



Review

Mass spectrometry-based proteomic quest for diabetes biomarkers[☆]Shiying Shao^{a,b,*}, Tiannan Guo^b, Ruedi Aebersold^{b,c,**}^a Division of Endocrinology, Tongji Hospital, Huazhong University of Science & Technology, Wuhan 430030, PR China^b Department of Biology, Institute of Molecular Systems Biology, ETH Zurich, Wolfgang-Pauli-Str. 16, 8093, Switzerland^c Faculty of Science, University of Zurich, 8057 Zurich, Switzerland

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ABSTRACT

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia, which affects hundreds of millions of individuals worldwide. Early diagnosis and complication prevention of DM are helpful for disease treatment. However, currently available DM diagnostic markers fail to achieve the goals. Identification of new diabetic biomarkers assisted by mass spectrometry (MS)-based proteomics may offer solution for the clinical challenges. Here, we review the current status of biomarker discovery in DM, and describe the pressure cycling technology (PCT)—Sequential Window Acquisition of all Theoretical fragment-ion (SWATH) workflow for sample-processing, biomarker discovery and validation, which may accelerate the current quest for DM biomarkers. This article is part of a Special Issue entitled: Medical Proteomics.

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1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia. Individuals suffering from DM are estimated to increase worldwide from 171 million in 2000 to 366 million in 2030 [1]. There are two main subgroups of DM, type 1 (T1DM) and type 2 diabetes mellitus (T2DM) [2]. T1DM results from selective autoimmune damage to insulin-producing β cells which lead to irreversible dysfunction of the cells [3]. T2DM presents two major defects— β -cell dysfunction and insulin resistance in peripheral tissues, resulting from various causes including glucose toxicity and lipotoxicity [4,5]. Chronic hyperglycemia of diabetes can cause long-term damages to different organs, especially eyes (diabetic retinopathy), kidney (diabetic nephropathy), and nerves (diabetic neuropathy) [6]. The prevalence of diabetic complications rises up to 98% for patients diagnosed with diabetes for 10 years or more and the complications severely affect patient's quality of life and can ultimately lead to death [6,7].

Although great advances have been achieved the field of diabetes research over the past decades, a multitude of clinical problems persist.

The identification of new biomarkers for early diagnosis and prediction of complications, particularly those in easily accessible clinical samples, would be useful to improved clinic outcome. Herein, we review the current status of diabetic biomarker research and provide some insights into the limitations and possible solutions for biomarker discovery and validation.

2. Clinical challenges of diabetes

Diagnosis of diabetes regularly relies on the measurement of blood glucose and insulin/C-peptide levels. However, blood glucose often rises temporarily under certain conditions of stresses such as myocardial infarction, infections, and surgery [8]. The use of medications can affect glucose levels as well [9]. Additionally, all the tests are exclusively dependent on the precise threshold values used which makes these tests relatively difficult to interpret and somewhat arbitrary [6,9]. It is not rare that some DM patients, who do not fulfill formal diagnostic criteria, may be already in the disease progression with certain degree of insulin resistance or inadequate insulin secretion [10].

Prediction and early detection of diabetes have potential to delay or reverse the diabetic progress. The pre-diabetic condition of T2DM is determined according to the plasma glucose measurement. However, many individuals in a pre-diabetic condition may have already acquired certain symptoms, while some of these pre-diabetic individuals can also remain in pre-diabetic status without progressing to diabetes [10,11]. It is not possible to personalize treatment for T2DM patients simply based on glucose measurement. For T1DM, the appearance of one or more autoantibodies targeting β -cells is among the first detectable clues of

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immune related β -cell attack [12]. However, not all islet autoantibody-positive subjects progress to T1DM [13]. A more precise prediction for diabetes is thus highly desirable.

Diabetic patients are prone to develop renal, retinal, or neurological complications. Diabetic nephropathy is the leading cause of chronic kidney disease (CKD) [14]. Early diagnosis and medical intervention (e.g. angiotensin-converting-enzyme inhibitor, ACEI) of this complication can prevent its development to CKD and uremia [15]. Microalbuminuria has been used as a biomarker for decades. However, debate emerges about the predictive value of microalbuminuria because 1) only a small percentage of patients with microalbuminuria develop to proteinuria and eventually diabetic nephropathy, 2) progressive renal dysfunction can already be present in some patients with normal urinary albumin levels, and 3) many other nephropathies can cause microalbuminuria in diabetic individuals [16]. These limitations may be attributed to the routine immunoassay-based albumin measurement, which can detect only the immunoreactive forms of albumin, whereas other forms of albumin remain undetectable. It is indispensable to identify some predictive markers which enable clinicians to evaluate the necessity of medical intervention, especially for patients in CKD phase but with normal urinary albumin.

