THEMATIC REVIEW

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 on 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 highlight 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 has revealed new biomarkers. Recent epidemiological studies first 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 summarises the current findings of metabolic research regarding diabetes in animal models and human investigations.

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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 on the health systems of many countries. The World Health Organization (WHO) reported that worldwide 171 million individuals were affected by diabetes in 2000, which is equivalent to a prevalence of 2·8%, and 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 from the International Diabetes Federation (IDF) revealed that this number has already been reached 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 β-cell function and consequently a lack of insulin production (type 1: T1DM) or to the development of insulin resistance and subsequently the loss of β-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 was reported to be the main genetic determinant (Polychronakos & Li 2011, Noble & Erlich 2012). 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 & Roden 2011). Moreover, a 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 & Roden 2011, Polychronakos & Li 2011).

The diagnosis of diabetes is mainly based on the results of blood tests examining fasting plasma glucose or 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 healthy diet, performing regular physical activity, achieving a normal body weight and smoking cessation, are recommended for diabetic patients.

This paper is one of three papers that form part of a thematic review section on Metabolomics. The Guest Editor for this section was Henri Wallasschofski, Ernst-Moritz-Arndt University, Greifswald, Germany.

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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 an 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 *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 ~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 & Wilson 2007) and can provide complementary snapshots of the metabolome of body fluids such as plasma, urine or cerebrospinal fluid (Bictash *et al.* 2010).

NMR spectroscopy

NMR is a widely used spectroscopic technique for metabolomics that is based on the magnetic properties of the atomic nucleus (e.g. ^{1}H, ^{13}C or ^{31}P). This method was first used for the analysis of body fluids in the 1980s (Nicholson *et al.* 1984, Iles *et al.* 1985, Bell *et al.* 1989). 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 ^{1}H nucleus, ^{1}H-NMR spectroscopy has been heavily used to investigate biological fluids such as plasma and urine as well as tissues. Each separate signal in an ^{1}H-NMR spectrum corresponds to a particular compound. Exemplary ^{1}H-NMR spectra for human urine and plasma are shown in Fig. 1. Based on measurements of the following:

i) chemical shift,
ii) 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,
iii) relaxation: describes the return to equilibrium of net magnetisation and included two types of relaxation: spin-lattice (T_1, also called longitudinal relaxation) and spin-spin (T_2, also called transverse relaxation),
iv) 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 & 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

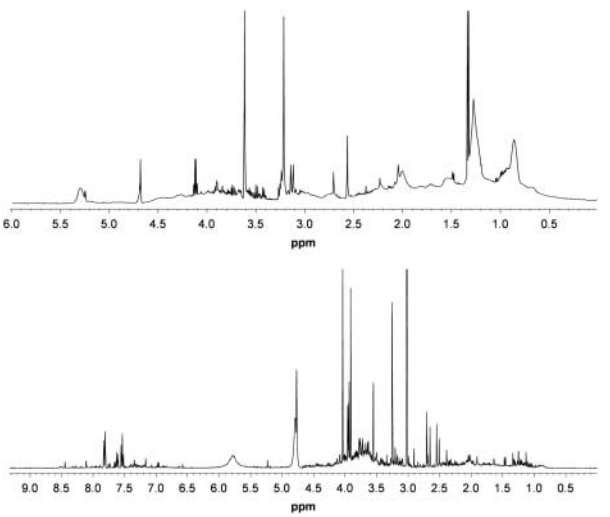

Figure 1 Assigned high-resolution ^{1}H-NMR spectrum of a human plasma (600 MHz) and human urine (400 MHz) sample.

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 & 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 and LC–MS demonstrate high separation efficiency and are excellent tools for metabolic profiling. Exemplary MS spectrum for human urine is shown in Fig. 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 & 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.* 2006*a*).

