

Metabolomics in Diabetes Research

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ABSTRACT

Diabetes represents one of the most important global health problems because it is associated with a large economic burden for the health systems of many countries. Whereas the diagnosis and treatment of manifest diabetes have been well investigated, the identification of novel pathways or early biomarkers indicative of metabolic alterations or insulin resistance related to the development of diabetes is still in progress. Over half of the type 2 diabetes patients show manifestations of diabetes-related diseases, which highlights the need for early screening markers of diabetes. During the last decade, the rapidly growing research field of metabolomics has introduced new insights into the pathology of diabetes as well as methods to predict disease onset and revealed new biomarker. A recent epidemiological studies firstly used metabolism to predict incident diabetes and revealed branched-chain and aromatic amino acids including isoleucine, leucine, valine, tyrosine and phenylalanine as highly-significant predictors of future diabetes. This review summarizes the current findings of metabolic research in animal models and human investigations.

INTRODUCTION

Diabetes mellitus, particularly type 2 diabetes (T2DM), represents one of the most significant global health problems because it is associated with a large economic burden for the health systems of many countries. The World Health Organization (WHO) reported that worldwide 171 million subjects are affected by diabetes in 2000, which is equivalent to a prevalence of 2.8%, and these reports predicted an estimated future number of 366 million affected individuals in 2030, which would be equivalent to a diabetes prevalence of 4.4% (Wild, et al. 2004). However, recent data of the International Diabetes Federation (IDF) revealed that this number has already been achieved in 2011. The IDF expected an even higher number of 552 million affected persons in 2030. Due to the broad range of diabetes-related complications, including diabetic nephropathy, peripheral neuropathy and cardiovascular disease, diabetes is a major cause of both morbidity and mortality.

Diabetes mellitus is a chronic disease that is characterised by high blood glucose levels, which may be due either to the progressive failure of pancreatic beta-cell function and consequently a lack of insulin production (type 1: T1DM) or to the development of insulin resistance and subsequently the loss in beta-cell function (T2DM). Approximately 90% of patients with diabetes have T2DM. T1DM is an autoimmune disease with a strong genetic component. Genetic susceptibility to T1DM has been intensively investigated, and the major histocompatibility complex (MHC) was reported to be the main genetic determinant (Noble and Erlich 2012; Polychronakos and Li 2011). The predominant cause of T2DM is related to lifestyle factors including diet, insufficient physical activity, an overweight or obese state and stress. Furthermore, at least 36 genes, accounting for 10% of the total genetic component, have been significantly associated with an increased risk for T2DM (Herder and Roden 2011). Moreover, further 18 genes were related to glucose and HbA1c levels as well as insulin resistance. Detailed reviews on the genetics of T1DM and T2DM have been presented elsewhere (Herder and Roden 2011; Polychronakos and Li 2011).

The diagnosis of diabetes is mainly based on the results of blood tests examining fasting plasma glucose or glycated haemoglobin (HbA1c) levels (World Health Organization 2006).

Additionally, the treatment of diabetes is linked to these measures, as the main goal of antidiabetic therapy is to reduce blood glucose and HbA1c levels via the administration of insulin (T1DM) or antidiabetic medication (T2DM). Furthermore, lifestyle changes, such as eating a more healthful diet, performing regular physical activity, achieving a normal body weight and smoking cessation, are recommended for diabetic patients.

Whereas the diagnosis and treatment of manifest diabetes have been thoroughly investigated, the identification of novel pathways or early biomarkers indicative of metabolic alterations or insulin resistance related to the development of T2DM is still underway. Data from the National Health and Nutrition Examination Survey (NHANES) showed that estimated 57.9 % of subjects with diagnosed diabetes are affected by one or more macro- or microvascular complications (American Association of Clinical Endocrinologists 2007), which highlights the need for early screening markers to monitor the development of T2DM. During the last decade, the rapidly growing research field of metabolomics has introduced new insights into the pathology of diabetes as well as methods to predict disease onset. In accordance with the other “omics” techniques, such as genomics, transcriptomics or proteomics, the term metabonomics, which is synonymous with metabolomics or metabolic profiling, was first introduced by Nicholson and colleagues in 1999 (Nicholson, et al. 1999). Metabolomics relates to measurements of the metabolome, which represents the entire collection of all small-molecule metabolites present in any biological organism (Oliver, et al. 1998; Tweeddale, et al. 1998). The advantages of metabolomics over other “omics” technologies include its high level of sensitivity and its ability to enable the analysis of relatively few metabolites compared with the unwieldy number of corresponding genes or mRNA molecules. The comprehensive Human Metabolome Database (HMDB) contained approximately 7900 metabolite entries in January 2009. Another advantage of metabolomics is that metabolites are the final downstream products of the interaction between genes and influences like environmental factors, health behaviour or pharmaceutical interventions, and metabolite levels reflect the activity of metabolic pathways. Therefore, metabolomics enables

the detection of short-term as well long-term physiological or pathological changes in cells, tissues or body fluids and represents a useful tool for biomarker detection.

The purpose of the present review is to summarise current metabolomics technologies and to provide an overview of the contribution of metabolomics to diabetes research.

METABOLOMICS TECHNOLOGIES

The two main high-throughput metabolomics tools consist of nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Both methods enable the comprehensive investigation of metabolic profiles (Dunn, et al. 2005; Hollywood, et al. 2006; Lenz and Wilson 2007) and can provide complementary snapshots of the metabolome of body fluids such as plasma, urine or cerebrospinal fluid (Bictash, et al. 2010).

Nuclear magnetic resonance spectroscopy

NMR is a widely used spectroscopic technique for metabolomics that is based on the magnetic properties of the atomic nucleus (e.g., ^1H , ^{13}C , or ^{31}P). This method was first used for the analysis of body fluids in the 1980s (Bell, et al. 1989; Iles, et al. 1985; Nicholson, et al. 1984). The behaviour of NMR active nuclei in a strong magnetic field provides information about the structural and chemical properties of a molecule. Due to the high abundance of the ^1H nucleus, ^1H -NMR spectroscopy has been heavily utilised to investigate biological fluids such as plasma and urine as well as tissues. Each separate signal in a ^1H -NMR spectrum corresponds to a particular compound. Exemplary ^1H -NMR spectra for human urine and plasma are shown in figure 1. Based on measurements of

- chemical shift
- spin–spin coupling: *Neighbouring nuclei influence the effective magnetic field which results in spin interaction. The so called spin-spin coupling can cause splitting of the signal into two or more peaks.*
- relaxation: *Describes the return to equilibrium of net magnetization and included two types of relaxation: Spin-Lattice (T_1 , also called longitudinal relaxation) and Spin-*

Spin (T_2 , also called transverse relaxation).

