Expert Opinion

1. Introduction
2. Diabetic complications
3. Disease biomarkers
4. Proteomic studies
5. Expert opinion

Proteomics as a tool to discover biomarkers for the prediction of diabetic complications

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Background: The incidence of diabetes is increasing rapidly, owing to unhealthy lifestyles. The human suffering and healthcare burden caused by diabetes are mainly a consequence of its microvascular and macrovascular complications. Late diagnosis and lack of strict metabolic control lead to increased morbidity and mortality. A problem is the insidious course of the complications, diagnosed in advanced stages, when end organ damage is already present. **Objective:** Discovery of early markers of microangiopathy and macroangiopathy may help to identify patients at risk of organ damage. **Methods:** This review focuses on recent developments in proteomics, performed on body fluids and different tissues implicated in microvascular and macrovascular complications. **Results:** The results provide important information for the early detection of diabetic complications as well as for better understanding of their pathophysiology.

Keywords: biomarkers, diabetic complications, proteomics, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, two-dimensional gel electrophoresis

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1. Introduction

Diabetes mellitus (DM) is a complex multi-organ disease that is characterized by defects in insulin action and secretion. These defects result in hyperglycemia but also in disturbances in the metabolism of fatty acids and proteins [1]. Type 1 and Type 2 diabetes are the two most common forms of the disease. Type 1 diabetes (T1D) results from an autoimmune destruction of pancreatic islet β -cells causing loss of insulin production. This leads to hyperglycemia and absolute dependence on insulin treatment for survival. Type 2 diabetes (T2D) is a dual disease where both defects in the insulin-secreting β -cell and increased insulin resistance in the target organs (liver, skeletal muscles and adipocytes) are present [2-4]. The cause of the progressive β -cell failure remains elusive, but especially unhealthy lifestyles (high-calorie foods and low exercise levels) leading to weight excess are the cause for the increase in insulin resistance. About 194 million people worldwide were estimated to have diabetes in 2003, a number expected to increase to 333 million, or 6.3% of the adult population, by 2025.

Diabetes places a particularly heavy burden on the health services, not so much because of the cost of treatment of hyperglycemia itself, but especially due to the high cost of treating the diabetic complications. If predictions of diabetes prevalence are fulfilled, the proportion of the world's healthcare budget spent on diabetes care will be 7 – 13% [5].

Landmark studies have shown a direct correlation between hyperglycemia (expressed as elevated HbA1c) and diabetic complications in both Type 1 and Type 2 diabetes (Figure 1) [6-9], but for the individual patient the appearance of complications is not predictable, forcing patients to undergo regular screening

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Figure 1. Schematic overview of the onset and progress of diabetes mellitus.

FFA: Free fatty acids.

tests for retinopathy, neuropathy, nephropathy and cardiovascular disease. These tests are cumbersome and often screening is not performed, with complications being detected at a late stage when intensification of therapy has only minor effects. Availability of biomarkers allowing detection of predisposition to development of complications or early stages of complications would allow detection of patients at risk or at a stage when intensification of therapy can still stop progression. Having these tools would allow intensification of efforts to be prioritized specifically in the patients with a high risk.

Recent developments in proteomics play a dominant role in the discovery of such robust biomarkers. Furthermore, these results can also achieve a better understanding of the pathways causing micro- and macroangiopathies [10]. This review focuses on the use of proteomics in the search for new disease biomarkers to detect diabetic complications at an early stage (Table 1). Mainly human studies are focused on, since biomarkers that are initially identified in animals have to be confirmed in human patients before they can be used as a valuable biomarker.

2. Diabetic complications

2.1 Macrovascular complications

Atherosclerosis is a chronic inflammatory condition initiated by an oxidative stress in the endothelium. It has been found before that hyperglycemia, dyslipidemia and hypertension all increase oxidative stress in endothelial cells and this stress is maintained through interactions between modified lipoproteins, macrophages and arterial wall constituents [11,12]. The ultimate problems of atherosclerosis are plaque rupture, thrombosis and major vessel occlusion. The driving factor for this increased risk is pro-coagulability, an increase in platelet aggregation, coupled with an increase in plasma concentrations of plasminogen activator inhibitor 1 (PAI-1) and other thrombotic factors [13].