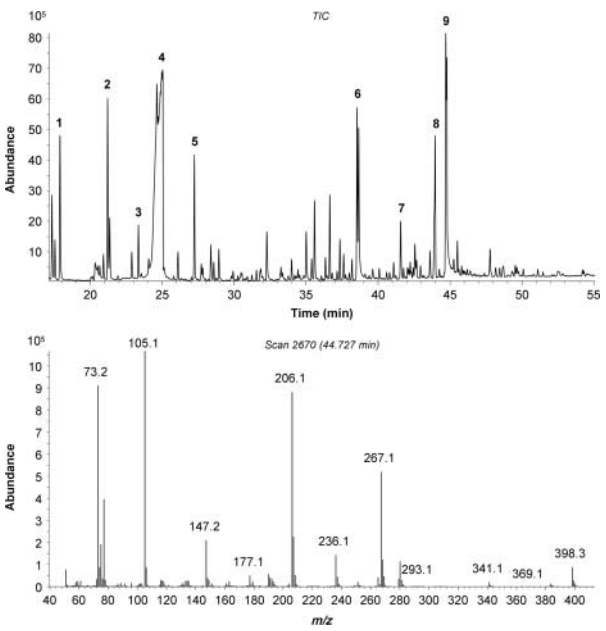

Figure 2 GC–MS analysis of organic acids in human urine. Above – total ion current (TIC) chromatogram: (1) glycolic, (2) p-cresol, (3) 3-hydroxyisovaleric, (4) urea, (5) succinate, (6) hydroxyphenylacetic, (7) aconitic, (8) citric, and (9) 4-(3-hydroxyprop-1-enyl)phenol. Below – mass spectrum at retention time 44·727 min.

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 (Shearer *et al.* 2008, Li *et al.* 2010*a*) or tissue (Li *et al.* 2010*a*, 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 ^{1}H-NMR-based metabolite concentrations in comparison with 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). ^{1}H-NMR spectroscopy also revealed diet-related variations in the levels of betaine, taurine, acetone/acetoacetate, phenylacetylglycine, pyruvate, lactate and citrate. MS-based studies can also detect additional diet-induced changes in metabolites (Li *et al.* 2010*a*, 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-deficient mice, which remain insulin sensitive independent of their diet (Li *et al.* 2010*a*). Assuming that metabolic changes identified in insulin-resistant WT 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*-acetylglutamate, 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- 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 on 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 ^{1}H-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, both are characterised by an autosomal recessive defect in the leptin receptor gene that results in obesity and subsequent insulin resistance (Chen & Wang 2005). Based on these models, differences in urinary (Williams *et al.* 2005*a*, 2006*b*, Salek *et al.* 2007, Gipson *et al.* 2008, Connor *et al.* 2010, Zhao *et al.* 2010*a*, Patterson *et al.* 2011), plasma (Major *et al.* 2006, Williams *et al.* 2006*a*) and tissue (Xu *et al.* 2009, Connor *et al.* 2010) 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.* 2005*a*, 2006*b*, Zhao *et al.* 2010*a*) typically have decreased urinary concentrations of TCA metabolites, such as citrate, malate, fumarate, 2-ketoglutarate or succinate, the *db/db* mouse (Salek *et al.* 2007, Connor *et al.* 2010) 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 twofold higher levels of citrate compared with 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 *et al.* (2006*b*) reported that the urinary ratios of α-ketoglutarate and succinate to citrate were in favour of citrate at an age of 4 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 8 weeks, whereas taurine was absent in 50% of the Zucker animals at 20 weeks (Williams *et al.* 2006*b*). 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 (Salek *et al.* 2007, Gipson *et al.* 2008, Connor *et al.* 2010), *db/db* mice (Gipson *et al.* 2008, Connor *et al.* 2010) and monkeys (Patterson *et al.* 2011) than in control animals, although lower concentrations of these compounds have also been reported (Williams *et al.* 2006*b*, Salek *et al.* 2007). 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 used 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. Furthermore, 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 β-cell adaptation to hyperglycaemia (Um *et al.* 2004, Khamzina *et al.* 2005, Fraenkel *et al.* 2008). 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 (Williams *et al.* 2006*b*, Salek *et al.* 2007). 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 (Williams *et al.* 2006*b*, Connor *et al.* 2010). 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 in the liver and higher urinary carnitine levels, 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.* 2005*b*). Increased fatty acid metabolism results from a higher rate of lipolysis in adipose tissue and might exacerbate insulin resistance in liver and muscle tissue (Delarue & 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, compared with controls (Salek *et al.* 2007, Zhao *et al.* 2010*a*). 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 (Williams *et al.* 2005*a*, 2006*b*, Salek *et al.* 2007, Gipson *et al.* 2008, Zhang *et al.* 2008, Zhao *et al.* 2010*a*, Patterson *et al.* 2011).