Effective monitoring of glucose levels is required for diabetic patients to achieve greater glycemic control. The blood glucose can be measured using either home self-monitoring of blood glucose (SMBG) or continuous monitoring of blood glucose (CMBG) [17]. Although SMBG is effective, patient compliance is poor mainly due to the requirement of blood sampling. Only about a quarter of diabetic subjects who require close glucose monitoring checked their glucose regularly [18]. CMBG includes a glucose sensor placed under the skin, which measures plasma glucose every a few minutes. However, CMBG is only applied in hospitalized patients, leading to a few drawbacks including high cost and invasive surgery [17,18]. Non-invasive specimens (e.g., salivary and tears) and assessments may benefit patients for better glucose monitoring.

To overcome these and other clinical challenges associated with DM, new biomarkers are highly desirable. Theoretically, genetic alterations (DNA-based), differentially expressed transcripts (RNA-based), and differentially regulated proteins (protein-based) can all be used as biomarkers. Recent genome-wide association studies (GWAS) have reported many loci implicated in T2DM pathophysiology. Saxena et al. identified and confirmed three loci associated with T2DM by analyzing 386,731 common single-nucleotide polymorphisms (SNPs) in 1464 T2DM patients [19]. However, establishing a clear and direct causal relationship between common genetic variations and disease development is not trivial [20]. It is evident that RNA levels do not necessarily correlate with protein levels and that protein levels are difficult to predict from genomic patterns [21]. The protein patterns are highly dynamic and are tightly regulated by intra- and extra-cellular stimuli without any change at genetic level [22]. Proteins are the final products of the gene expression process and they are therefore thought to be more direct reflection of disease status than nucleic acid-based markers. Therefore, proteins offer high potential to serve as biomarkers for clinical application [23].

Currently, enormous efforts have been invested to protein-based biomarker research, triggering rapid progress on MS-based proteomics in recent years. Nowadays, proteomics has penetrated into various field of biomedical research, including the exploration of diabetic biomarkers from a variety of biospecimens. In this article we review the quest for DM biomarker from sample-processing to discovery and validation using MS-based proteomics.

3. Specimens in DM biomarker research

3.1. Biofluids samples

Easily accessible human body fluids such as plasma and urine are thought to contain tens of thousands of different proteins [24] and

they have become the most widely used samples for diabetic biomarker studies. New technologies of sample collection and preparation allow us to explore biomarkers in non-invasively obtained samples other than blood and urine. Bencharit et al. proposed that salivary proteomes of patients with DM can vary along with changes in their HbA1C levels [25], which may be used for glucose monitoring and help patients to achieve greater control on their diabetes. Kim et al. identified some tear proteins differently expressed in diabetic patients with retinopathy compared to control subjects [26], a finding that might be useful as diagnostic biomarkers of diabetic retinopathy. Moreover, vitreous humor is a highly hydrated extracellular matrix of the eye and is in close contact with the retina. It therefore reflects the physiological and pathological conditions of the retina and replaces blood fluid as a new source of for diabetic retinopathy research [27].

However, these biofluids share some common limitations. Take plasma for example, proteins in one clinical sample can span across a large dynamic concentration range of up to 12 orders of magnitude, which increases the difficulty of detecting low-abundance proteins [24]. The presence of very high abundance proteins such as serum albumin (35–50 mg/ml) which mask the lower abundance plasma proteins presents major challenges for comprehensive plasma proteome analysis [24]. The plasma flows through all organs; therefore tissue derived proteins get highly diluted in the systemic circulation to a concentration range of ng/ml and below [24]. Based on the information of HUPO plasma proteome collaborative study [28] and currently used plasma biomarkers [29], it is evident that the concentration ranges of the two populations minimally overlap [30], suggesting that the proteomic strategies used lacked the sensitivity to reliably detect potential biomarker proteins in the lower concentration ranges. These considerations remind us to re-consider the value of these newly identified diabetic biomarkers from biofluids. The new diabetic biomarkers discovered by MS-based methods are in the range of $\mu\text{g/ml}$ to mg/ml , i.e. Complement C3 [31], Apolipoprotein (Apo) A-I [32], Apo C-II [33], Apo E [34], C-reactive protein (CRP) [34], and transferrin [35]. In contrast, the concentrations of C-peptide and insulin (routinely clinical used biomarkers) in blood plasma of healthy individuals are around 0.9 ng/ml and 0.36 ng/ml (Fig. 1). The two plasma biomarkers are thus situated below the region which traditional proteomic technology can reliably detect proteins and the same applies to many other clinically used biomarkers known today.