- diffusion

the identification of single metabolites and absolute quantification are possible. Detailed information regarding NMR theory, its application and typical chemical shift values is available elsewhere (Blümich 2005). The application of NMR spectroscopy in metabolomics ranges from pharmaceutical studies (Lindon, et al. 2007) to cardiovascular biomarker identification (Griffin, et al. 2011; Rhee and Gerszten 2012).

Advantages of NMR spectroscopy include the non-destructive nature of the analysis, the robust and reproducible measurements and the minimal preparation requirements, as no separation or ionisation steps are necessary. However, in comparison to MS, the analytical sensitivity of NMR is relatively low, even if stronger magnetic fields are used to increase the analytical sensitivity.

Mass spectrometry

MS is a powerful tool for investigating molecular structure as well as for detecting and quantifying metabolites (Lenz and Wilson 2007). The first step in MS consists of the ionisation of the analyte, which is followed by the separation of the ions according to their mass-to-charge (m/z) ratio using an analyser with an electromagnetic field. In metabolomics, MS is often combined with other suitable methods for the analytical separation of compounds, including gas chromatography (GC) or liquid chromatography (LC), to achieve detection of distinct metabolite classes (Buscher, et al. 2009). One study comparing GC, LC and capillary electrophoresis (CE) revealed that LC was the most robust method and enabled the broadest coverage of metabolite classes (Buscher et al. 2009). However, both GC-MS und LC-MS demonstrate high separation efficiency and are excellent tools for metabolic profiling. Exemplary MS spectrum for human urine is shown in figure 2. The application of MS in the metabolomics field ranges from studies of microorganisms and plants to biomarker detection. The greatest advantage of the MS methods is their high level of sensitivity, although disadvantages arise from the destruction of the sample and the long sample preparation time

required. Detailed descriptions of MS methods have been provided elsewhere (de Hoffmann and Stroobant 2007).

Due to the limited overlap for metabolite detection and thus the complementary nature of MS and NMR spectroscopy, studies using a multiplatform approach may provide a more comprehensive understanding of metabolic alterations than studies using only one of these tools (Williams, et al. 2006a).

EXPERIMENTAL STUDIES APPLIED TO GLUCOSE METABOLISM AND DIABETES

Dietary-induced obesity and insulin resistance

A series of experimental studies have been conducted using dietary-induced obesity or insulin resistance rodent models to investigate the metabolic profiles of urine (Kim, et al. 2009), blood (Li, et al. 2010a; Shearer, et al. 2008) or tissue (Li et al. 2010a; Lin, et al. 2011), and these results have led to new insights into the development of diabetes. Dietary-induced obesity models have the advantage of being **more** similar to the development of human obesity **in comparison to genetic models**, and as a result, these models mirror the progression of insulin resistance and diabetes after a prolonged period of development (Fearnside, et al. 2008). High-fat-fed C57BL/6J mice become obese and insulin resistant and demonstrate different serum levels of ¹H-NMR-based metabolite concentrations in comparison to chow-fed mice (Shearer et al. 2008). Whereas the citrate concentration was higher in high-fat-fed mice than in chow-fed mice, the concentrations of glycine, lysine, suberate, acetate, leucine, valine or trimethylamine N-oxide were significantly lower in high-fat-fed mice. Furthermore, dietary-induced insulin resistance could be predicted according to various metabolite levels, specifically those of lysine, glycine, citrate, leucine, suberate and acetate, and these metabolite levels could also be used to discriminate between chow- and high-fat-fed animals. Additionally, the urinary metabolic profiles of high-fat-fed rats were significantly different from those of normal-diet-fed rats (Kim et al. 2009). ¹H-NMR spectroscopy also revealed diet-related variations in the levels of betaine, taurine, acetone/acetoacetate, phenylacetylglutamine, pyruvate, lactate and citrate. MS-based studies

can also detect additional diet-induced changes in metabolites (Li et al. 2010a; Lin et al. 2011); one study investigated the diet-induced inhibition of insulin in the liver tissue and plasma of wild-type and glycerol-3-phosphate acyltransferase 1 (GPAT-1)-deficient mice, which remain insulin sensitive independent of their diet (Li et al. 2010a). Assuming that metabolic changes identified in insulin-resistant wild-type mice are specifically related to hepatic insulin resistance and may therefore indicate a causative pathway, these authors demonstrated alterations in the concentrations of 43 liver and 19 plasma metabolites. The identified increases in urea cycle-related metabolites, such as citrulline, aspartate or N-acetyl-glutamate, were indicative of early up-regulation of the urea cycle, whereas the altered levels of liver metabolites suggested the existence of variations in glucose metabolism (1,5-anhydroglucitol decrease), bile acid metabolism (taurocholate decrease) and pyrimidine metabolism (2'-deoxyuridine increase). Moreover, the increase in pyrimidine metabolites and the decreases in bradykinin, kynurenine and α -ketoglutarate concentrations were also confirmed in the plasma. A separate MS study extended this approach of diet-induced insulin resistance to include a metabolic oral glucose tolerance test (OGTT) and additionally examined liver, brain and skeletal tissues (Lin et al. 2011). These MS data enabled the authors to discriminate between both the 120-min and 0-min time points for both standard-fed (SD) and high-fructose-fed (HFrD) rats, and these data also identified specific metabolic effects in insulin-resistant rats. As expected, insulin administration-related up-regulation of lysophosphatidylcholines (Lin et al. 2011) was observed in SD rats but not in HFrD rats. However, the levels of the branched-chain amino acids (BCAA) proline, tryptophan and methionine were decreased in HFrD rats at 120 min but were unchanged in SD rats, and opposite effects were observed for the amino acids leucine and isoleucine, which had previously been shown to be related to insulin sensitivity (Shaham, et al. 2008) and were present at lower levels in HFrD rats. By comparing SD and HFrD rats at the 0-min time point, differences were identified for various compounds, including phospholipids, amino acids, bile acids, fatty acids and metabolites. Moreover, regarding purine metabolism and the Krebs cycle, a complex metabolic perturbation in HFrD rats was observed. Increased levels of

phospholipids and fatty acid were also found in high-fat-fed mice in combination with lower levels of betaine, carnitine and acylcarnitines, which are metabolites involved in lipid metabolism (Kim, et al. 2011). In liver and skeletal muscle tissue, a high-fructose diet leads to oxidative stress, elevated levels of amino acids and alterations in fatty acid biosynthesis, whereas this type of diet is related to decreased amino acid levels and the up-regulation of purine biosynthesis in the cerebral cortex and hippocampus (Lin et al. 2011).

