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REVIEW ARTICLE

Metabolomics in diabetes, a review

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ABSTRACT

Metabolomics is a promising approach for the identification of chemical compounds that serve for early detection, diagnosis, prediction of therapeutic response and prognosis of disease. Moreover, metabolomics has shown to increase the diagnostic threshold and prediction of type 2 diabetes. Evidence suggests that branched-chain amino acids, acylcarnitines and aromatic amino acids may play an early role on insulin resistance, exposing defects on amino acid metabolism, β-oxidation, and tricarboxylic acid cycle. This review aims to provide a panoramic view of the metabolic shifts that antecede or follow type 2 diabetes.

KEY MESSAGES

- BCAAs, AAAs and acylcarnitines are strongly associated with early insulin resistance.
- Diabetes risk prediction has been improved when adding metabolomic markers of dysglycemia to standard clinical and biochemical factors.

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Introduction

Metabolomics is a promising approach to understand and explore the body cell homeostasis; it is the metabolic complement of functional genomics and has proven to be very useful in medical and life sciences research (1). Metabolomics allows the profiling of numerous small endogenous molecules (<1500 Da) in cells, body fluids and tissues, most of these molecules were previously known. The whole ensemble of low weight molecules that serve for the total compound of reactions for maintenance, growth, and normal function, is known as metabolome (2). Metabolites are markers of biochemical, physiological, or pathological reactions and are able to show the interaction among different pathways that develop within a living cell (3,4). Metabolic pathways in the cell can be described qualitatively and quantitatively and show the endpoints of gene expression and cellular environmental changes (5), providing an understanding of the physiology of the cell and by this the general status of the living organism.

One of the goals of metabolomics besides the understanding of physiologic pathways is to develop diagnostic biomarkers that could serve as tools for clinical practice, diagnosis, prognosis, and predictors of therapeutic response (6). The human metabolome database contains >40,000 metabolite entries (7). According to the Human Metabolome Database browser filters, ~1900 endogenous compounds have been detected and quantified in blood while ~1200 have been detected and quantified in urine; however, only 43 metabolites associated with type 2 diabetes (T2D) have been detected and quantified in blood and urine, this data is available at the human metabolome database webpage (7). The metabolic profile is dynamic, it varies continuously in response to changes in gene expression or changes induced by exogenous metabolites such as those provided by food or drugs (8).

The metabolic phenotype is ruled by the central dogma of cellular biology, DNA provides mRNA that serves as material for the translation and expression for protein synthesis, thus metabolic phenotype is determined by concentrations of biological products inside and outside the cell, tissue, or fluid. Metabolomics has an important role in this organic interplay; however, metabolites may imply a negative feedback on DNA, contrasting the directionality of the central dogma (9). The objective of this review is to provide a panoramic view of the findings that have been made in the

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metabolomics field in association with T2D, showing consistency and differences in results between studies.

Metabolomics: the role in diabetes and other metabolic conditions

Several areas of opportunities are open to apply metabolomics in clinical practice. For example, new diagnostic and predictive markers are required. In addition, emphasis has been placed in a patient-centered approach for treatment of T2D, creating the need for having indicators for future response for individual therapies (10).

Dysglycemic states and diabetes

Metabolomics has completed many aspects about the pathologic pathways generated in diabetes; Carnitines, branched chain amino acids (BCAAs), aromatic amino acids (AAAs), and free fatty acids (FFAs) could be potential markers associated with dysglycemic states, but although there are many answers about their function in relation to diabetes, its precise role is not well defined yet. Observations on β-oxidation dysregulation have given new milestones for the understanding of the dysglycemic metabolic phenotype (11).

It is well known that high concentrations of insulin or its inability to regulate intracellular responses cause a metabolic shift of energy obtainment through β-oxidation. Zhao et al. (11) show that palmitate (C16:0) concentrations were associated with insulin resistance when normal glucose tolerance-insulin sensitive subjects, normal glucose tolerance-insulin resistant subjects and two dysglycemic states, impaired fasting glucose (IFG), and impaired glucose tolerance (IGT) were evaluated. Palmitate is an indicator of FFA availability; the latter can be measured by the palmitate rate of appearance (Ra). Palmitate Ra has been negatively correlated with skeletal muscle insulin sensitivity, in basal and insulin infusion conditions in non-diabetic women (12). There is no certainty that these metabolic modifications precede or follow insulin resistance, however, a prior study that evaluated subjects either with IGT or diabetes compared to obese non-diabetic subjects, Ra of FFAs was found to be decreased on IGT and T2D compared to obese subjects, supporting the idea that hyperinsulinemia causes a decrease in FFA Ra and suggesting that intramuscular triglyceride deposits, present on IGT and T2D, could affect the transportation of FFAs into the cytosol and consequently decrease FFA oxidation rate, demonstrating that the same defects in FFA metabolism and adiposity signals exist between subjects with IGT and T2D (13,14).