To comprehensively analyze plasma and other body-fluid samples at the required concentration range, specific sample preparation strategies have been developed. First, fractionation methods prior to MS analysis are introduced to allow the identification of lower-abundance proteins in serum and plasma [36,37]. However, such techniques can be problematic. Although sample fractionation is effective in increasing the depth of coverage of identified proteins, it also increases the number of samples to be analyzed per sample, which is time and labor intensive and thus prohibits comparative measurements of larger patient groups. Additionally, a multi-step protein separation workflow will add another level of bioinformatic complexity towards the detection of disease related patterns. Another strategy to achieve higher sensitivity has been the selective removal of high-abundance proteins by selective immunodepletion. This method is now routinely used and several reagents depleting different numbers of proteins are commercially available and quite robust (Table 1). Brand et al. reported that removal of the six most abundant plasma proteins leads to an estimated five-fold enrichment of a potential biomarker [38]. A third approach focuses on the in-depth analysis of sub-proteomes, for example, the identification of N-linked glycopeptides in complex biological samples (glycosylation enrichment) [39]. With this method, Liu et al. reported that 273 unique N-linked glycopeptides can be identified in plasma sample and the quantification of plasma glycoproteins was in the low ng/ml concentration range [40].

Besides sample preparation strategies, new MS technology has to be developed to be more sensitive to identify and quantify minute amounts of proteins in plasma (this will be discussed below).

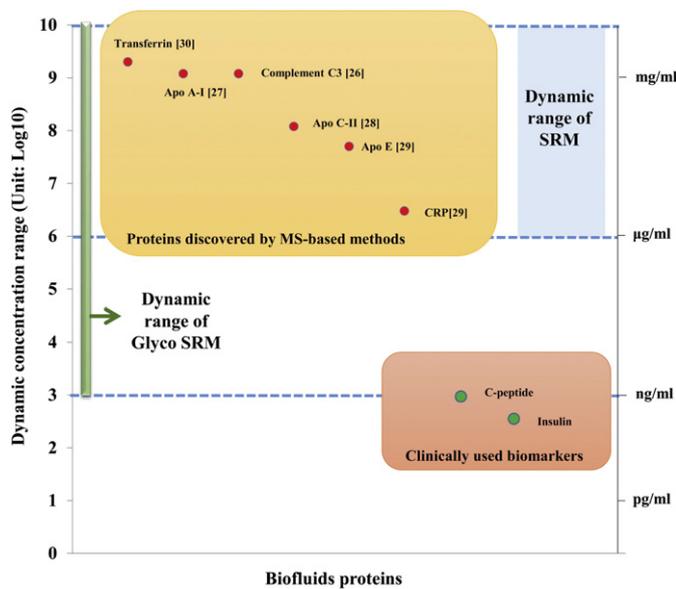


Fig. 1. Diagram of the plasma protein concentration range including a portion of DM biomarkers identified by MS-based methods (red dots) and two clinically used proteins (insulin and C-peptide, green dots). Blue area represents the dynamic range of S/MRM MS and green area indicates the dynamic range of glycosylation enrichment S/MRM MS (Glyco-S/MRM).

3.2. Tissue samples

It is well known that some tissue-specific proteins are highly diluted in blood and only represent marginal levels of the total plasma protein content [24]. Therefore, tissue samples should be more direct and appropriate although they are not easily accessible and always minute in size (such as biopsy samples for diabetic microangiopathy and nephropathy detection). Therefore, sample preparation needs to be improved and optimized for low-abundance protein analysis.