End stage organ damage involves myocardial infarctions, stroke and peripheral vascular damage, leading to foot problems. These complications account for 70% of the deaths of diabetic patients and are responsible for major human suffering and healthcare costs. Again, when this damage is detected, intensifying metabolic control can only

Table 1. List of the clinical studies of diabetic complications and replicated studies.

Biomarker	Regulation	Tissue	Ref.	Replicated studies
Macrovascular complications				
Apolipoprotein C-III	Increase	Serum	Davidsson <i>et al.</i> [46]	Zhang <i>et al.</i> [39]
Microvascular complications				
Retinopathy				
Pigment epithelium derived factor	Decrease	Vitreous humor	Garcia-Ramirez <i>et al.</i> [59]	Spranger <i>et al.</i> [60] Ogata <i>et al.</i> [61]
Zinc- α -2-glycoprotein	Increase	Vitreous humor	Garcia-Ramirez <i>et al.</i> [59]	Gohda <i>et al.</i> [64]
Nephropathy				
Transport protein vitamin D-binding protein	Increase	Urine	Rao <i>et al.</i> [66]	
α 1-Antitrypsin	Increase	Urine	Rao <i>et al.</i> [66]	
Calgranulin B	Increase	Urine	Rao <i>et al.</i> [66]	
α 1-Microglobulin/bikunin precursor	Decrease	Urine	Rao <i>et al.</i> [66]	
Apolipoprotein A-I	Decrease	Urine	Rao <i>et al.</i> [66]	
β 2-Microglobulin	Increase	Urine	Dihaza <i>et al.</i> [68]	Schaub <i>et al.</i> [69] Hong <i>et al.</i> [70]
Ubiquitin A-52 residue ribosomal protein fusion product 1	Increase	Urine	Dihaza <i>et al.</i> [68]	Schaub <i>et al.</i> [69] Hong <i>et al.</i> [70]
Tamm-Horsfall protein	Decrease	Urine	Mischak <i>et al.</i> [73]	Torffvit <i>et al.</i> [74]

slow progression, not reverse damage. At present, detection before damage involves associated risk factors (e.g., low-density lipoprotein [LDL] cholesterol), but again only cumbersome examinations such as cycloergometry or even heart catheterization are available. The main attention is therefore focused on development of surrogate markers. Some of these, for example sensitive C-reactive protein, have been shown to be useful, but their clinical applicability is still awaited.

2.2 Microvascular complications

Microangiopathies refer to damage of the small blood vessels, especially supplying the kidney (nephropathy) and the eye (retinopathy). Also, the damage to neurons seen in diabetes is partly due to microangiopathy [14].

Diabetic nephropathy (DN) associated with diabetes is the most common cause of end stage renal disease. The earliest putative diagnostic sign for the presence of diabetic renal damage is a stable increase in albumin excretion rate, that is, microalbuminuria [15,16], which can progress to macroalbuminuria and subsequently to kidney failure [17]. Multiple studies suggest that both genetic and environmental factors (hypertension, poor glycemic and lipid control, smoking) play a role in the development and progression of DN [18–20]. In T1D, microalbuminuria is the best predictor for subsequent development of nephropathy, with ~ 50% of patients with microalbuminuria progressing to overt

nephropathy. Conversely, in T2D patients, microalbuminuria is not specific for diabetic renal damage because it may also reflect generalized vascular injury or renal damage from arterial hypertension or heart failure [15,21,22]. As a consequence, the presence of microalbuminuria in T2D patients is often a better predictor of cardiovascular disease than of DN [23]. Owing to the association of DN with cardiovascular disease risk, there is an enormous public health burden associated with this complication [24].

Diabetic retinopathy (DR) is one of the most common microvascular complications associated with diabetes and is the leading cause of blindness and visual impairment in adults aged < 40 years [25,26]. The development of DR is associated with long duration of diabetes, chronic hyperglycemia (expressed as elevated HbA1c levels) and high blood pressure [27]. Intervening at a late stage does not reverse the damage and, as in diabetic nephropathy, screening examinations at an asymptomatic stage are needed. This involves performing funduscopy of the eyes at regular intervals, again an examination dreaded by patients, since to visualize the periphery of the retina, a dilatation of the pupil needs to be achieved, impairing visual functioning for several hours. Landmark studies have demonstrated that when tight glycemic control, especially when combined with tight blood pressure control and other measures, for example stopping smoking, are initiated early on, at the stage of background DR progression, even reversal of retinopathy can be seen [6].