Adiposity signals also influence the expression of enzymes related to BCAA catabolism, particularly the Branched-chain alpha-ketoacid dehydrogenase complex (BCKD). Analysis of omental adipose tissue from obese women with or without metabolic syndrome that underwent bariatric surgery showed that obese women without metabolic syndrome had higher concentrations of enzymes associated with BCAA catabolism when compared to obese women with metabolic syndrome, supporting the fact that in metabolic syndrome or associated conditions, BCKD complex is impaired by other mechanisms different from obesity (15). Alpha-hydroxybutyrate (α-HB), a byproduct of α-ketobutyrate (α-KB) synthesis, identifies insulin resistant and normal glucose-insulin sensitive subjects, furthermore, it may be a good diagnostic tool when used conjointly with other biomarkers to identify IR and IGT earlier than clinical tests available to date (16,17). Palmitate, glycine, and long chain acylcarnitines as decanoylcarnitine also identify insulin resistance however its usefulness has been seen in later stages in comparison to α-HB (16).

α-HB and Linoleoyl-glycerophosphocholine (L-GPC) have opposite effects in β-cell function, α-HB decreases glucose-mediated insulin secretion and is a positive independent predictor of insulin sensitivity, L-GPC on the other way, increases glucose-mediated insulin secretion and is a negative predictor of insulin sensibility; this was observed during follow-up of subjects that progressed to dysglycemia and T2D which showed higher concentrations of α-HB and lower concentrations of L-GPC whereas an inverse behavior was observed in subjects that remained euglucemic during the following of the RISC and BOTNIA cohorts (18). Elevated concentrations of BCAA, gluconeogenic amino acids and decreased glycine concentrations appear to predict progression to dysglycemia. Studies points show that increased fasting concentrations of α-HB and decreased L-GPC are indicators for glucose intolerance and have a similar power regarding traditional tests like the 2-h glucose challenge (18).

In a recent study, low serum glycine concentrations have shown a strong relationship with red meat intake and T2D risk on a subset of the EPIC-Potsdam cohort when followed for a mean of 7 years. The stated mechanism is due activation of glycine-dependent pathways in response to red meat ingestion, thereby glycine is related to glutathione synthesis in response to oxidative stress an insulin resistance. Furthermore, Heme production in response to iron availability is a pathway that consumes glycine (19). However, red meat role on diabetes should be taken cautiously and studied more thoroughly although there's evidence that supports this

hypothesis; insulin-resistance leads to expression of ALAS-H enzyme which catalyzes conversion of glycine to succinyl-CoA and 5-aminolevulinic acid leading to Heme synthesis (20). In addition to glycine, biomarkers that are consistent with other recent studies were associated with diabetes, diacyl-phosphatidylcholine, acyl-alkyl-phosphatidylcholines, lysophosphatidylcholines, and sphingomyelins, which will be discussed further in this review (19,21,22). Other glycine intermediary metabolite as β-Hydroxypyruvate has been recently observed as diabetes predictor, β-Hydroxypyruvate alters the excitatory properties of myenteric neurons and reduce islet insulin content on mice, furthermore, β-Hydroxypyruvate-to-D-Serine ratios have been found decreased on subjects with impaired tolerance glucose compared to subjects with normal glucose tolerance and T2D, exposing the neural relay that amplifies insulin secretion in response to an altered glucose homeostasis (23).

Batch et al. (24) assessed metabolic wellness in the WLM, CATHGEN, and STEDMAN cohorts; patients were classified as MW (metabolically well) and MUW (metabolically unwell) (two or more of: impaired fasting glucose, hypertension, hypertriglyceridemia, low HDL-C, and insulin resistance based on HOMA). The metabolic profile could discriminate metabolic wellness independently of BMI. According to BMI stratification, BCAA (Val, Leu, Ile) Phe, Tyr, Met, Ala, and His showed differences between groups. Importantly, BCAA also separated MW from MUW patients independently of BMI, along with acylcarnitines C3, C4, C5, and C5:1, non-esterified fatty acids, glutamine and ornithine. Consistent results with the association of BCAAs and high risk for insulin resistance have been reported; adding dietary BCAA within a background of obesity can affect mitochondrial function, which also generates accumulation of incompletely oxidized metabolites derived from lipids (25). Increased levels of BCAA on insulin-resistant states indicate a reduced BCAA catabolism however, in healthy subjects BCAA intake may increase insulin sensitivity (17,26). BCAA relate and predict insulin resistance but there's no certainty that they affect insulin functioning mechanisms (27).

Control on dietary BCAAs could be used as a treatment modality on insulin-resistance prone patients. High dietary intake of BCAAs has been associated with a decrease in diabetes risk in women and men from the Takayama Study cohort, leucine was the strongest factor that reduced diabetes risk, since it has an important role in stimulating insulin release (28). In subjects with poor metabolic control (HbA1c $>$ 7%) and normal weight, HOMA-IR, basal parameters of glucose and postprandial parameters of glucose and insulin decreased importantly with supplementation of dietary BCAAs; in the long term glucose control improved with a decrease in HbA1c. BCAAs can improve protein anabolism and muscle synthesis resulting in greater glucose uptake by insulin-sensitive tissues (29). Furthermore, BCAA profiles can identify subjects that would benefit better from weight loss and improve their insulin-sensibility (30). Serum BCAAs are not entirely dependent on dietary intake, increased serum BCAA on obese subjects is due to a decrease in the quantity and activity of the BCKD complex, which lowers BCAA catabolism and clearance (28).