Pressure cycling technology (PCT), based on the principle that programmed cycles oscillating between ultra-high and ambient pressure induces dissolution of matrix from sample, has recently been developed as a new technology to ameliorate the complex proteomic sample handling for minute sample amounts [41,42]. Our data showed that 0.2–0.5 mg human wet kidney tissue can generate about 20 µg peptides, which is enough for 20 injections of MS analysis (Fig. 2, manuscript in preparation). Additionally, proteomes generated from these minute samples are similar to that from large samples (2–3 mg), illustrating no information loss by sample amount limitation. With this technology, minute kidney biopsy may be analyzed for the biomarker study of diabetic nephropathy.

Biospecimens are commonly stored in the form of formalin-fixed paraffin-embedded (FFPE) tissues and such samples represent a large

collection of pathological samples worldwide. They are highly stable at room temperature, and hence an attractive sample resource, especially for retrospective biomarker studies. It is expected that valuable biomarkers from human pancreas FFPE sections could be identified. However, formaldehyde treatment and histological processing significantly reduce protein extraction efficiency and may lead to protein misidentification, which limit the use of FFPE tissues for proteomic analyses. Aged FFPE tissues are also hardened, making proteins difficult to be extracted and solubilized. With PCT-based sample process (heat augmented with high hydrostatic pressure), the number of non-redundant proteins identified in the FFPE tissue was nearly identical to that of matched fresh-frozen tissue, reported by Fowler and coauthors [43].

4. Protein measurement in DM biomarker research

4.1. Antibody-based methods

Antibody-based immunoassays, such as Western blotting (WB), immunofluorescence (IF), immunohistochemistry (IHC) staining technique, or enzyme-linked immunosorbent assay (ELISA), are the traditional protein measurement methods. They are routinely employed in the clinic because they are convenient, rapid, sensitive and provide high sample throughput. The high-quality tests based on automated analyzers are now routinely used for some well-established DM biomarker detection including insulin, C-peptide, and some T1DM specific autoantibodies against islet-cell (ICA), glutamic acid decarboxylase (GADA), insulin (IAA), etc [44]. Moreover, protein microarrays provide a high-throughput platform for protein profiling. With this method, Miersch et al. revealed 26 novel autoantigens and a known T1DM-associated autoantigen, which expanded the current T1DM “autoantigenome” [45].

However, antibody-based methods are accompanied with several limitations. First, the antibody-based measurements rely on two highly specific monoclonal antibodies. The development of an ELISA assay is an expensive (>\$100,000 per antibody) and time-consuming process, typically requiring development times of 1–2 years [46]. Second, antibody monospecificity may rather represent a good expectation than fact. It was reported that only 531 antibodies from a collection of 11,000 antibodies detected a single protein band in western blots of human plasma [47]. Third, IHC has its own limitations. The data are usually scored by expert opinion across multiple tissue sections to offer a semi-quantitative measurement for protein abundances and objective, e.g. machine-learning based scoring schema are largely missing. In light of the above, alternative protein measurement approaches such as MS-based technologies are gaining popularity.

4.2. MS-based proteomics

Many proteomic techniques, including two-dimensional electrophoresis (2-DE), Matrix-Assisted Laser Desorption Ionization mass

Table 1

List of targeted proteins by selective immunodepletion kits.

Kit	Number of depletion	Targeted proteins
ProteoExtract™ Albumin/IgG removal kit by Merck	2	Albumin, IgG
POROS® Affinity Albumin/IgG depletion cartridges by Applied Biosystems	2	Albumin, IgG
ProteoPrep® plasma immunodepletion kit by Sigma	20	Albumin, IgG, transferrin, fibrinogen, IgA, α2-macroglobulin, IgM, α1-antitrypsin, complement C3, haptoglobin, Apo A-I, A-III and B; α1-acid glycoprotein, ceruloplasmin, complement C4, C1q; IgD, prealbumin, and plasminogen
Multiple Affinity Removal System (MARS®) by Agilent Technologies	14	Albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, α2-macroglobulin, α1-acid glycoprotein, IgM, Apo A-I and A-II, complement C3, and transthyretin
IgY-12 spin column by Beckman Coulter	12	Albumin, IgG, transferrin, fibrinogen, α1-antitrypsin, IgA, IgM, α2-macroglobulin, haptoglobin, Apo A-I and A-II, and α1-acid glycoprotein