In general, the distinction between diet-related effects and obesity-related effects represents a common problem in dietary-induced diabetes models. One recent study in mice investigated the long- and short-term consequences of various types of diets and aimed to distinguish the specific effects of each diet from those of obesity in general (Duggan, et al. 2011). The results revealed that diet has a major impact on the metabolic profiles measured by ^1H -NMR; whereas diet influenced metabolites related to energy and glucose metabolism, obesity mainly caused alterations in amino acids and large non-polar molecules.

Genetic rodent models of diabetes

Genetic T2DM models have several advantages over diet-induced models, including a short generation time, heritable traits and lower cost, although the “natural” development of diabetes over a prolonged period of time is lacking in these animals. The two most popular obesity/T2DM models include the db/db model and the obese Zucker (fa/fa) model, which are both characterised by an autosomal recessive defect in the leptin receptor gene that results in obesity and subsequent insulin resistance (Chen and Wang 2005). Based on these models, differences in urinary (Connor, et al. 2010; Gipson, et al. 2008; Patterson, et al. 2011; Salek, et al. 2007; Williams, et al. 2005a; Williams, et al. 2006b; Zhao, et al. 2010a), plasma (Major, et al. 2006; Williams et al. 2006a) and tissue (Connor et al. 2010; Xu, et al. 2009) metabolic profiles have been reported between affected and control rodents. Several studies have demonstrated profound alterations in metabolites involved in the tricarboxylic acid (TCA) cycle. The TCA cycle is an amphibolic pathway that occurs in the inner mitochondrial membrane and plays an important role in energy metabolism. The final

products of fatty acid degradation and glycolysis are included in the TCA cycle, and TCA cycle intermediates are involved in amino acid synthesis and degradation as well as gluconeogenesis. Whereas Zucker rats (Williams et al. 2005a; Williams et al. 2006b; Zhao et al. 2010a) typically have decreased urinary concentrations of TCA metabolites, such as citrate, malate, fumarate, 2-ketoglutarate or succinate, the db/db mouse (Connor et al. 2010; Salek et al. 2007) has exhibited increased levels of TCA metabolites, and these changes are indicative of the down- and up-regulation of the TCA cycle, respectively. In a study with rhesus macaques, animals with T2DM demonstrated 2-fold higher levels of citrate compared to normal animals (Patterson et al. 2011). Additionally, Sprague–Dawley rats with T1DM induced by streptozotocin demonstrated higher levels of pyruvate, succinate and fumarate (Zhang, et al. 2008). This study further showed strong associations between TCA cycle intermediates and components of glucose metabolism in normal rats, specifically between pyruvate, as the end product of glycolysis, and 2-oxoglutarate, fumarate or citrate. In diabetic rats, there was evidence for a disturbed balance between the TCA cycle and glucose metabolism, as the glucose levels were not associated with those of lactate or various TCA cycle intermediates. Thus, the concentrations of succinate were not correlated with those of 2-oxoglutarate or citrate (Zhang et al. 2008). Beside disturbances in intermediate correlations, the metabolite composition demonstrated a strong age-dependent variation. Williams and colleagues (Williams et al. 2006b) reported that the urinary ratios of α -ketoglutarate and succinate to citrate were in favour of citrate at an age of four weeks in Zucker rats, although this finding was no longer apparent at 20 weeks. In db/db mice, a decrease in the concentration of both TCA and non-TCA metabolites was also reported with age (Salek et al. 2007). Regarding taurine, the urinary levels in control and Zucker rats were comparable at eight weeks, whereas taurine was absent in 50% of the Zucker animals at 20 weeks (Williams et al. 2006b). Other metabolites involved in amino acid metabolism have been shown to be associated with T2DM. For example, amino acids, such as phenylalanine, valine, tryptophan, lysine and glutamic acid, and amino acid metabolites, such as 2-hydroxyisobutyrate, 2-hydroxyisovalerate and kynurenic acid, were present at higher

concentrations in the urine of Zucker rats (Connor et al. 2010; Gipson et al. 2008; Salek et al. 2007), db/db mice (Connor et al. 2010; Gipson et al. 2008) and monkeys (Patterson et al. 2011) than in control animals, although lower concentrations of these compounds have also been reported (Salek et al. 2007; Williams et al. 2006b). These results provide evidence for the complex perturbation of amino acid metabolism in diabetic disease. The largest portion of amino acid metabolism takes place in the liver, and although a broad range of amino acids are glucogenic and are utilised for hepatic gluconeogenesis, a smaller number of amino acids are ketogenic and are converted to ketone bodies. An additional rodent study (Mochida, et al. 2011) on T1DM using the AKITA mouse model confirmed these previous urine findings and demonstrated higher amino acid, BCAA, alanine, citrulline and proline levels in the plasma of T1DM rats indicative of disease state-dependent changes. As hyperglycaemia progressed, the differences regarding the mentioned amino acids and BCAA became more pronounced. Additionally, these authors demonstrated a relation between increased blood glucose levels and increases in plasma levels of valine, leucine, isoleucine, BCAA and alanine (Mochida et al. 2011). In addition to insulin resistance, BCAA supplementation to the high-fat diet leads to chronic increased activation of mTOR in rats (Newgard, et al. 2009), and overactivation of the mTOR/S6K1 pathway has been linked to the development of insulin resistance via beta-cell adaptation to hyperglycaemia (Fraenkel, et al. 2008; Khamzina, et al. 2005; Um, et al. 2004). Further details concerning the relation between amino acids and diabetes are provided in the human studies section of this review.