Assessment of insulin resistance with HOMA-IR in the Finnish cohorts (31) showed that sex-dependent hormones affect the metabolomic signature between men and women. When women were obese BCAAs and Tyr were associated with insulin resistance whereas in obese men, Tyr, Ala, and ketone bodies showed a more important association. Gln and β-Hydroxybutirate were inversely related with insulin resistance. In addition, genetic variants were assessed. Only the already known GCKR protein gene showed association with insulin resistance when the rs1260326 SNP was present. Ile, Ala, α1-acid glycoprotein, total fatty acids, and n-9 saturated fatty acids showed the strongest association with this gene in relation to insulin resistance. Previous studies have reported that large neutral amino acids show important differences between obese men and women when it comes to insulin resistance (32,33). High concentrations of neutral amino acids induce protein synthesis and inhibition of proteolysis and also decrease glucose uptake and glycogen synthesis, causing a disturbance in glucose uptake. These processes are bound by the m-TOR signaling pathway for protein synthesis and insulin-mediated glucose uptake (33).

Novel observations were noticed on saliva (34) when compared conjointly with serum and urine of T2D patients and healthy controls. 1,5-Anhydroglucitol (1,5-AG) was found decreased in saliva of T2D patients and positively correlated well with blood levels of 1,5-AG. Urine levels of 1,5-AG in patients with T2D were increased. A negative correlation was noted between 1,5-AG and both blood glucose and HbA1c. When the renal threshold for glucose gets exceeded, the 1,5-AG reabsorption gets impaired, explaining the observation that low 1,5-AG in saliva or blood correlated with glucosuria in patients with T2D. 1,5-AG is indicative of glucose control 5–7 days prior to the test, putting forth the possibility that it can be a useful tool for short-term glucose control monitoring and diagnosis of T2D. A summary of the metabolites that relate to dysglycemia is presented on Table 1.

Table 1. Differential metabolites in dysglycemia/T2D.

	Strategy	Approach	Observations	Study
Dysglycemic states	Analysis of serum and urine of subjects with impaired glucose tolerance versus controls	UPLC-QTOF MS	Blood: Increased FFAs, C16:0 and C18:0. Decreased lisophosphatidylcolines. Urine: Increased tryptophan, Xantine. Decreased hippuric acid	Zhao et al. (11)
	Normal glucose tolerant insulin-sensitive subjects versus normal glucose tolerant insulin-resistant and dysglycemia	UHPLC MS/MS	Blood: Increased C16:0, glycine, long chain acylcarnitines, α-HB	Gall et al. (16)
	Follow of RISC and BOTNIA cohorts to T2D or dysglycemia progression	UHPLC MS/MS and LC-ESI MS/MS	Blood: Increased α-HB, BCAA and decreased L-GPC	Ferranini et al. (18)
	Metabolically well versus metabolically unwell subjects of the WLM, CATHGEN, and STEDMAN cohorts	MS/MS	Blood: Increased BCAA stratified by BMI. Increased acylcarnitines independently of BMI.	Batch et al. (24)
	Insulin resistance assessment on obese versus lean subjects	NMR-S	Blood: Obese woman with increased BCAAs, Val, Tyr. Obese Man with increased Tyr, Ala, and ketones. Both: Decreased β-HB and Gln	Würtz et al. (31)
	Saliva versus urine and serum of T2D versus controls	UHPLC-GC MS/MS	Saliva: Decreased 1,5 AG that correlated with blood levels. Urine: Increased 1,5 AG.	Mook-Kanamori et al. (34)
	Serum after 75 g OGTT to classify as NGT, IGT, and T2D.	HPLC, EI-MS/MS	β-Hydroxypyruvate-to-D-serine ratio on IGT	Sheng Zhang et al. (23)
Metabolic challenges	Subjects undergoing 2-h OGTT versus water	LC-MS/MS	Blood: Post 2-h challenge, decreased BCAA, and glycerol	Shaham et al. (35)
	2-h 75 g OGTT of 377 non-diabetic subjects	LC-MS/MS	Blood: Decreased β-HB, TCA cycle intermediates, serotonin, and vitamin B compounds after the challenge	Ho et al. (38)
	4-day study challenge in healthy subjects	NMR-S, LC-MS, FIA-MS	Blood: Increased acylcarnitines in catabolic states.	Krug et al. (42)
	High calorie intake on weight discordant monozygotic twins.	GC/GC-TOFMS, UPLC-QTOFMS, UPLC-TQMS	BCAAs, FA, Hydroxybenzoic acids	Bondia-Pons et al. (60)
Diabetes risk prediction	EPIC-Postdam cohot prospective evaluation for T2D	FIA MS/MS	Blood: Increased Diacyl-Alkyl-phosphatidylcholine, BCAAs, AAA, Propionylcarnitine, glycine	Floegel et al. (21)
	FHS-Offspring study and MDC cardiovascular cohort assessment for T2D	LC-MS/MS	Blood: Increased 2-AAA	Wang et al. (61)
	Twins UK cohort controls versus T2D and IFG	MS	Blood: Increased long chain lipids, BCAAs, methyl-2-oxovalerate, 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutyrate.	Menni et al. (64)
Obesity	Metabolomic profile of obese versus lean patients	GC-MS	Blood: Increased BCAAs, Methionine, Ala, Glutamate/Glutamine, AAA, C3 & C5	Newgard et al. (25)

LC: Liquid chromatography; MS: Mass spectrometry; UHPLC: Ultra high performance liquid chromatography; MS/MS: Tandem mass spectrometry; ESI: Electrospray ionization; NMS: Nuclear magnetic spectroscopy; FIA: Flow injection analysis; L-GPC: L-glycerolphosphocoline; TCA: Tricarboxylic acid; β-HB: β-Hydroxybutyrate; 2-AAA: 2-aminoadipic acid; 1,5-AG: 1,5-Anhydroglucitol; FFAs: Free fatty acids.