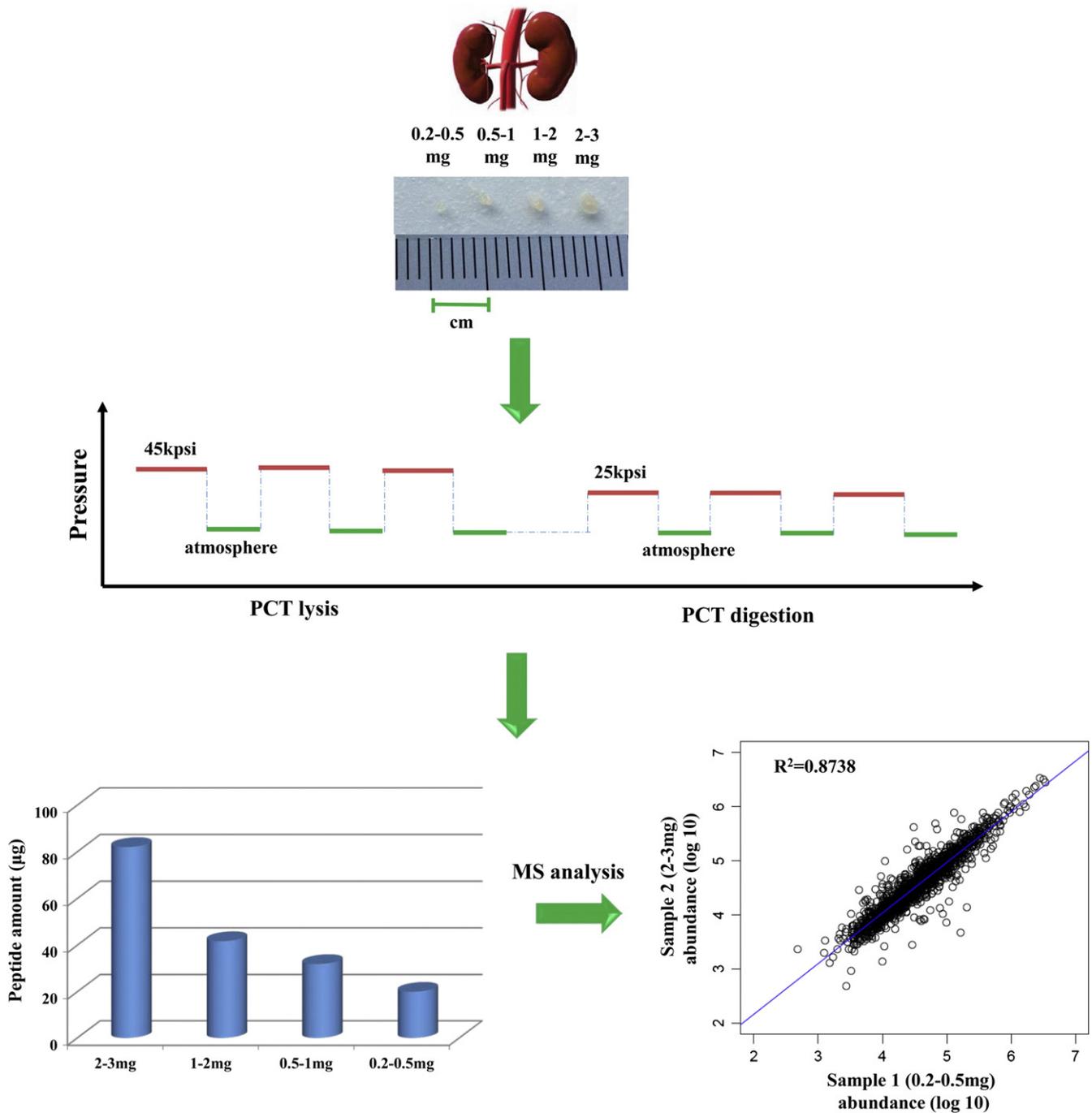


Fig. 2. Schematic diagram of PCT-based sample-processing steps. Human kidney tissues in small size (from 0.2 to 3 mg) are placed in Microtubes® (Pressure BioSciences) for lysis and digestion under programmed cycles oscillating between ultra-high and ambient pressure in barocycler. 20 µg peptides can be obtained from 0.2 to 0.5 mg kidney tissue. The R^2 of the protein abundance between sample 1 (0.2–0.5 mg) and sample 2 (2–3 mg) is 0.88.

spectrometer (MALDI-MS)/Surface-enhanced laser desorption/ionization (SELDI)-MS, Liquid chromatography (LC)-MS/MS shotgun analysis, and selected reaction monitoring/Multiple reaction monitoring (S/MRM), have been applied for diabetic biomarker discovery. However, these reported studies achieved only modest success, mainly due to technical limitations with respect to reproducibility, sensitivity, proteome coverage, quantitative accuracy, sample throughput, and dynamic range (Fig. 3) [48].