3. Disease biomarkers

A disease biomarker can be defined as a physical, functional or biochemical indicator of a disease process that is quantifiable and correlates well with clinical and biochemical measures of the disease progression. The ability to study and treat diabetic complications is hampered by a lack of these biomarkers. As people with T2D may not show any symptoms of complications for many years, the diagnosis is often made at a later stage of disease development, when interventions are less effective. Therefore, there is an urgent need for patient-friendly and early disease markers. Robust biomarkers would also reduce the need to achieve hard clinical end points in clinical trials and would help to lower the associated costs. So far, the two most important proteomic techniques to detect biomarkers are the classical two-dimensional gel electrophoresis (2-DE) and the surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) technique.

3.1 Two-dimensional gel electrophoresis

In 1994, the term proteomics was introduced and defined as 'the field that attempts the systematic analysis, identification and quantification of the proteins present in one or more biological samples' [28]. Classical 2-DE is a well-established technique for the simultaneous separation and display of hundreds to thousands of proteins [29]. The method separates proteins in two dimensions according to their isoelectric point and molecular mass. After protein visualization, the proteins of interest are excised and identified by mass spectrometry (MS).

The renewed 2-DE technique, called two-dimensional differential gel electrophoresis (2-D DIGE), makes it possible to analyze differential protein levels in protein samples with high statistical certitude (e.g., control versus disease), visualized by tagging the protein samples with two different fluorescent dyes (Cy3 and Cy5). A third dye is used to label an internal standard (comprises equal amounts of each sample to be compared) that will be used in every gel of the experiment to reduce the gel-to-gel variation and to increase the statistical power (Figure 2) [30,31]. Afterwards, the differentially expressed spots can be excised from the gel and identified by mass spectrometry [32]. The main disadvantages of this technique are the limitation of detecting only proteins with molecular masses in the range 15 – 150 kDa, the low throughput and the manual labor-intensive work.

The most troublesome step so far remains the procedure for sample preparation and mainly the protein degradation due to endogenous proteolytic activity [33]. Proteases must be inactivated to prevent protein degradation that otherwise may result in artefactual spots, loss of high molecular mass proteins or even a complete disappearance of proteins. In some tissues, such as the pancreas, exo-proteinases are present at high concentrations and are responsible for

artefactual protein heterogeneity. Proteolysis was tackled in the first place by the addition of proteinase inhibitors and thiourea in the lysis solution. Thiourea increases the efficiency in solubilizing proteins but also induces a protective effect on sensitive substrates towards endogenous proteolysis. Also, the use of basic pH during protein extraction, inactivating proteases by low pH (e.g., precipitation with ice-cold trichloroacetic acid) or boiling the sample in sodium dodecyl sulfate (SDS) for a few minutes, could be efficient in the reduction of unwanted proteolysis [34,35]. However, one should keep in mind that complete inactivation of all proteases may be rather difficult. In summary, inhibition of any artefactual modification by using highly reliable procedures during sample processing is of crucial importance to obtain biologically relevant data.

3.2 Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry

As the 2-D DIGE technique cannot detect proteins with low molecular masses and as disease biomarkers are often small proteins/peptides (< 20 kDa), SELDI-TOF MS is an interesting technique that has recently gained importance in studying these relatively small biomarker proteins and peptides. The technique combines the capture of proteins to a solid phase, a protein chip array, through a physiochemical interaction and subsequent TOF MS. The biological fluid of interest is pre-fractionated or loaded onto chemically treated supports with specific chromatographic features. After removal of unbound proteins, salts and lipids, the immobilized proteins are desorbed and ionized by a laser source and mass spectrometry spectra are acquired by a TOF mass spectrometer. The end result of a SELDI-TOF MS analysis is a list of molecular masses of proteins whose relative abundance differs significantly between two or more samples (e.g., control versus disease) (Figure 3) [36,37]. The possibility of applying biological samples without previous protein separation and the quantitiveness of the method allow rapid determination and analysis of the differential expression of sample protein profiles. The most important criticism of the use of this methodology is that proteins are usually not identified. Without this identification, it is difficult to support the hypothesis that these proteins are part of the affected tissue or its microenvironment, thus it is difficult to relate a protein peak to a disease state [38].