A series of rodent studies found increased urinary levels of fatty acids in Zucker rats (Salek et al. 2007; Williams et al. 2006b). Furthermore, higher levels of carnitine, an essential compound for the transport of fatty acids from the cytosol into the mitochondria, and one that is related to T2DM (De Palo, et al. 1981), have been observed in diabetic rodents (Connor et al. 2010; Williams et al. 2006b). A multiplatform study confirmed the enriched fatty acid metabolism of the db/db mouse by revealing increased transcript levels of fatty acid metabolism-associated carnitine palmitoyltransferase (Cpt) in the liver and higher urinary carnitine levels, as measured using LC-MS (Gipson et al. 2008). Furthermore, increased

carnitine levels with age were observed in db/db but not in control mice, and similar age-dependent increases in carnitine were also reported in non-diabetic rats (Williams, et al. 2005b). Increased fatty acid metabolism result from a higher rate of lipolysis in adipose tissue and might exacerbate insulin resistance in liver and muscle tissue (Delarue and Magnan 2007). High fatty acid levels subsequently lead to a higher oxidation rate and therefore to the induction of ketogenesis. Thus, diabetic animals typically exhibit higher levels of ketone bodies, including β -hydroxybutyrate or acetone, as compared to controls (Salek et al. 2007; Zhao et al. 2010a). Similar to changes in amino acid levels, increased levels of ketone bodies have been associated with the diseased state as well as age (Salek et al. 2007). Taken together, the shift from euglycaemia towards hyperglycaemia is likely linked to pronounced metabolic perturbations and mitochondrial metabolic dysfunction. Beside the previously mentioned metabolites, a wide range of additional urine or tissue metabolites, e.g., choline, allantoin, glycine or betaine, has also been related to obesity or diabetes in different studies (Gipson et al. 2008; Patterson et al. 2011; Salek et al. 2007; Williams et al. 2005a; Williams et al. 2006b; Zhang et al. 2008; Zhao et al. 2010a).

Several studies have also detected differences in metabolites known to originate from the gut microflora. For example, hippurate, which is mainly produced via gut microbial metabolism (Nicholson, et al. 2005), was shown to be elevated in db/db mice (Connor et al. 2010; Salek et al. 2007) but decreased in Zucker rats (Salek et al. 2007; Williams et al. 2006b; Zhao et al. 2010a), whereas microbiota-derived methylamines such as dimethylamine or trimethylamine-N-oxide were shown to be increased in both types of rodents (Connor et al. 2010; Gipson et al. 2008; Salek et al. 2007; Zhang et al. 2008; Zhao et al. 2010a). Reasons for this discrepancy may be related to differences in the composition of the intestinal microflora.

HUMAN STUDIES APPLIED TO GLUCOSE METABOLISM AND DIABETES

Glucose ingestion

The OGTT measures the body's ability to metabolise glucose and clear excess glucose from the bloodstream. Since the 1970s, the OGTT has been a standard diagnostic tool in

diabetology. A 2-h OGTT is routinely performed in fasting patients; patients drink a beverage containing a specific amount of glucose according to their body weight, and 2 h after the glucose load, the blood glucose concentration is measured and provides information on glucose tolerance. According to the WHO, in healthy individuals, the venous plasma glucose level should be below 7.8 mmol/l; values greater than this can be used to diagnose impaired glucose tolerance (7.8 - 11.1 mmol/l) or diabetes mellitus (≥ 11.1 mmol/l) (World Health Organization 2006).

Several studies have investigated changes in the metabolic profile in relation to glucose ingestion (Matysik, et al. 2011; Shaham et al. 2008; Spegel, et al. 2010; Zhao, et al. 2009). An investigation (Shaham et al. 2008) among participants in the Metabolic Abnormalities in College Students (MACS) study who demonstrated normal glucose tolerance revealed significant kinetic alterations in 21 out of 191 plasma metabolites, as measured by LC-MS, in response to an OGTT. Eighteen of these metabolites were independently identified in subjects from the Framingham Offspring Study, and several metabolites, including glucose, lactate, hippurate and glycerol, had also been previously related to glucose metabolism (Pelkonen, et al. 1967). However, this study demonstrated novel changes in the levels of bile acids following the OGTT; the levels of three bile acids, glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA) and taurochenodeoxycholic acid (TCDCA), were increased within the first 30 min following glucose ingestion and remained stable thereafter. Another study (Zhao et al. 2009) examining healthy individuals revealed similar results concerning these bile acids and reported as much as a 6-fold increase in these levels after 30 min (followed by a subsequent decrease). Both findings were confirmed by a third study (Matysik et al. 2011) that investigated bile acid signalling during the course of an OGTT in relation to 15 conjugated and unconjugated bile acids. In normal subjects, the levels of GCDCA, the bile acid with the highest plasma levels, and chenodeoxycholic acid (CDCA) increased within the first 60 min of the OGTT. Furthermore, this study found that in response to oral glucose ingestion, the levels of all of the examined glycine- and taurine-conjugated bile acids were increased at 60 min and declined slightly over the following 60 min, whereas

the levels of unconjugated bile acids, e.g., cholic acid, lithocholic acid and ursodeoxycholic acid, declined throughout the course of the OGTT. Bile acids are produced in the liver by the oxidation of cholesterol and are stored in the gall bladder. Upon food intake, bile acids are released into the duodenum and small intestine and facilitate the intestinal absorption of nutrients, particularly dietary fat, drugs and steroids. The majority of excreted bile acids are reabsorbed in the terminal ileum and return to the liver via the enterohepatic circulation, and very low levels of bile acids are found in the systemic circulation. Beside their major role in dietary lipid absorption, bile acids are metabolic factors that play regulatory roles in fat, glucose and energy metabolism (Houten, et al. 2006; Lefebvre, et al. 2009). The reported increase in bile acids in response to glucose ingestion is in concordance with a 3-fold increase in the levels of bile acids in human serum following a standard liquid meal (De Barros, et al. 1982). Furthermore, oral glucose ingestion leads to increased levels of cholecystokinin, a hormone that stimulates the production of hepatic bile and gall bladder contractions (Liddle, et al. 1985). The link between bile acids and glucose homeostasis was further confirmed by the demonstration of enhanced CYP7A1 mRNA expression following insulin injection or oral glucose administration in fasting mice (Li, et al. 2012). Additionally, in primary human hepatocytes, insulin and glucose were shown to stimulate CYP7A1 mRNA expression (Li, et al. 2010b; Li, et al. 2006), which suggests the existence of glucose/insulin-regulated gene transcription in the liver. The CYP7A1 gene encodes the enzyme cholesterol 7 α -hydroxylase, which is the rate-limiting enzyme in the cholesterol catabolic pathway and the conversion of cholesterol to bile acids and therefore represents a major point of regulation during bile acid synthesis. The direct glucose/insulin-stimulated expression of CYP7A1 leads to an increased bile acid pool size. Taken together, these findings indicate an important connection between bile acid metabolism and glucose homeostasis. Hence, it is not surprising that the bile acid metabolism is altered in patients with diabetes (Prawitt, et al. 2011). In addition, a metabonomic study revealed higher plasma levels of GCDCA in subjects with impaired glucose tolerance, as compared to subjects with normal glucose tolerance (Zhao, et al. 2010b). Another study detected differences in the composition of the