4.2.1. 2-DE based proteomics and its potential in diabetic research

The traditional approach for quantitative protein profiling relied on 2-DE for protein separation followed by the mass spectrometric analysis

of selected proteins. In 2DE proteins are separated by isoelectric focusing and SDS-PAGE [49] and the separated proteins are detected by staining. Usually, a selected subset of the detected proteins is isolated from the gel, digested and the peptides are spotted on the sample plate of a MALDI-MS or SELDI-MS for further analysis.

Since its introduction in 1975, 2-DE has been widely used in proteomic research, including the DM biomarker discovery. Prediction or early diagnosis of diabetes is crucial to avoid its progression toward severe organ damage. By combining SDS-PAGE fractionation and SELDI-TOF MS, Sundsten et al. demonstrated that Apo H and transferrin were down-regulated from serum of T2DM patients compared to individuals of normal glucose tolerance [35]. Furthermore, in 2009, Liu et al.

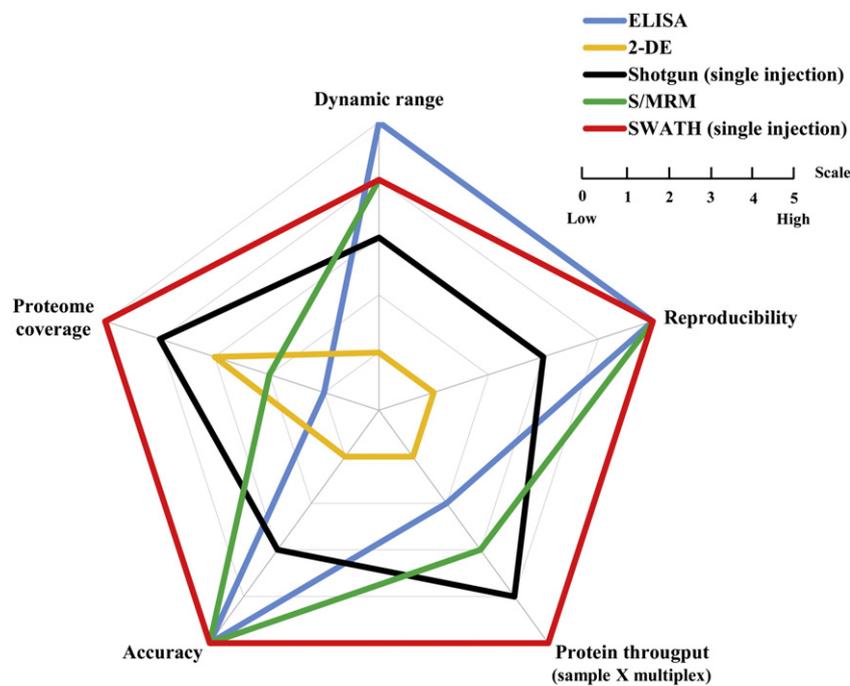


Fig. 3. Technical performance of MS-based methods in terms of proteome coverage, accuracy, dynamic range, protein throughput (sample X multiplex), and reproducibility. Axis shows the magnitude of respective variable. Note: All comparisons are qualitative assessment.

employed 2-DE coupled with MALDI-TOF MS to identify novel T2DM diagnostic markers [32]. In this study 57 from more than 200 spots were found differentially expressed between controls and diabetic patients, including up-regulated galectin-1 and down-regulated Apo A-I. More recently, using the similar MS technology (2D-LC-MALDI-TOF) [34], Apo A-I was also found to decrease in T2DM plasma samples, which is in agreement with the study from Liu [32]; while Apo E, leptin, and CRP were found to significantly increase in T2DM patients [34]. Additionally, proteomics were applied in differential DM diagnosis. Maahs et al. verified that some specific collagen fragments (collagen α -1 (I) chain and collagen alpha-1 (III) chain) in urine support the distinction between T1DM from T2DM [50]. These data demonstrate the potential of even the first generation proteomics technologies for novel diagnostic biomarker discovery which might complement to the existing diabetic biomarkers.