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 (Salek *et al.* 2007, Connor *et al.* 2010) but decreased in Zucker rats (Williams *et al.* 2006*b*, Salek *et al.* 2007, Zhao *et al.* 2010*a*), whereas microbiota-derived methylamines such as dimethylamine or trimethylamine-*N*-oxide were shown to be increased in both types of rodents (Salek *et al.* 2007, Gipson *et al.* 2008, Zhang *et al.* 2008, Connor *et al.* 2010, Zhao *et al.* 2010*a*). 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 (Shaham *et al.* 2008, Zhao *et al.* 2009, Spegel *et al.* 2010, Matysik *et al.* 2011). 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, 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, glycochenodeoxycholic acid (GCDCA) and taurochenodeoxycholic acid, 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 sixfold 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. On 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 threefold 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 homoeostasis 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.* 2006, 2010*b*), which suggests the existence of glucose/insulin-regulated gene transcription in the liver. The *CYP7A1* gene encodes the enzyme cholesterol 7α-hydroxylase, which is the rate-limiting enzyme in the cholesterol catabolic pathway and in 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 homoeostasis. Hence, it is not surprising that 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 compared with subjects with normal glucose tolerance (Zhao *et al.* 2010*b*). 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 cholesterol as well as improvements in glycaemic control in T2DM patients (Garg & Grundy 1994, Suzuki *et al.* 2006, Zieve *et al.* 2007, Kondo & Kadowaki 2010). Compared with patients who received control treatments or placebos, T2DM patients given bile acid sequestrants demonstrated greater reductions in the levels of plasma glucose and HbA1c.

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; Zhao *et al.* 2009, Spegel *et al.* 2010) were reported. The study by 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 β-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:

i) 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 compared with healthy controls. Furthermore, in both T1DM and T2DM

Table 1 List of altered amino acids in patients with diabetes or during an OGTT. Pathways were listed according to the Human Metabolome Database

Amino acids	Essential (E) non-essential (NE)	Alanine metabolism	Glutathione metabolism	Glycine and serine metabolism	Valine, leucine/ isoleucine 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	Nicholson *et al.* (1984), Messana *et al.* (1998), Zuppi *et al.* (2002) and Oresic *et al.* (2008)	
Arginine	E	X		X			X					Shaham *et al.* (2008)	Shaham *et al.* (2008)
Citrulline	NA						X				Arginine and proline metabolism, aspartate metabolism	Lanza *et al.* (2010)	
Glutamate	NE	X	X	X		X	X			X	Arginine and proline metabolism, amino sugar metabolism, cysteine metabolism, folate metabolism	Messana *et al.* (1998) and Lanza *et al.* (2010)	Spegel *et al.* (2010)
Glycine	NE	X	X	X				X	X		Carnitine synthesis, porphyrin metabolism	Shaham *et al.* (2008)	Suhre *et al.* (2010)
Histidine	E									X			Lanza *et al.* (2010) and Wang *et al.* (2011)
Homocitrulline	NS											Shaham *et al.* (2008) and Spegel *et al.* (2010)	Lanza *et al.* (2010) and Wang *et al.* (2011)
Isoleucine	E				X							Shaham *et al.* (2008)	Shaham *et al.* (2008)
Leucine	E				X							Shaham *et al.* (2008) and Spegel *et al.* (2010)	Shaham *et al.* (2008) and Spegel *et al.* (2010)
Lysine	E										Carnitine synthesis, biotin metabolism	Lanza *et al.* (2010), Suhre *et al.* (2010) and Wang *et al.* (2011)	Shaham *et al.* (2008), Zhao *et al.* (2009) and Spegel *et al.* (2010)
Methionine	E			X					X		Spermidine/spermine biosynthesis, betaine metabolism	Spegel *et al.* (2010)	Lanza *et al.* (2010)

(continued)