Application of metabolomics in challenge studies

Metabolomics with regard to other ''omics'' reflects a ''snapshot'' of what is happening at a metabolic level. When a controlled physiologic challenge is applied, the metabolic network is exposed to extreme conditions in which it must act quickly to maintain homeostasis, revealing metabolomic fingerprints that otherwise may stay unnoticed at baseline.

Glycerol, leucine, and isoleucine are strong predictors of fasting insulin levels; this was observed in 22 healthy young adults that underwent a 2-h oral glucose tolerance test (OGTT) and compared with 25 subjects ingesting plain water (35). BCAAs such as leucine/isoleucine and glycerol were significantly reduced after the test according to the basal levels, explaining that the effect that insulin exerts in proteolysis and lipolysis has different power; during the 2-hour challenge there is a more rapid and important decrease in glycerol than in amino acid concentrations showing that insulin's inhibitory action is stronger on ketogenesis than proteolysis. When IR and hyperinsulinemia are present, SREBP-1c transcription factor (responsible for triglyceride and fatty acid biosynthesis) is overstimulated by insulin, FFA synthesis is accelerated and serum FFA increases in an uncontrolled fashion, explaining the classic hypertriglyceridemia of diabetes, and the increase in glycerol in obese insulin-resistant subjects (36,37). In healthy subjects, the insulin excursion and the decrease on glycerol shows the well-regulated insulin function on inhibition of fat breakdown.

Biochemical shifts of an OGTT of non-diabetic subjects were assessed in the Framingham Offspring cohort. The insulin excursion during the curve shifts metabolism from a catabolic state to an anabolic state after the 2 h; β-HB, isoleucine, lactate, orotate, and pyridoxate changes related with insulin excursion during the challenge. Blunting of the course was noticed on insulin-resistant subjects when compared to insulin-sensitive subjects. Serotonin and Vitamin B derivatives showed a differential excursion on insulin resistant subjects (38). Vitamin B1, is an essential cofactor of pyruvate dehydrogenase, B5 is associated with the TCA cycle and B6 with amino acid metabolism. Serotonin is an important neurotransmitter that plays an important role on glucose homeostasis. It has been observed that deletion of the serotonin receptor gene 5-HT_2 in rats produced insulin resistance and T2D (39). Cysteine metabolism is dependent on vitamin B-6, cysteine is important for the synthesis of glutathione, an important antioxidant that counters the oxidative stress of diabetes (40). Glutathione deficiency has been linked with impaired NEFA oxidation and insulin resistance in old people, these same conditions have been reversed by glutathione restoration in plasma (41).

Six metabolic challenges were performed by Krug et al. (42) in 15 healthy Caucasian subjects volunteered to a 4-day study. NMTR, LC-MS, FIA-MS, and NMR spectroscopy were used for evaluation of sera and breath. Subjects went through a 36-h fasting, liquid standard diet (LSD), OGTT, oral lipid tolerance test (OLTT), a physical activity test (PAT), and a cold pressure stress test. As expected, during 36-h fasting NEFAs and BCAAs showed high concentrations in plasma. Propionylcarnitine (C3), proline, and C0 were positively correlated with vareylcarnitine but negatively with C2. During the 36-h fasting, C2 concentrations increased whereas C0 decreased. Insulin and glucose were used as parameters of anabolism, values showed high amplitude between subjects in postprandial states such as OGTT, SLD, or OLTT. We detailed this study because we consider important the demonstration of the malleability of the metabolic response in its two primary modes, anabolism (OGTT, OLTT, and LSD) and catabolism (fasting and exercise). It is known that during fasting, lipolysis, and β-oxidation satisfy the need of energy, CoA–esther by-products enter the mitochondria via the palmitoyl-CoA carnitine transferase II shuttle, this transporter requires C0 for transport into de mitochondria, this explains the decrease in C0 during fasting; all acylcarnitines except, C3, C4, and C5 showed an increase when β-oxidation was enhanced; C0 and C2 show an opposite effect in anabolic states where insulin is in high concentrations. BCAA-derived acylcarnitines C3, and C5 have been reported to be increased on obese subjects (25), C4 which is also derived from BCAA metabolism has shown a positive correlation with basal glucose and HbA1c (43). Acylcarnitines in general reflect FA, amino acid oxidation rate and mitochondrial shuttle capability, during catabolic states like fasting, acylcarnitines increase in plasma indicating a greater lipid oxidation rate (44). Some of the studies mentioned in this section can be found on Table 1.