One major advantage of 2-DE is its ability to separate intact isomeric forms of protein for the study of posttranslational modifications [49]. However, 2-DE is of limited utility for small proteins, very basic or acidic proteins, or for hydrophobic proteins and, depending on the detection method used, 2-DE has limited dynamic range of 2–3 orders of magnitude. Moreover, the complicated process limits 2-DE to a relatively low-throughput scale of research [51]. Therefore, although 2-DE combined MS technology was once widely used in diabetic biomarker discovery and in proteomics in general, it has largely been succeeded by more powerful second generation proteomic methods (see below) (Fig. 3). We summarized the MS methods applied in biofluids derived DM biomarker discovery from 2005 till now (Fig. 4). Most of these publications applied 2-DE combined MS strategy until shotgun and other more recently developed technologies were widely used for proteomic analysis. As the performance of proteomic technologies has rapidly progressed over the last decade, the value of reported biomarker candidates should be assessed in view of the method used to identify them.

4.2.2. Shotgun based proteomics and its applications in diabetic research

Shotgun proteomics, also referred to as discovery proteomic, is a universally used proteomic method for identifying proteins in complex

mixtures. It is based on operating a mass spectrometer in data-dependent acquisition (DDA) mode. This measurement scheme is called DDA because peptide fragmentation is guided by the abundance of detectable precursor ions. In this mode, precursor ions are detected in a survey scan and specific detected peptide ions are then selected for collision induced dissociation (CID) and fragment ion (MS2) spectra recording. The recorded information is then searched against protein sequence database to derive the sequence of the peptides and the corresponding proteins. Herein, it is noted that this mode only allows the peptide ions with the intensity above a predefined threshold value to be stochastically selected and sequenced [52].

Label-free protein quantification includes two broad approaches, that is, absolute and relative quantification. The latter approach is used to describe protein profile changes in comparison with another sample (e.g., control) [53]. The relative protein abundance can be analyzed by either spectrum counts or peptide peak intensities. The spectrum counting method simply counts the number of spectra identified for a given peptide across multiple MS analyses; while peptide peak intensity method uses the area under or the chromatographic peak of precursor or fragment ion to estimate peptide abundance [53], which is considered a more direct and advantageous approach than spectrum counts [54]. Therefore, most of comparative studies use peak intensity method for relative peptide quantification.

Using global LC-MS-based proteomic analyses, Zhang et al. identified 24 serum proteins that were significantly differently abundant between controls and patients with T1DM. The identified proteins included platelet basic protein and C1 inhibitor, which were expected to be discriminators distinguishing T1DM from healthy controls [55]. Overgaard et al. investigated the plasma proteome from 123 T1DM patients with iTRAQ labeling MS proteomics to understand early determinants of diabetic nephropathy [56]. Apo C-III was found to over-expressed in patients with macroalbuminuria, whereas Apo A-I level decreased, although not to a statistically significant degree. It is interesting to note that members of Apo family are identified in most of these proteomic studies, which attracts more and more researchers to investigate the roles of these Apo proteins in diabetic diagnosis and prediction. However, the assured clinical value of these proteins remains to be