Table 1 *Continued*

Amino acids	Essential (E) non-essential (NE)	Alanine metabolism	Glutathione metabolism	Glycine and serine metabolism	Valine, leucine/ isoleucine degradation	Glucose–alanine cycle	Urea cycle	Bile acid biosynthesis	Methionine metabolism	Histidine metabolism	Further pathways	Reference patient studies	Reference OGTT studies
Ornithine	NE	X		X			X				Spermidine/spermine biosynthesis	Spegel *et al.* (2010)	Oresic *et al.* (2008), Zhao *et al.* (2010*b*) and Wang *et al.* (2011)
Phenylalanine	E										Phenylalanine and tyrosine metabolism	Zhao *et al.* (2009)	Lanza *et al.* (2010) and Wang *et al.* (2011)
Pyroglutamate	NA		X									Shaham *et al.* (2008)	Nicholson *et al.* (1984), Lanza *et al.* (2010) and Wang *et al.* (2011)
Threonine	E			X							Threonine and 2-oxobutanoate degradation	Shaham *et al.* (2008) and Spegel *et al.* (2010)	
Tryptophan	E										Tryptophan metabolism	Nicholson *et al.* (1984), Messana *et al.* (1998), Zuppi *et al.* (2002) and Oresic *et al.* (2008)	
Tyrosine	NE										Phenylalanine and tyrosine metabolism, catecholamine biosynthesis	Shaham *et al.* (2008)	Shaham *et al.* (2008)
Valine	E				X						Propanoate metabolism	Lanza *et al.* (2010)	

NS, not specified, NA, not applicable.

Table 2 List of altered fatty acids in patients with diabetes or during an OGTT. Pathways were listed according to the Human Metabolome Database

		Pathways	Reference patient studies	Reference OGTT studies
Saturated fatty acid (SFA)				
C6:0	Caproic acid	β-Oxidation of very long-chain FA, FA biosynthesis, mitochondrial β-oxidation of short-chain saturated FA	Suhre *et al.* (2010)	
C7:0	Heptanoic acid	NS	Suhre *et al.* (2010)	
C9:0	Pelargonic acid	NS	Suhre *et al.* (2010)	
C12:0	Lauric acid	β-Oxidation of very long-chain FA, FA biosynthesis, mitochondrial β-oxidation of medium-chain saturated FA	Spegel *et al.* (2010)	Yi *et al.* (2007, 2008)
C14:0	Myristic acid	FA biosynthesis	Zhao *et al.* (2009)	Yi *et al.* (2007, 2008)
C15:0	Pentadecanoic acid	NS		Yi *et al.* (2006, 2007, 2008), Li *et al.* (2009) and Zhao *et al.* (2010*b*)
C16:0	Palmitic acid	FA biosynthesis, FA elongation in mitochondria, FA metabolism, glycerolipid metabolism	Zhao *et al.* (2009)	Yi *et al.* (2006, 2007, 2008) and Zhao *et al.* (2010*b*)
C18:0	Stearic acid	Mitochondrial β-oxidation of long-chain saturated FA	Zhao *et al.* (2009) and Spegel *et al.* (2010)	Yi *et al.* (2007, 2008)
C20:0	Arachidic acid	NS		Yi *et al.* (2006, 2007, 2008)
C24:0	Lignoceric acid	β-Oxidation of very long-chain FA		
Monounsaturated fatty acid (MUFA)				
C14:1, C20:1	–	NS	Spegel *et al.* (2010)	Yi *et al.* (2006, 2007, 2008) and Zhao *et al.* (2010*b*)
C16:1	C16:1n-7: palmitoleic acid C16:1n-9: hexadecenoic acid	NS	Zhao *et al.* (2009) and Spegel *et al.* (2010)	
C18:1	C18:1n-7: *cis*-vaccenic acid C18:1n-9: oleic acid	NS	Yi *et al.* (2007, 2008), Li *et al.* (2009) and Zhao *et al.* (2010*b*)	Zhao *et al.* (2009) and Spegel *et al.* (2010)
Polyunsaturated fatty acid (PUFA)				
C18:2	C18:2n-6: linoleic acid	α-Linolenic acid/linoleic acid metabolism	Zhao *et al.* (2010*b*)	
C18:3	C18:3n-3: α-linolenic acid C18:3n-6: γ-linolenic acid	α-Linolenic acid/linoleic acid metabolism	Yi *et al.* (2007, 2008) and Zhao *et al.* (2010*b*)	Zhao *et al.* (2009)
C20:2	–	NS	Zhao *et al.* (2009)	Yi *et al.* (2007, 2008)
C20:3, C22:4	–	NS	Zhao *et al.* (2009)	Yi *et al.* (2008) and Zhao *et al.* (2010*b*)
C20:4	C20:4n-6: arachidonic acid	α-Linolenic acid/linoleic acid metabolism	Zhao *et al.* (2009)	Zhao *et al.* (2010*b*)
C20:5, C22:6	–	NS		
C22:5	–	NS	Mihalik *et al.* (2010)	
Carnitine	–	Oxidation of branched-chain FA, carnitine synthesis, β-oxidation of very long-chain FA, mitochondrial β-oxidation of long/short-chain saturated FA	Mihalik *et al.* (2010)	
Acylcarnitines				
C3	Propionylcarnitine	Oxidation of branched-chain FA	Mihalik *et al.* (2010)	
C4, C4OH	(Hydroxy-)butyrylcarnitine	NS	Mihalik *et al.* (2010)	