Diabetes risk prediction

Diabetes risk scores have a variable predictive ability depending on the study population in which they are developed. Traditional risk scores are based on clinical features as age, familiar background, BMI, waist circumference, and arterial blood pressure (45). Biochemical features and chemical biomarkers such as fasting plasma glucose, HDL, triglycerides, and liver enzymes can increase the performance of risk scores. There are various models for risk assessment, whether using clinical, biochemical or genetic features, or a

combination of these. The Finnish Diabetes Risk Score for clinical features developed on the FINRISK studies had an aROC curve of 0.85 and 0.87 in the 1987 and 1992 cohorts, respectively (46). The Cooperative Health Research in the Region of Augsburg (KORA) survey 2000, assessed four screening questionnaires for undiagnosed diabetes, aROC curves showed low validity when the former questionnaires were applied to different populations; 61% for the Rotterdam Diabetes Study, 65% for the Finnish Diabetes Risk Score, 67% for the Cambridge Risk Score, and 90% for the San Antonio Heart Study (47). The German Diabetes Risk Score applied in the European Prospective Investigation into cancer and Nutrition (EPIC)-Postdam Study achieved an aROC curve of 0.84, however, this was designed and applied to Caucasian population (48). When biochemical features such as HDL, triglycerides and fasting glucose were added to clinical features, the aROC curve increased, as observed on the Atherosclerosis Risk in Communities (ARIC) study (49) in which the aROC curve increased from 0.71 to 0.80; similarly in the Framingham Offspring Study and the German Diabetes Risk Score (DRS) adding biochemical models (HDL, triglycerides, HbA1c, fasting glucose) improved the aROC curves from 0.72 to 0.85 and 0.84 to 0.90, respectively. Interestingly, the DRS used gamma-glutamyltransferase and alanine aminotransferase to increase the aROC curve (50,51). Subjects from the Botnia Study (52) where assessed with a 2-h OGTT, 1-h glucose concentration showed to be the best predictor for future diabetes, the aROC curve increased from 0.67 (fasting plasma glucose) to 0.79 (1-h glucose concentration) (53).

Although many risk models based on genetic factors have shown poor prediction over clinical factors, some have shown increased ability to predict diabetes (54). Various SNPs have been identified on GWAS to be associated with T2D, such as TCF7L2, KCNJ11, PPARG, CDKAL1, IGF2BP2, CDKN2A/2B, FTO, and HHEX (55). In a Japanese study, prediction improved with the construction of a risk model that included SNPs different from those reported on GWAS studies, they achieved an aROC curve of 0.80, regardless that they had little effect of association with a phenotype (54). A predictive test applied on the Framingham Heart Study dataset showed an improved aROC curve of 0.606 when comparing 18 loci to a former 3 loci test (AUC 0.596), furthermore, this risk score had better prediction of diabetes on individuals under 50 years than those who were over this age (56). Risk assessment on the Inter99 and ADDITION studies, allowed to observe that genetic variants used conjointly with conventional factors (BMI, age, and sex) do not increase significantly the AUC over traditional factors alone (0.92–0.93) (57–59). To evaluate the metabolic phenotype on obesity independently of the genetic load, a metabolic challenge with a high-calorie meal was given to 16 weight-discrepant monozygotic twins (>3 kg/m^2 of BMI) from two of the Finnish Twin cohorts (FT16 and FT12). BCAAs, fatty acids (oleic acid principally) and 3-hydroxybenzoic acid were higher at baseline levels on obese subjects, the interesting fact is that after the challenge the values converged on heavy and lean twins, suggesting underlying mechanisms that allow a strict homeostatic control after a high-caloric load on obesity independently of the genetic load (60).

A prospective evaluation for T2D risk prediction in the EPIC-Postdam cohort associated L-GPC with metabolic imbalance. Acyl-alkyl-phosphatidylcholines, sphingomyelins, and lysophosphatidylcholines showed a negative association with T2D. On the other hand, diacyl-alkyl phosphatidylcholines BCAA, AAA, C3, and glycine showed a positive association with risk for T2D development (19,22), Follow-up for a mean of 7 years, consistently correlated L-GPC with insulin resistance. Comparison of these metabolites with other risk scores substantially increased the AUC for diabetes prediction; AUC for DRS alone was 0.84, metabolites alone 0.84, and for the DRS + fasting glucose + HbA1c + Metabolites the aROC curve increased to 0.912 (21). 2-Aminoadipic acid (2-AAA), a lysine degradation product, was found to be a strong predictor of diabetes in normoglycemic subjects of the FHS offspring study, and the MDC cardiovascular cohort. 2-AAA showed strong association with insulin resistance and β-cell function. Adjusting 2-AAA to diet, BCAA and AAA did not change results; showing that 2-AAA is an independent predictor for T2D development. Administration of 2-AAA to experimental-mice eating a standard diet versus mice eating high fat diet showed lower glucose concentrations when 2-AAA was added as supplementation, showing that 2-AAA increases insulin secretion (61) (Table 1), however, 2-AAA has not yet been tested in risk scores although it could be useful as it does not directly correlate with other metabolomic biomarkers of insulin resistance like BCAAs or AAA which means its metabolic interplay may be somehow different.

Women from the TwinsUK cohort (62) were metabolically assessed; observations showed elevated concentrations of long chain lipids on women with IFG compared to controls and short chain lipids were decreased in T2D compared to controls. BCAAs and their metabolites, 3-methyl-2-oxovalerate, 4-methyl-2-oxopentanoate, and 3-methyl-2-oxobutyrate were increased on IFG and T2D. 3-methyl-2-oxovalerate, a product of isoleucine, showed the strongest association with IFG and T2D. The association was able to be replicated on an independent cohort from the KORA F4

study (63) and on urine of different women from the Twins UK (64) (Table 1). The findings reflect impairment of mitochondrial function for BCAA catabolism, the high amounts of by-products of BCAA may affect mitochondrial oxidation of glucose and lipids resulting in stress and accumulation of BCAAs and intermediates (Table 1). While Wang et al. (3) suggest causality between increased BCAA and diabetes; other authors have observed an opposite BCAA behavior (65). A likely explanation may be in the differences between study subjects, since obtainment of low BCAA concentrations was observed in patients that had established diabetes and treatment, suggesting that physiologic responses may not be representative of early metabolic changes. Even so, follow-up of patients with increased BCAA showed no added differences over baseline results, keeping its prediction value the same from base-line examination through 12-year follow up (65).