4. Proteomic studies

As complications of diabetes affect multiple organs, it becomes important to identify biomarkers associated with the different affected organs and tissues. As the affected organs secrete many proteins, blood is the primary source for the detection of potential biomarkers of different tissues in the body. Nevertheless, it has been demonstrated that specimen collection is a crucial step for successful biomarker discovery in human blood samples. Plasma is obtained by collecting blood in an anticoagulant solution and

Figure 2. Principle of 2-D DIGE technique. Two samples (e.g., control versus experimental) are labeled with two different fluorescent dyes (Cy3 and Cy5). A third fluorescent dye (Cy2) is used to label an internal standard (comprises equal amounts of each sample to be compared) that will be used in every gel of the experiment to reduce the gel-to-gel variation and to increase the statistical power. Fluorescent imaging of the gel at the wavelengths specific for each dye generates images that can be overlaid directly by the DeCyder software for identification of any differentially expressed proteins between the samples. Afterwards, these differentially expressed spots can be excised from the gel and identified by mass spectrometry.

2-D DIGE: Two-dimensional differential gel electrophoresis.

subsequent centrifugation. For analysis and display of circulating low molecular mass peptides and proteins, the use of platelet-depleted EDTA or citrate plasma is recommended, as platelet contamination or activation leads to distinct and multiple peptide signals in plasma samples. In contrast, a large number of peptides, many of them in rather high abundance, are present only in serum and are not detectable in plasma. Owing to the high levels of albumin, IgG and transferrin in sera, it is also very difficult to mine low abundance biomarkers by traditional proteomic approaches. Zhang *et al.* [39] used multicolumn liquid chromatography (LC) before classical 1-D and 2-D to separate sera samples into different groups according to their biochemical and biophysical properties. Serum is obtained after coagulation,

a process that involves the activation of proteases in cascade. This can result to the formation of a clot containing activated leukocytes and platelets that can change the proteome pattern. Biological, individual and inter-individual variations can also affect proteomes and require careful consideration to find valid biomarkers in human blood [40]. Similar attention must be paid when isolating circulating cells, as many cells, such as polymorphonuclear neutrophils or platelets, may be activated during the isolation process [41]. Besides blood, vitreous liquid and urine can also be collected to detect disease biomarkers, respectively, for retinopathy and nephropathy. Alternatively, proteomic analysis can be performed directly on affected tissue samples. However, this approach is not always feasible because of the heterogeneity

Figure 3. Principle of SELDI-TOF technique. The biological fluid of interest is loaded onto chemically treated supports with specific chromatographic features. After removal of unbound proteins, salts and lipids, the immobilized proteins are desorbed and ionized by a laser source and mass spectrometry spectra are acquired by a TOF mass spectrometer. The end result of a SELDI-TOF MS analysis is a list of molecular masses of proteins whose relative abundance differs significantly between two or more samples (e.g., control versus disease).

SELDI-TOF MS: Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry.

of many tissues (e.g., an atherosclerotic plaque), as well as the problem of obtaining samples from healthy control subjects. In the next sections, the search for biomarkers by the use of proteomic techniques, related to microvascular and macrovascular complications, are discussed.

4.1 Macrovascular complications

Macrovascular complications are the main cause of DM-induced mortality. As the aorta represents tissue of the macrovasculature, the proteome of the descending aorta may provide an index for the development of diabetic macroangiopathy. As it is not possible to isolate aorta lysates of healthy controls, Cho *et al.* [42] used a rat model, where

they isolated aorta lysates and sera from streptozotocin-induced diabetic and control male rats to discover potential biomarkers. Using the SELDI-TOF MS technique, they detected 0, 4 and 3 biomarkers in the descending aorta to differentiate the DM group from the control group, respectively, at 4, 8 and 12 weeks of age. Five other biomarkers were found to have high classification values in the sera to differentiate the DM group and the control group at all time intervals. One serum biomarker was matched with islet amyloid polypeptide that was already positively correlated with DM [43,44]. Another potential serum biomarker was matched with resistin-like alpha and this was also in line with a previous report, which correlated resistin with DM [45].