bile acid pool between T2DM patients and controls (Brufau, et al. 2010). Whereas the size of the total bile acid pool was not different, T2DM subjects demonstrated increased deoxycholic acid input rates and cholic acid synthesis rates but exhibited a lower proportion of CDCA. Furthermore, therapy with bile acid sequestrants leads to the expected reductions in both total cholesterol and low-density lipoprotein (LDL) cholesterol as well as improvements in glycaemic control in T2DM patients (Garg and Grundy 1994; Kondo and Kadowaki 2010; Suzuki, et al. 2006; Zieve, et al. 2007). Compared to patients who received control treatments or placebos, T2DM patients given bile acid sequestrants demonstrated greater reductions in the levels of plasma glucose and glycosylated haemoglobin.

In regards to the OGTT studies, metabolites beside bile acids were also altered during the OGTT in healthy subjects. In fact, increases in lysophosphatidylcholine (Zhao et al. 2009) and decreases in amino acids (Table 1) (Shaham et al. 2008; Spegel et al. 2010), acylcarnitines (Zhao et al. 2009) and fatty acids (Table 2) (Spegel et al. 2010; Zhao et al. 2009) were reported. The study by Zhao et al. (Zhao et al. 2009) provided a more systematic overview of fatty acid plasma changes during an OGTT; although the levels of fatty acids declined during an OGTT, the levels of saturated (SFA) and monounsaturated fatty acids (MUFA) were more significantly decreased than those of polyunsaturated fatty acids (PUFA). Moreover, a substantial reduction in the SFA/MUFA ratio was observed, consisting of a shift from MUFA towards SFA. These findings indicate a change in fatty acid composition following an OGTT. A more detailed discussion on this topic is presented in the next chapter. Overall, metabolic studies have revealed alterations in metabolites related to pathways involved in the action of insulin, including lipolysis, ketogenesis, proteolysis and glucose metabolism. These results indicate a change from beta-oxidation to glycolysis and fat storage in response to glucose ingestion.

Patient investigations

Comparisons between the levels of various metabolites in diabetic patients and healthy controls have confirmed many of the findings from animal studies as well as studies

investigating metabolic changes during an OGTT. These findings from human patients can be summarised as follows:

- 1) As expected, diabetic patients exhibited differences in glucose metabolism. Patients with T1DM under insulin deprivation (Lanza, et al. 2010) or in T2DM (Li, et al. 2009; Suhre, et al. 2010) demonstrated elevated glucose or mannose levels as compared to healthy controls. Furthermore, in both T1DM and T2DM patients (Messana, et al. 1998; Zuppi, et al. 2002) increased lactate levels were observed, at which these levels increased with the grade of glucosuria in T2DM patients (Messana et al. 1998).
- 2) With respect to TCA cycle metabolites (and similar to db/db mice), patients with T1DM (Zuppi et al. 2002) and T2DM (Messana et al. 1998) had higher levels of citrate, and these levels were also associated with increasing glucosuria (Messana et al. 1998). An additional study investigated the metabolic profiles of children who later progressed to T1DM and found decreased succinate and citrate levels at the time of birth (Oresic, et al. 2008).
- 3) The above-mentioned results were further confirmed in regards to the levels of ketone bodies during diabetes. Higher levels of acetone, acetoacetate and β -hydroxybutyrate were observed in insulin-deprived T1DM (Lanza et al. 2010) and T2DM (Messana et al. 1998; Nicholson et al. 1984; Suhre et al. 2010) subjects, indicating ketoacidotic metabolic decompensation.
- 4) Additionally, alterations in intestinal microflora-associated metabolites have been detected. Insulin-deprived T1DM and T2DM patients exhibited elevated levels of hippurate, dimethylamine or trimethylamine-N-oxide (Messana et al. 1998; Zuppi et al. 2002), although a separate study observed lower levels of hippurate and 3-hydroxyhippurate among pre-diabetic individuals with impaired glucose tolerance (Zhao et al. 2010b). The human gut microbiota has an important role in health, which has been comprehensively discussed by several authors (Fujimura, et al. 2010; Nicholson et al. 2005; Prakash, et al. 2011) and is outside the focus of the present review.