Protective tools have been also evaluated; ingestion of fruit and vegetables (FV) was assessed with a three biomarker score on a sample from the EPIC-Norfolk study; vitamin C, carotenoids (beta- and alpha-carotene), and lutein correlated negatively with T2D incidence. Vitamin C and carotenoids are responsive to changes on total ingestion of FV, it was found that BMI and waist circumference adjustment attenuated results, meaning that a healthier diet which contains mayor quantities of FV associates with body weight, an important modifiable risk factor for T2D development (66).

Obesity

Sera and urine of obese subjects revealed a metabolic signature of increased BCAA (Leucine/isoleucine, valine), methionine, alanine, glutamate/glutamine, AAA (phenylalanine and tyrosine), and C3, C5 acylcarnitines. Moreover low levels of α-ketoglutarate and glycine compounds were observed in urine. An important relationship was observed regarding insulin resistance; elevation of BCAAs modifies the function of the large neutral amino acid transporter 1 receptor (LAT1), which is needed for the entrance of BCAAs and AAA into the cell (25) (Table 1). Evidence indicates that short chain fatty acids (SCFAs) and BCAAs play an important role between obesity and diabetes since obese people are specially prone to develop diabetes when they have increased concentrations of BCAA (67). It is speculated that increased BCAAs concentrations stimulate the production of SCFAs that act as modulators of fatty acid metabolism by the stimulation of leptin production, in this way SCFAs will exert inhibitory effects on lipolysis of adipocytes and contribute to obesity. Furthermore, BCAAs boost the conversion of pyruvate to alanine, a highly glycogenic amino acid that has been found clearly elevated in obese patients (68). There is not much difference between the metabotypes of obese patients and dysglycemic/diabetic states, suggesting the metabolic mesh of these states is part of a closely related entity, in which they share abnormal BCAA concentrations. The metabolic mesh that links obesity and insulin resistance was assessed by Gaussian graphical modeling on a subcohort of the EPIC-Potsdam study (22), different clusters segregated acylcarnitines and phospholipids (Sphingomyelins, lysophosphatidylcholines, diacyl-phosphatidylcholines and acyl–alkyl-phosphatidylcholines) in one group, and in other groups amino acids and C6-Sugars as expected. Findings showed that acylcarnitines were positively correlated with obesity, diacyl-phosphatidylcholines with higher triglyceride levels and a greater risk for T2D and obesity, whereas acyl–alkyl-phosphatidylcholines correlated negatively with obesity (22). These findings were consistent with a previous study performed on a cohort of the EPIC-Potsdam study referred above (21). Interpreting the metabolome by clustering different metabolites and using statistical tools to assess their connection and role in disease may be useful to understand and visualize the metabolite network and build prediction tools with differential metabolites that characterize disease.

Short chain fatty acids (SCFA) with straight chain have been related to altered gut microbiota metabolism, gut microbiota is the main responsible for the production of these compounds which are derived from the fermentation of carbohydrates and BCAA catabolism (68,69). Proteins that reach the colon are metabolized by the gut microbiota and generate by-products such as α-HB, other products such as hippuric acid and 3-hydroxyhippuric acid are produced by the degradation of AAA, these metabolites can be measured in urine and have been related to the phenotypic changes of gut microbiota that characterize IGT and obesity (11,70,71). Amino acids support bacterial growth and regulate energetic homeostasis; gut bacteria contain mayor quantities of BCAA than other amino acids. Lysine, arginine, glycine, and BCAAs generate a mixture of metabolic end-products that contribute to the metabolic profile of dysglycemia, among them ammonia, SCFA (acetate, propionate, butyrate), and branched-chain fatty acids (valerate, isobutyrate, and isovalerate), these metabolites appear to modulate the bacterial physiology by signaling pathways that influence the gut epithelial cell integrity and immune system performance (72–74). Faecal samples of obese donors, as expected, show higher concentrations of SCFA, however this may reflect a decrease in the absorption of SCFA or an increase of

SCFA production by gut microbiota, a higher conversion of AA to SCFA may contribute to this finding, reflecting higher amino acid catabolism by gut microbiota. *Clostridium* are the most important bacteria related to amino acid generation and have been previously reported on T2D (75), high-fat diet (HFD)-fed mice with high representations of *Clostridium ramosum* led to mayor weight increase when compared to HFD fed mice without this bacteria (76).

About 90% of the ''lean'' gut microbiota is represented by *Firmicutes* (Gram-positive), *Bacteroidetes* (Gram-Negative), *Actinobacteria* (gram-positive), and *Proteobacteria* phyla (77,78). Obesity and T2D cause an increase of the *Firmicutes* and decrease of the *Bacteroidetes* representation (79–81). The gut microbiota participates in the metabolic interplay that links bile acid metabolism with glucose and lipid homeostasis (82,83). Primary biliary acids (BA) are converted to secondary BA mainly by *Firmicutes*, The increase of the *Firmicutes* representation on T2D and overweight patients has been related with higher conversion of secondary BA and a decrease of primary BA in serum (84). Interesting is that secondary BA inhibit the expression of gluconeogenic genes through farnesoid X receptor FXR and enhance insulin secretion and sensitivity in the pancreas (85), and that primary BA promote the secretion of GLP1 on intestinal cells by TGR-5 activation and has a protective effect on diet-induced obesity (86,87). Although this could be a response mechanism to maintain homeostasis on obesity and insulin resistance, this contrasting data needs to be studied further.