Davidsson *et al.* [46] isolated different LDL subclasses from serum samples of T2D patients with subclinical atherosclerosis. The LDL fractions were analyzed further by SELDI-TOF MS. These T2D patients showed high circulating levels of small, dense LDL (sdLDL), enriched in apolipoprotein C-III (apoC-III) and deprived in ApoA-I. These sdLDL have an increased affinity for arterial proteoglycans and, once associated, they are modified by enzymatic and oxidative processes, causing increased uptake by macrophages [47]. Such alterations may lead to entrapment and modifications of sdLDL in the intima proteoglycan layer, a critical initial step in atherogenesis [48,49]. In addition, the diminished concentration of ApoA-I has been demonstrated to lower the protection of the arterial wall against atherogenic modifications [50]. This study proves that it is possible to use sdLDL concentrations and especially the composition of these particles as a biomarker of macroangiopathies at an early stage of disease. These observations were in accordance with the study of Zhang *et al.* [39], who also showed an upregulation of apoC-III in serum of T2D patients.

Nevertheless, these results are in contrast to the observations of Matsumura *et al.* [51], who reported an increased level of apoA-I and apoA-IV and a decreased level of apoE in the OLETF rats, an animal model of T2D with obesity [52]. The elevated level of ApoA-I, an anti-atherogenic protein in high-density lipoproteins, differs from human diabetic patients [53]. Also apoA-IV, a satiety signal mediator, was paradoxically increased in the hyperphagic OLETF rat. As there could be a difference in lipoprotein metabolism between humans and rats, it is clear that careful assessment of data is needed. Given that a biomarker is of great importance for clinical applications, it may be more relevant to search for and identify potential biomarkers directly in human samples.

In a study by Cho *et al.* [54], SELDI-TOF MS was applied to serum and tissues of diabetic rats after streptozotocin injection in a search for biomarkers. One potential biomarker was found in the kidney and the eye, three were discovered in the aorta and eight potential biomarkers could be detected in serum. One of these serum biomarker

candidates was found to match the C-reactive protein, which is associated with inflammation. This observation is in line with previous studies, demonstrating that elevated serum C-reactive protein levels correlate with diabetes development [55,56] and diabetic atherosclerosis [57]. This demonstrates that the development of DM is associated with inflammation, a finding that is confirmed by Sundsten *et al.* [10], who showed a lowering in albumin and tranferrin levels, both associated with inflammation [58], in serum of T2D patients compared with healthy people. Zhang *et al.* [39] also showed an upregulation of inflammation related proteins, such as haptoglobin and hemoglobin.

4.2 Microvascular complications

4.2.1 Retinopathy

Garcia-Ramirez *et al.* [59] used the 2-D DIGE technique to screen vitreous humor for new potential candidates involved in the pathogenesis of DR. They were able to identify 11 proteins in the vitreous fluid of DR patients compared with non-diabetic subjects: eight proteins were increased, whereas three were decreased. In line with previous reports [60,61], pigment epithelium-derived factor was decreased in DR patients. There is growing evidence to suggest that pigment epithelium-derived factor is the most important natural inhibitor of angiogenesis and is the main factor accounting for the anti-angiogenic activity of the vitreous fluid, where it is found in high quantities [62,63]. The zinc- α -2-glycoprotein 1 (ZAG) showed an increased abundance, but its exact role in the vitreous fluid of diabetic patients with DR is not yet clear. Interestingly however, it should be mentioned that hyperglycemia has been involved in ZAG production [64] and gene expression of ZAG has been found to be upregulated in mice with diabetic nephropathy [65].

As no differences were observed between the two groups in terms of serum levels of five of the identified proteins, eye production rather than serum diffusion seems to be the main factor accounting for the different protein abundances detected in diabetic patients. It is obvious that further studies are necessary to address the precise role of the candidate biomarkers in the pathogenesis of DR and their potential as therapeutic targets.