Fatty acid alterations in patients with diabetes have been extensively examined. In accordance with genetic rat models (Salek et al. 2007; Williams et al. 2006b), increased fatty acid levels were detected in T2DM patients (Li et al. 2009; Suhre et al. 2010; Yi, et al. 2007; Yi, et al. 2008; Yi, et al. 2006) as well as in subjects with impaired glucose tolerance (Zhao et al. 2010b). Changes in as many as 18 fatty acids, including SFA, MUFA and PUFA, were found in one study. Furthermore, the metabolic profile of plasma acylcarnitines revealed higher fasting levels of long-chain saturated and monounsaturated acylcarnitines in obese and T2DM subjects as compared to lean controls (Mihalik, et al. 2010). Moreover, the levels of free carnitine were increased in both groups, although differences between groups were observed for short- and medium-chain acylcarnitine species as well as hydroxyacylcarnitines, where higher levels were observed in T2DM patients. Similar to an OGTT-induced reduction in acylcarnitines (Zhao et al. 2009), an insulin-stimulated euglycaemic clamp led to a decrease in all acylcarnitine species for all three of the investigated groups, although this reduction was blunted in patients with T2DM (Mihalik et al. 2010). All of the investigated fatty acids and acylcarnitines are listed in table 2. Many studies have demonstrated that obese subjects often exhibit elevated fatty acid levels due to the enlarged volume of adipose tissue (Jensen, et al. 1989; Newgard et al. 2009; Opie and Walfish 1963). Furthermore, higher levels of fatty acids are related to a greater risk for diabetes (Charles, et al. 1997; Pankow, et al. 2004; Paolisso, et al. 1995), although the underlying mechanisms are not completely understood. However, elevated levels of free fatty acids induce insulin resistance in muscle and liver tissue by decreasing insulin-stimulated glucose uptake and glycogenesis (Boden 2003; Griffin, et al. 1999; Wilding 2007). Moreover, the improvement in insulin sensitivity caused by a reduction in fatty acid levels supports these findings (Boden, et al. 1998; Cusi, et al. 2007; Santomauro, et al. 1999). At present, there are several hypotheses as to how free fatty acids interfere with insulin signalling; these are related to oxidative stress or inflammatory lipid pathways and have been reviewed by Boden (Boden 2011).

An additional observation from patient studies is the change in amino acid levels in diabetic patients. A broad range of amino acids, including leucine, isoleucine, valine, phenylalanine,

tyrosine, alanine, tryptophan and homocitrulline, has been shown to be substantially increased in T1DM (Lanza et al. 2010) or T2DM patients (Messana et al. 1998; Suhre et al. 2010) as well as among subjects with obesity (Newgard et al. 2009) or impaired glucose tolerance (Zhao et al. 2010b). In addition, these findings have been confirmed by studies that revealed positive associations between amino acid levels and the homeostasis model assessment index (Newgard et al. 2009) and insulin resistance of obese subjects, according to Bergman's minimal model (Huffman, et al. 2009). However, lower levels of glycine, glutamate and threonine have been observed in diabetic patients and obese subjects (Lanza et al. 2010; Messana et al. 1998; Newgard et al. 2009). A recent investigation (Wang, et al. 2011) of the Framingham Offspring Study firstly examined the predictive ability of fasting plasma metabolite levels for incident T2DM and showed that the amino acids isoleucine, leucine, valine, tyrosine and phenylalanine were elevated 12 years prior to the onset of diabetes and were also linked to a higher diabetes risk. In fact, the combination of increased fasting isoleucine, tyrosine and phenylalanine levels at baseline was related to a greater than 5-fold higher risk of incident diabetes. Moreover, these results were independently replicated in the Malmo Diet and Cancer Study. These studies have highlighted the impact of amino acids on the actions of insulin and consequently glucose metabolism. In the 1940s, Luetscher (Luetscher 1942) already reported higher amino acid levels among diabetic patients, and this finding was later confirmed by the demonstration of the positive correlation between amino acid levels and insulin (Felig, et al. 1969). Furthermore, the intravenous administration of amino acids leads to the stimulation of insulin secretion. This insulinotropic effect differs depending on the specific amino acids in question (Floyd, et al. 1966; Floyd, et al. 1968). However, the underlying mechanisms are complex and are related to the inhibition of glucose transport and gluconeogenesis (Krebs, et al. 2002; Langenberg and Savage 2011; Patti, et al. 1998).

CONCLUSION AND CHALLENGES FOR THE FUTURE

The application of metabolomics in diabetes studies has rapidly evolved during the last decade and provides researchers with the opportunity to gain new insights in metabolic profiling and pathophysiological mechanisms. Thus, several metabolites were identified to be related to diabetes or insulin resistance and represent the basis for the identification of novel diabetes biomarkers. Some findings were newly discovered altered metabolites e.g. bile acids, whereas other metabolic variations were already known e.g. fatty acids or amino acids. However, the results often led to a revalue of knowledge. Langenberg and colleague (Langenberg and Savage 2011) currently discuss the potential of an amino acid profile as predictor of T2DM and highlighted the fact that the addition of amino acids to established risk factors only minimal improved the risk predication. This general problem is also apparent for genetic variants and other clinical novel biomarkers of diabetes whose power to add considerable improvement in risk assessment is limited (Lyssenko, et al. 2008; Meigs, et al. 2008; Salomaa, et al. 2010). Nevertheless, metabolomics increase the knowledge of disease progression and provide approaches for therapy.

DECLARATION OF INTEREST

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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FIGURE LEGENDS

Figure 1. Assigned high resolution ^1H nuclear magnetic resonance spectrum of a human plasma (600 MHz) and human urine (400MHz) sample.

Figure 2. GC-MS analysis of organic acids in human urine. Above - total ion current (TIC) chromatogram; below - mass spectrum at retention time 44.727min. 1. glycolic; 2. p-cresol; 3. 3-hydroxyisovaleric; 4. urea; 5. succinate; 6. hydroxyphenylacetic; 7. aconitic; 8. citric; 9. 4-(3-hydroxyprop-1-enyl)phenol.

Table 1. List of altered amino acids in patients with diabetes or during an oral glucose tolerance test (OGTT).