Among the *Firmicutes phyla*, an important genus called *Blautia* is the most representative; *Blautia coccoides* can activate TNFα and cytokine secretion greater than lipopolisacharide (LPS) (88), triggering low grade inflammation by means of ''metabolic endotoxemia'' through activation of CD14/toll-like receptor 4 (TLR-4) dependent mechanisms (89–91). The increase of endotoxemia and high-fat diet alters intestinal permeability and reduces the expression of tight junction proteins; chylomicrons carry LPS and deliver it to blood, contributing to this state of metabolic endotoxemia (92,93). Supporting this fact, it has been observed that continuous subcutaneous infusion of LPS for 4 weeks on murine models results in higher fasting glycemia, hyperinsulinemia, weight gain, and kytokine expression in a similar fashion as mice fed with high-fat diet (94).

While Firmicutes phyla increases on obesity and insulin resistance conditions, butyrate-producing bacteria (Roseburia and Faecalibacterium Prausnitzii most importantly) show an inverse behavior, showing lesser representations when compared to healthy subjects (95). Butyrate is the most important energy source of colonic bacteria, butyrate, and its derivatives promote GLP-1 and PYY secretion by L-cells, decrease of butyrate-producing bacteria on obese subjects can explain some of the deterioration on GLP-1 related functions (96,97). Gut microbiota transplant from obese mice to germ-free mice leads to a significant increase of body fat and insulin resistance on the later compared with lean mice, supporting the fact that obesity may be a consequence of an altered gut microbiota and not otherwise (98). On the other hand, transplant of ''healthy'' gut microbiota to obese subjects appears to be associated with metabolic improvement and better insulin sensitivity (99). Diet may be the most important factor that influences the representation of gut microbiota. A decrease of *Bifidobacterium* abundance has been related with high-calorie diet, high cholesterol, and ethanol consumption, moreover administration of *Bifidobacterium* or probiotics improves glucose metabolism and decreases inflammation (88), whereas *Akkermansia Muciniphila* administration correlates negatively with weight (100) and its abundance decreases in obesity and T2D (101).

Branched chain amino acids (BCAA) and AAA (Phe, Tyr) are increased on insulin-resistant obese individuals (102) and in non-diabetic individuals that progress to T2D (30), moreover they are useful to predict T2D development. Threonine, tryptophan, lysine, and histidine have been also related with insulin-resistance associated to obesity and T2D, however, they do not predict development of disease (3). Obese individuals with hyperinsulinemia tend to have greater BCAA and AAAs concentrations. Gastric Bypass has shown to decrease plasma insulin, improve HOMA-IR, and T2D control; together with this, BCAA/AAAs levels decrease and relate with insulin sensitivity (67). Methionine and catabolic derivatives (cysteine and cystine) increase in obesity, insulin resistance, and T2D; increases in the concentration of cystine directly correlate with BMI and body fat in the Hordaland Homocysteine Study (103,104). The amino acid pattern on blood of obese subjects could be due to a limited catabolism of BCAA by an impaired function of BCKD complex on adipose tissue, liver, and muscle (40). To assess if this pattern of BCAA is influenced by obesity or insulin resistance alone, PPARγ agonists were tested on white adipose tissue (WAT) of C57BL/6K healthy mice and obese db/db mice, obese mice had reduced BCKD complex proteins on retroperitoneal WAT, suggesting an alteration of insulin signaling pathways that impairs BCAA utilization on WAT (15). PPAR-gamma treatment in humans increases mRNA expression of BCKD complex on WAT and increases insulin sensibility possibly by an improvement on insulin action or glucose usage (105), it is possible that these effects on insulin action may also affect BCAA

metabolism. Omental adipose tissue may be a major responsible for the BCAA phenotype on obesity, it has been observed that BCAA catabolic enzymes are lowered on omental WAT but not subcutaneous WAT of obese women with metabolic syndrome, this suggest that subcutaneous adipose tissue may not play an important role on the BCAA signature and that the metabolic syndrome may affect BCAA enzyme expression (15). Altogether, the metabolic phenotype that consistently repeats, shows that obesity, metabolic syndrome, and type 2 diabetes share to a greater extent, perturbations on BCAA, acylcarnitines (medium and long) and lipids, directing to common behaviors, lifestyles, and risk factors that trigger disease development (106).

The large neutral amino acid transporter (LAT1) introduces large neutral amino acids into the cell, high concentrations of BCAA compete for this transporter and impair AAA uptake, explaining the increase on plasma AAAs (107), this uncoupling of AAA entrance into the cell affects neurotransmitter production since serotonin is derived from tryptophan and catecholamines are derived from phenylalanine and tyrosine. Neurotransmitter perturbations are strongly associated with depression and obesity (108).