4.2.2 Nephropathy

Rao *et al.* [66] used 2-D DIGE to identify new disease biomarkers in urine samples of T2D patients. To improve the detection of low abundance proteins, the six most abundant serum proteins, such as immune globulin and albumin, were removed. Seven urine proteins were upregulated and four proteins were downregulated in diabetic nephropathy with macroalbuminuria when compared with samples from diabetic patients without albuminuria. Most of the upregulated biomarkers were glycoproteins that could potentially be representative of the hyperglycosylated state of these proteins in the serum

proteome. There was also an elevation in the transport protein vitamin D-binding protein and in two defense response proteins (α 1-antitrypsin and calgranulin B) in the urine of diabetic nephropathy patients. Among the down-regulated proteins, α 1-microglobulin/bikunin precursor and ApoA-1 were identified. Urinary α 1-microglobulin indicates proximal tubular dysfunction and could be a useful biomarker for the early detection of nephropathy in diabetic subjects in addition to albuminuria, which indicates glomerular dysfunction. It has already been demonstrated that ApoA-1 is an important cardiovascular risk factors [67], but, so far, its association with diabetic nephropathy has not been studied.

Dihazi *et al.* [68] used the SELDI-TOF MS technique to screen urine samples from diabetic nephropathy patients for diagnostic biomarkers. After separation of the sample fraction of interest by SDS – PAGE and in-gel digestion and mass spectrometry analysis, β 2-microglobulin could be identified and was found in larger amounts in the urine of patients with nephropathy compared with controls. This is in accordance with previous studies showing an association of altered β 2-microglobulin concentrations with diabetic nephropathy [69,70]. An alteration of ubiquitin A-52 residue ribosomal protein fusion product 1 (UbA52) concentration was also found in the urine of diabetic nephropathy patients: UbA52 is a ubiquitin fusion protein made of 60S ribosomal protein attached to a ubiquitin peptide [71]. These data support the hypothesis that the increased expression of UbA52 in tubular cells of diabetic nephropathy patients combined with acute tubular injury and cell apoptosis results in an alteration of the UbA52 concentration in urine of diabetic nephropathy patients. Next to the two upregulated proteins, a processed form of ubiquitin with m/z 6188 was missing in the urine of diabetic nephropathy patients. As a consequence, this ubiquitin form could also be used as a prognostic marker for diabetic nephropathy.

Otu *et al.* [72] examined in a 10-year follow-up study urine samples of Pima Indians with T2D and control subjects that were both normoalbuminuric and had normal renal function. They used the SELDI-TOF MS technology to distinguish patients who went on to develop diabetic nephropathy from those who did not within the 10-year period by producing a 12-peak urine protein signature. This protein signature appeared to be independently associated with the development of diabetic nephropathy. The main criticism of this study is that this 12-peak protein signature was investigated only for a unique subset of Pima Indians and needs to be validated in a larger group of patients with diabetes. It will also be necessary to identify the proteins in the signature to be sure that they are biologically important in the development of nephropathy.

Mischak *et al.* [73] used capillary electrophoresis coupled with mass spectrometry for a fast and accurate evaluation of 2000 polypeptides in the urine samples from healthy individuals and from patients with T2D with different

degrees of albumin excretion rate. By doing this, they could establish a normal and a specific diabetic pattern of polypeptide excretion. In patients with higher-grade albuminuria, they were able to detect a polypeptide pattern indicative of 'diabetic renal damage'. Surprisingly, almost all diabetic patients with retinopathy were also found in the group of patients with a urinary polypeptide pattern indicative for diabetic renal damage, leading to higher-grade albuminuria. Using mass spectrometry analysis, a fragment of the relaxin-like factor, a polypeptide from Tamm-Horsfall protein and an albumin fragment were identified. These polypeptides are protein fragments known to be affected when renal disease is present. The specific fragments might serve as better markers of diabetic nephropathy because the parental proteins are found only in urine once advanced renal damage has occurred. This result was in accordance with the findings of Torffvit *et al.* [74], who also reported a decrease in Tamm-Horsfall protein urinary excretion rate in patients with diabetic nephropathy.