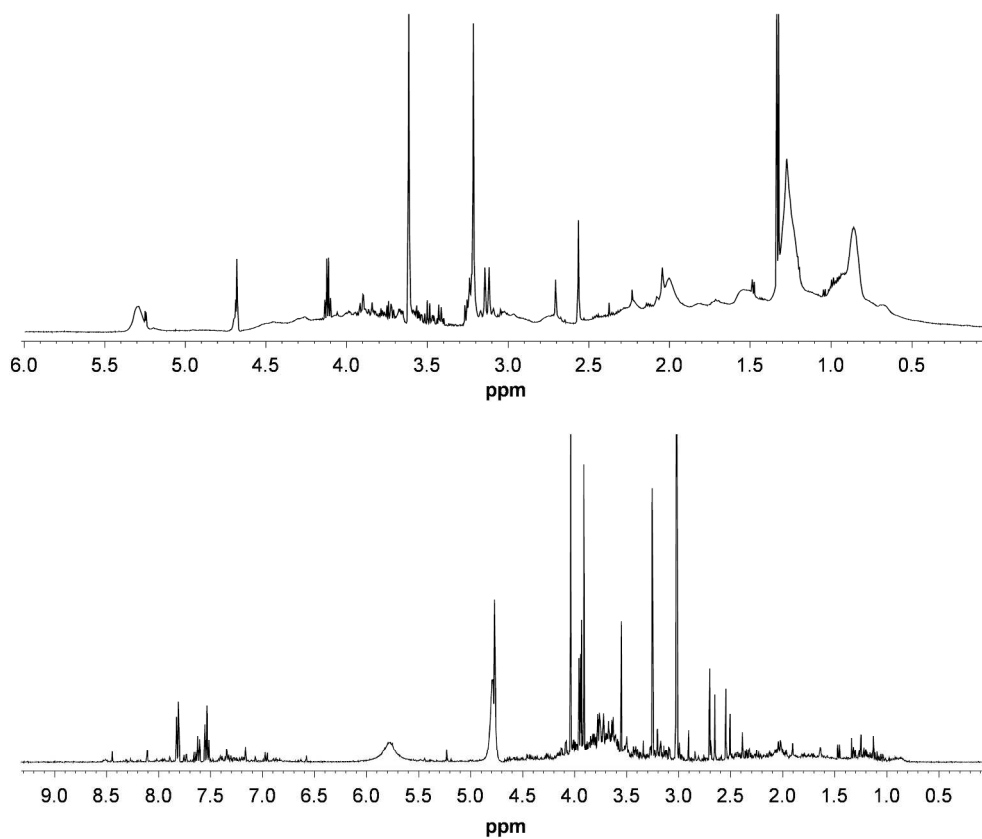
Amino acids	Essential (E) Nonessential (NE)	Alanine Metabolism	Glutathione Metabolism	Glycine and Serine Metabolism	Valine, Leucine / Iso- leucine Degradation	Glucose-Alanine Cycle	Urea Cycle	Bile Acid Biosynthesis	Methionine Metabolism	Histidine Metabolism	Further pathways	Reference Patient studies	Reference OGTT studies
Alanine	NE	X		X		X	X				Selenoamino Acid Metabolism	Messana 1998; Nicholson 1984; Oresic 2008; Zuppi 2002	
Arginine	E	X		X			X					Shaham 2008	Shaham 2008
Citrulline	na						X				Arginine and Proline Metabolism, Aspartate Metabolism	Lanza 2010	
Glutamate	NE	X	X	X		X	X		X		Arginine and Proline Metabolism, Amino Sugar Metabolism, Cysteine Metabolism, Folate Metabolism	Lanza 2010; Messana 1998	Spegel 2010
Glycine	NE	X	X	X				X	X		Carnitine Synthesis, Porphyrin Metabolism	Shaham 2008	Suhre 2010
Histidine	E									X			Lanza 2010; Wang 2011
Homocitrulline	n. s.											Shaham 2008; Spegel 2010	Lanza 2010; Wang 2011
Isoleucine	E				X							Shaham 2008	Shaham 2008
Leucine	E				X							Shaham 2008; Spegel 2010	Shaham 2008; Spegel 2010
Lysine	E										Carnitine Synthesis, Biotin Metabolism	Lanza 2010; Suhre 2010; Wang 2011	Shaham 2008; Spegel 2010; Zhao 2009
Methionine	E			X					X		Spermidine / Spermine Biosynthesis, Betaine Metabolism	Spegel 2010	Lanza 2010
Ornithine	NE	X		X			X				Spermidine / Spermine Biosynthesis	Spegel 2010	Oresic 2008; Wang 2011; Zhao 2010b
Phenylalanine	E										Phenylalanine and Tyrosine Metabolism	Zhao 2009	Lanza 2010; Wang 2011
Pyroglutamate	n.a.		X									Shaham 200	Lanza 2010; Nicholson 1984; Wang 2011
Threonine	E			X							Threonine and 2-Oxobutanoate Degradation	Shaham 2008; Spegel 2010	
Tryptophan	E										Tryptophan Metabolism	Messana 1998; Nicholson 1984; Oresic 2008; Zuppi 2002	
Tyrosine	NE										Phenylalanine and Tyrosine Metabolism, Catecholamine Biosynthesis	Shaham 2008	Shaham 2008
Valine	E				X						Propanoate Metabolism	Lanza 2010	

Pathways were listed according to the Human Metabolome Database. N. s. = not specified.

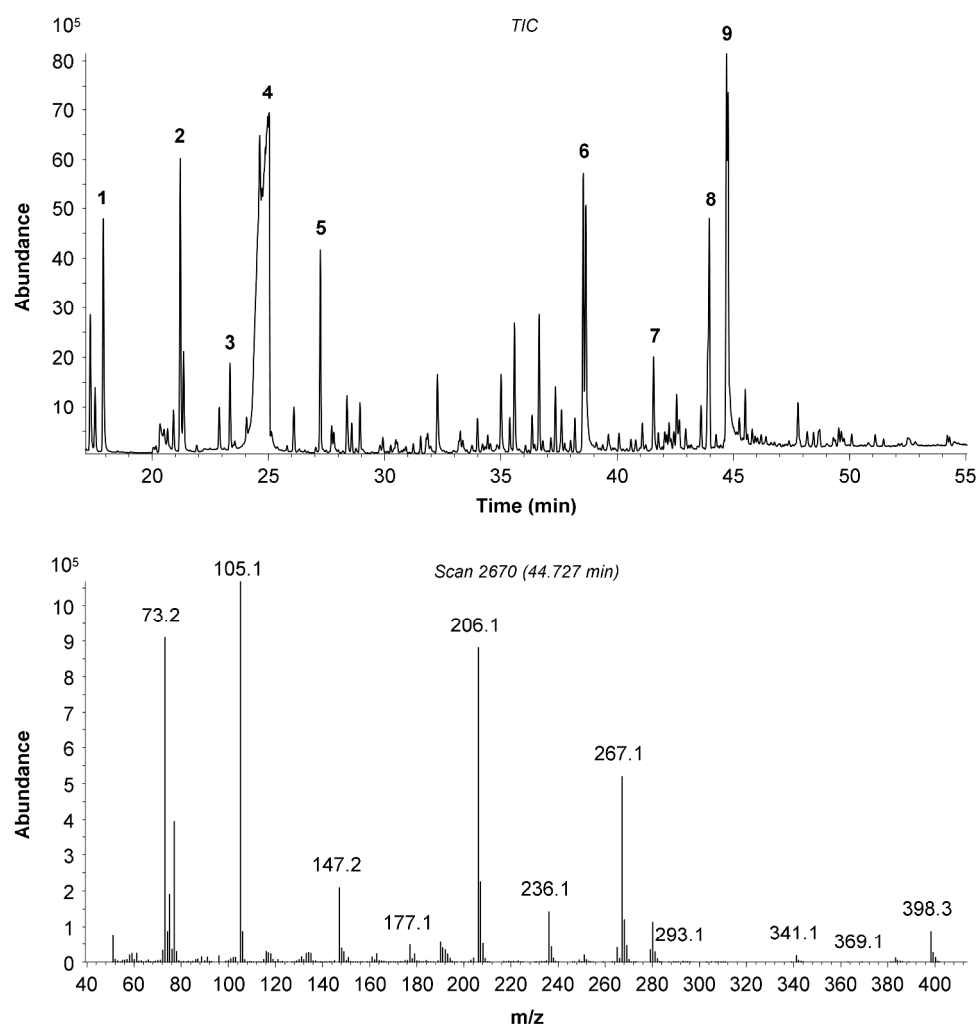
Table 2. List of altered fatty acids in patients with diabetes or during an oral glucose tolerance test (OGTT).