Saturation of fatty acid oxidation (FAO) in obesity leads to an accumulation of acylcarnitines and depletion of TCA cycle intermediaries (malate, succinate, and citrate), the high FAO flux does not match with TCA cycle flux and results in an incomplete FAO (109). Alterations on CPT1 may decrease FAO on obese people, since obese subjects have decreased CPT1, the lipid-rich intracellular ambient affects insulin signaling and FAO rate, this mitochondrial overload leads to an incomplete β-oxidation which overpasses the TCA cycle capacity to oxidize products from FAO and causes accumulation of intermediary metabolism products such as acylcarnitines (33,110).

Discussion and perspectives

Evidence shows that the most important changes have been observed on intermediary metabolism (Table 1). Increased acylcarnitines suggest an overload of β-oxidation that cannot be matched by the TCA cycle (109). BCAAs and AAA have been found to be strongly related with early insulin resistance and T2D prediction independently from BMI (24). It is well-known that when insulin resistance is present some associated metabolites show sex-related associations, being women more prone to increase BCAAs and men prone to increase Tyr, Ala, and ketone bodies (31). There is not yet a causality established between BCAAs and insulin resistance, even though evidence points that the increase of BCAAs is the secondary event, this could be due to perturbations on the BCKD complex observed on hyperinsulinemia and obesity (15,28,40). Another mechanism that may affect BCAA metabolism is the relative IGF-1 deficiency on obese subjects that makes the circulating BCAA pool to be directed towards catabolic pathways, this can explain the decrease of α-KG and increase of glutamate, since α-KG is consumed and glutamate is synthetized in the first step of BCAA catabolism.

Challenge studies seem to be the best method to evaluate metabotypes, due to their ability to improve variability of the metabolome. Metabolic challenges have exposed the effects between insulin and the affected pathways of dysglycemic states, showing the existence of a metabolic shift from catabolism (fasting, exercise) to anabolism (hyperinsulinemia), this state of anabolism appears to be maintained on obesity and insulin resistance (111). The OGTT provides a dynamic scene of the interaction between insulin and glucose, additionally it offers the advantage of setting the individual as its own control. FFAs overload β-oxidation on skeletal muscle and liver in an attempt to maintain energy substrate when insulin resistance is present; the defective oxidation of fatty acids produces acylcarnitine accumulation, thus an increased blood and urine pattern can be observed (21,43,110). A fact that supports the role of acylcarnitines on dysglycemia is its interaction with NF-Kβ, promoting inflammation and thereby contributing to insulin resistance (112). Metabolites associated with TCA cycle, amino acid metabolism, β-oxidation, and glycolysis expose a different pattern between subjects with normal insulin concentrations and hyperinsulinemia. Alterations on carnitine palmitoyltransferase 1 (CPT1), may be the cause of the acylcarnitine pattern found on dysglycemia and diabetes. CPT1 catalyzes the esterification of carnitine with acyl-CoA to produce acylcarnitines and transport the acyl group into the mitochondria and proceed to β-oxidation, an increased glycolytic flux increases malonyl-CoA concentrations, malonyl-CoA is a potent inhibitor of CPT1, thereby disturbing acylcarnitine metabolism (113).

Potential tools for diagnosis have emerged from metabolomics studies (α-hydroxybutyrate, L-GPC) (16,18,21), as well as tools for normoglycemia compliance monitoring (1,5-anhydroglucitol) (34). However, further studies are needed to target the related metabolites with therapy strategies, and evaluate their power in predictive risk scores. To associate metabolites in a predictive matter, observations must be made before the development of disease so the risk for disease and comorbidities can be stablished. The full characterization

of the metabolome in T2D remains a mayor challenge because the large amounts of metabolites that have been related and its variability make a complex phenotype. Study designs for transversal approaches need to be more heterogeneous and adjust variables between groups to the greatest possible to make them more similar (114).

Few metabolomics studies report sensitivity, specificity or ROC curves, making difficult the agreement on which biomarkers can be translated to the clinical field, beyond reporting biomarkers in a qualitative or quantitative fashion information should be analyzed to assess the validity of a given biomarker when extrapolating it to the general population, ROC curves are the standard for the discovery and development of diagnostic and prognostic tests. When a metabolomics approach is made, various metabolites can be associated by chance with a given condition, however these false positives must be validated with different analytical and validation experiments to assure that the findings may be significant (115).

In the previously described study performed by Ferranini et al. (18), various ROC curve analyses were made comparing the standard diabetes predictors (familiar background, sex, age, and BMI) and 2-h plasma glucose with an increase on ROC curves by 0.044 for the RISC cohort and 0.017 for the Botnia cohort. Increases on ROC curve values in both cohorts were observed when adding α-HB and L-GPC to fasting plasma glucose and 2-h plasma glucose improving ROC curve values by 0.018 for the RISC cohort and 0.008 for the Botnia cohort. Limitations exist when calibrating of the tools for T2D risk prediction, most of it implies that the tool should be used on the population in which it has been developed or recalibrated for another population (116). In general, the metabolomic analyses increase ROC curves when added to classical clinical and biochemical markers, a more detailed discussion can be found on the diabetes risk prediction section.

With the development of metabolomics, it is expected to achieve a more personalized control of diabetes. Depending on the patient's metabolomic profile, it would be possible to perform more effective strategies with personalized decisions based on the individual behavior, phenotypic features, laboratory findings, gene sequences, and metabolic and proteomic profiles. The late advances in the field of omics offer new opportunities to improve early diagnosis, clinical outcome, prevention of complications, and decrease in disease progression (117).

Disclosure statement

The authors report no declarations of interest.

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