5. Expert opinion

The above-mentioned studies show that the identification and characterization of the differentially expressed peaks may contribute to an early detection of the development of diabetic complications, but also to a better understanding of the development and progression of the disease. These efforts may lead to a diagnosis before clinical complications are present and as a consequence will result in a lowering of morbidity, mortality and health/economic costs. A major point of criticism is that most biomarkers detected with the use of the SELDI-TOF MS technique show only the m/z ratio of proteins that are different between the disease and the control group. As a consequence, the exact protein ID is not known, while this ID is required to validate the detected biomarker and to provide new insights into the biological pathways involved in the disease processes. However, the exact identification of SELDI peaks is an extremely difficult task, as SELDI has low mass resolution and does not possess MS/MS capability. As a result, protein peaks of interest have to be isolated and analyzed by using mass spectrometers with higher resolution. This purification of proteins is tedious and it is uncertain whether the purified protein is truly the protein peak observed by SELDI. Nevertheless, in recent years there have been a lot of technical improvements concerning the SELDI-TOF MS technology and these could lead to the development of a technique that couples separation and identification by MS/MS. Another important technical improvement was made a couple years ago by the introduction of the liquid chromatography mass spectrometry technique. In this approach, peptides generated from an enzymatic cleavage of the total protein mixture are separated by liquid chromatography, followed by fragmentation and identification using a mass spectrometer. This technique allows a high-throughput identification of all proteins,

including not very abundant proteins and proteins with extreme properties (highly acidic or basic, hydrophobic, membrane proteins). As it will become important to screen a large group of patients for disease biomarkers, LC-MS combined with a further automation will most probably contribute to this issue. In this view, it is also important to identify biomarkers in body fluids, which can be easily isolated by a non-invasive method. As many manipulations can influence the proteome, a standard protocol for isolation and identification is essential for accurate analysis, and will hopefully be developed in the near future. Another important improvement would be the pre-fractionation of complex mixtures, such as serum or plasma, to simplify these mixtures into less complex components, leading to the identification of low abundance proteins that often play an important biological role.

Another issue is sample collection, as handling during isolation can influence the proteome pattern. When disease biomarkers are identified in plasma or serum, it is also difficult to identify the organic source of the biomarker as all organs secrete proteins and peptides into the blood stream. Accordingly, when studying tissues it is difficult to rule out the influence of the heterogeneity of the tissue. Also, the isolation of tissue biopsies, especially in healthy controls, is a very invasive procedure. It should be questioned which type of sample, blood or tissue, should be used to discover serologic biomarkers. At present, there is still debate whether plasma or serum can serve as a window into the state of a patient's disease. There is also no scientific evidence that a linear relationship exists between protein changes in diseased tissue and in blood. To move the field forward, the type of sample used for biomarker studies should undoubtedly be confirmed.

Many of the studies discussed in this review have been performed only on small subpopulations. It is obvious that future studies should focus on evaluating the identified biomarkers in a large and heterogeneous group of patients and healthy controls. It should also be taken into account that the excretion of biomarkers varies with sex, age, hypertension and medication. In this view, biomarkers validated for one patient group may not be diagnostic in another. Therefore, inclusion and exclusion criteria will have to be clearly defined to come to a well-defined 'gold standard' diagnostic test. Moreover, many studies are being performed in animal models of a certain disease. Although these studies reveal very valuable data, it is important to keep in mind that there could be a difference in metabolism between humans and animals. Therefore, careful assessment of data is needed and all identified biomarkers have to be confirmed in human samples.

In conclusion, it is clear that with the advancements in proteomics, a whole new area of research has been created, opening many new possibilities in the search for valuable biomarkers. Nevertheless, in future, research should reach a consensus tissue to study the detection of biomarkers, as

well as a standard protocol to isolate this tissue. In addition, the development of a system that will immediately couple the detection of biomarkers to the identification of the peak will be very important in moving the field forward. Although still in its initial stages, the authors believe that this area, as it is evolving very rapidly, will succeed in identifying new valuable clinical biomarkers in the near future, thereby improving the quality of life of diabetic patients.

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Declaration of interest

The authors have no conflict of interest to declare and no fee has been received for preparation of the manuscript.

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