Pathways			Reference Patient studies	Reference OGTT studies
Saturated fatty acid (SFA)				
C6:0	Caproic acid	Beta Oxidation of Very Long Chain FA, FA Biosynthesis, Mitochondrial Beta-Oxidation of Short Chain Saturated FA	Suhre 2010	
C7:0	Heptanoic acid	n. s.	Suhre 2010	
C9:0	Pelargonic acid	n. s.	Suhre 2010	
C12:0	Lauric acid	Beta Oxidation of Very Long Chain FA, FA Biosynthesis, Mitochondrial Beta-Oxidation of Medium Chain Saturated FA	Spegel 2010	Yi 2007; Yi 2008
C14:0	Myristic Acid	FA Biosynthesis	Zhao 2009	Yi 2007; Yi 2008
C15:0	Pentadecanoic acid	n. s.		Li 2009; Yi 2007; Yi 2008; Yi 2006; Zhao 2010b
C16:0	Palmitic acid	FA Biosynthesis, FA Elongation In Mitochondria, FA Metabolism, Glycerolipid Metabolism	Zhao 2009	Yi 2007; Yi 2008; Yi 2006; Zhao 2010b
C18:0	Stearic acid	Mitochondrial Beta-Oxidation of Long Chain Saturated FA	Spegel 2010; Zhao 2009	Yi 2007; Yi 2008
C20:0	Arachidic acid	n. s.		Yi 2007; Yi 2008; Yi 2006
C24:0	Lignoceric acid	Beta Oxidation of Very Long Chain FA		
Monounsaturated fatty acid (MUFA)				
C14:1, C20:1	-	n. s.	Spegel 2010	Yi 2007; Yi 2008; Yi 2006; Zhao 2010b
C16:1	C16:1n-7: Palmitoleic acid C16:1n-9: Hexadecenoic acid	n. s.	Spegel 2010; Zhao 2009	
C18:1	C18:1n-7: cis-Vaccenic acid C18:1n-9: Oleic acid	n. s.	Li 2009; Yi 2007; Yi 2008; Zhao 2010b	Spegel 2010; Zhao 2009
Polyunsaturated fatty acid (PUFA)				
C18:2	C18:2n-6: Linoleic acid	α -Linolenic Acid / Linoleic Acid Metabolism	Zhao 2010b	
C18:3	C18:3n-3: α -linolenic acid C18:3n-6: γ -linolenic acid	α -Linolenic Acid / Linoleic Acid Metabolism	Yi 2007; Yi 2008; Zhao 2010b	Zhao 2009
C20:2	-	n. s.	Zhao 2009	Yi 2007; Yi 2008
C20:3, C22:4	-	n. s.	Zhao 2009	Yi 2008; Zhao 2010b
C20:4	C20:4n-6: Arachidonic acid	α -Linolenic Acid / Linoleic Acid Metabolism	Zhao 2009	Zhao 2010b
C20:5, C22:6	-	n. s.		
C22:5	-	n. s.	Mihalik 2010	
Carnitine	-	Oxidation of Branched Chain FA, Carnitine Synthesis, Beta Oxidation of Very Long Chain FA, Mitochondrial Beta-Oxidation of Long/Short Chain Saturated FA	Mihalik 2010	
Acylcarnitines				
C3	Propionylcarnitine	Oxidation of Branched Chain FA	Mihalik 2010	
C4, C4OH	(Hydroxy-)Butyrylcarnitine	n. s.	Mihalik 2010	
C5, C5OH	(Hydroxy-)Valeryl carnitine	n. s.	Mihalik 2010	Zhao 2009
C6, C6OH	(Hydroxy-)Hexenoylcarnitine	n. s.	Zhao 2009	Mihalik 2010
C8	Octanoylcarnitine	Mitochondrial Beta-Oxidation of Short Chain Saturated FA	Zhao 2009	Mihalik 2010
C10, C10:1	Decanoylcarnitine	n. s.		Mihalik 2010
C12	Dodecanoylcarnitine	n. s.	Suhre 2010	
C14:1, C14OH	(Hydroxy-)Tetradecanoylcarnitine	n. s.	Suhre 2010	
C16, C16OH	(Hydroxy-)Hexadecanoylcarnitine	n. s.	Suhre 2010	
C18, C18:1	Octadecanoylcarnitine	n. s.	Spegel 2010	Yi 2007; Yi 2008

Pathways were listed according to the Human Metabolome Database. N. s. = not specified. FA = fatty acids.



Assigned high resolution ¹H nuclear magnetic resonance spectrum of a human plasma (600 MHz) and human urine (400MHz) sample.
910x806mm (96 x 96 DPI)



GC-MS analysis of organic acids in human urine. Above - total ion current (TIC) chromatogram; below - mass spectrum at retention time 44.727min.
888x937mm (96 x 96 DPI)