citrulline, dimethylarginine (SDMA + ADMA), 1,5-anhydroglucitol, octanoylcarnitine, 15-methylpalmitate, 10-heptadecenoate, myristate, myristoleate, palmitoleate, pentadecanoate, 5-dodecenoate, heptanoate, pelargonate, palmitoyl sphingomyelin, cholesterol | [29] |
| T2DM subjects ($n$=81) | UPLC-QTOF-HDMS | Urine | (↑): Acylcarnitines, 3-indoxylsulfate, glucose, glycine | [54] |

**Table 1** (continued)

| Study design | Analytic technique | Biofluid | Metabolites significantly affected by the disease* | Reference |
|---|---|---|---|---|
| Healthy subjects ($n$=42)<br>T2DM subjects ($n$=35)<br>Healthy subjects ($n$=35) | $^1$H NMR | Plasma | (↓): Citric acid, kynurenic acid, urate, glucuronolactone, lysine, phosphate<br>(↑): α-glucose, β-glucose<br>(↓): Isoleucine, leucine, valine, lactate, alanine, glutamate, creatine, creatinine, myo-inositol, scyllo-inositol, choline, tyrosine, phenylalanine, 1-methylhistidine | [55] |

*Increase (↑) or decrease (↓) of metabolites in the same line

$^1$ *H NMR* proton nuclear magnetic resonance, *AcylCN* acyl carnitine, *ADMA* asymmetric dimethylarginine, *DMA* dimethylamine, *DMG* dimethylglycine, *EFA* esterified fatty acids, *GC× GC-TOF-MS* two-dimensional gas chromatography-time-of-flight mass spectrometry, GC-MS gas chromatography-mass spectrometry, *GC-TOF-MS* gas chromatography-time-of-flight mass spectrometry, *HPLC* high-performance liquid chromatography, *HPLC-ESI-MS/MS* high-performance liquid chromatography-electrospray ionization with tandem mass spectrometry, *LC-MS/MS* liquid chromatography with tandem mass spectrometry, *LC-TOF-MS* liquid chromatography-time-of-flight mass spectrometry, *lysoPC* lysophosphatidylcholine, *lysoPE* lysophosphatidylethanolamine, *NEFA* non-esterified fatty acids, *NGT* normal glucose tolerance, *PC* phosphatidylcholine, *PG* phosphatidylglycerol, *PI* phosphatidylinositol, *SDMA* symmetric dimethylarginine, *SM* sphingomyelin, *T2DM* type 2 diabetes mellitus, *TMA* trimethylamine, *TMAO* trimethylamine *N*-oxide, *UHPLC/MS/MS2* ultra-high-performance liquid chromatography/tandem mass spectrometry, *UPLC/Q-TOF-MS* ultra-performance liquid chromatography-quadrupole/time-of-flight-mass spectrometry, *UPLC-ESI-QTOF-MS* ultra-performance liquid chromatography-electrospray ionization-quadrupole-time-of-flight-mass spectrometry, *UPLC-MS/MS* ultra-performance liquid chromatography with tandem mass spectrometry, *UPLC-oaTOF-MS* ultra-performance liquid chromatography coupled with orthogonal acceleration time-of-flight mass spectrometry, *UPLC-QTOF-HDMS* ultra-performance liquid chromatography-quadrupole time-of-flight high-definition mass spectrometry