(continued)

Table 2 *Continued*

		Pathways	Reference patient studies	Reference OGTT studies
C5, C5OH	(Hydroxy-)valerylcarnitine	NS	Mihalik *et al.* (2010)	Zhao *et al.* (2009)
C6, C6OH	(Hydroxy-)hexenoylcarnitine	NS	Zhao *et al.* (2009)	Mihalik *et al.* (2010)
C8	Octanoylcarnitine	Mitochondrial β-oxidation of short-chain saturated FA	Zhao *et al.* (2009)	Mihalik *et al.* (2010)
C10, C10:1	Decanoylcarnitine	NS		Mihalik *et al.* (2010)
C12	Dodecanoylcarnitine	NS	Suhre *et al.* (2010)	
C14:1, C14OH	(Hydroxy-)tetradecanoylcarnitine	NS	Suhre *et al.* (2010)	
C16, C16OH	(Hydroxy-)hexadecanoylcarnitine	NS	Suhre *et al.* (2010)	
C18, C18:1	Octadecanoylcarnitine	NS	Spegel *et al.* (2010)	Yi *et al.* (2007, 2008)

NS, not specified; FA, fatty acids.

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).

ii) 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).

iii) 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 (Nicholson *et al.* 1984, Messana *et al.* 1998, Suhre *et al.* 2010) subjects, indicating ketoacidotic metabolic decompensation.

iv) 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.* 2010*b*). The human gut microbiota has an important role in health, which has been comprehensively discussed by several authors (Nicholson *et al.* 2005, Fujimura *et al.* 2010, 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 (Williams *et al.* 2006*b*, Salek *et al.* 2007), increased fatty acid levels were detected in T2DM patients (Yi *et al.* 2006, 2007, 2008, Li *et al.* 2009, Suhre *et al.* 2010) as well as in subjects with impaired glucose tolerance (Zhao *et al.* 2010*b*). 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 compared with 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 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 (Opie & Walfish 1963, Jensen *et al.* 1989, Newgard *et al.* 2009). Furthermore, higher levels of fatty acids are related to a greater risk for diabetes (Paolisso *et al.* 1995, Charles *et al.* 1997, Pankow *et al.* 2004), 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 (Griffin *et al.* 1999, Boden 2003, Wilding 2007). Moreover, the improvement in insulin sensitivity caused by a reduction in fatty acid levels supports these findings (Boden *et al.* 1998, Santomauro *et al.* 1999, Cusi *et al.* 2007). 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 (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.* 2010*b*). In addition, these findings have been confirmed by studies that revealed positive associations between amino acid levels and the homoeostasis 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 (Messana *et al.* 1998, Newgard *et al.* 2009, Lanza *et al.* 2010). A recent investigation (Wang *et al.* 2011) of the Framingham Offspring Study first 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 before 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 fivefold 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 (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 i.v. 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, 1968). However, the underlying mechanisms are complex and are related to the inhibition of glucose transport and gluconeogenesis (Patti *et al.* 1998, Krebs *et al.* 2002, Langenberg & Savage 2011).

Conclusion and challenges for the future

The application of metabolomics in diabetes studies has rapidly evolved during the last decade and provides researchers the opportunity to gain new insights into 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 & Savage 2011) discussed the potential of an amino acid profile as a predictor of T2DM and highlighted the fact that the addition of amino acids to established risk factors only minimally 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 increases the knowledge of disease progression and provides approaches for therapy.

Declaration of interest

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