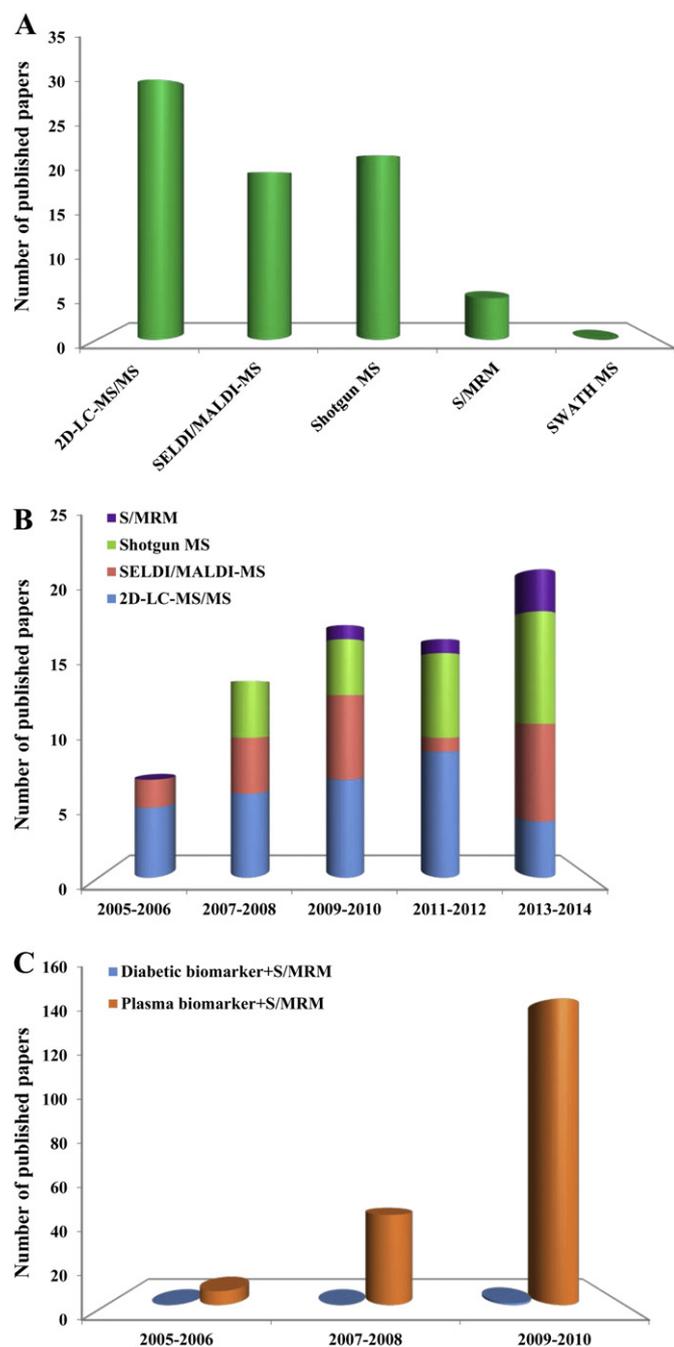


Fig. 4. Number of published articles related MS-based proteomic analysis in biofluids derived human DM biomarker discovery (focusing on diagnosis/prediction) between 2005 and July 2014 (A, B). The growth of diabetic biomarker publications using S/MRM method in comparison with published papers in S/MRM-based plasma biomarker identification (C).

established due to the plasma sample problem (discussed in Section 3.1) and shotgun technology limitations (Fig. 3).

Although most of the MS-based proteomic studies in the past decade were carried out using shotgun proteomics, mainly because of the robustness of the method and the impressive proteome coverage it achieves, the stochastic sampling of this technique markedly affects reproducible detection of proteins between different experiments and between samples in large clinical cohorts. As a result, every repeat analysis of the same or similar complex samples will result in only partially overlapping protein subsets being identified. Moreover, the poor accuracy, low sensitivity, and low sample throughput weaken the employability of shotgun in biomarker discovery.

4.2.3. S/MRM based proteomics and its applications and potential in diabetic research

S/MRM is a MS technique that utilizes multiple mass analysis steps to detect a series of predetermined ions. It has been developed to quantitatively analyze small molecules, but is now increasingly being used as a complement to the shotgun methods in proteomics [57]. SRM is usually carried out on triple quadrupole (QQQ) instruments where the first and third quadrupoles act as mass filters to select a specific precursor ion (Q1) and to record unique fragment ions (Q3), whereas the CID fragmentation is conducted in the second quadrupole used as a collision cell (Q2) [58]. In contrast to shotgun MS, S/MRM is well suited for the highly reproducible quantification of predetermined analytes across many samples and across different laboratories. In addition, the non-scanning nature of S/MRM allows a wide dynamic range up to five orders of magnitude. Moreover, S/MRM assays can be multiplexed so as to quantify hundreds of specific analytes in a single sample injection [57]. Importantly, S/MRM is, currently, the most sensitive MS-based method [57], which, together with high reproducibility, wide dynamic range and efficient throughput, enables S/MRM to be a good choice for plasma sample analysis.

However, in spite of the favorable performance profile of S/MRM, the sensitivity of LC-S/MRM is currently still too low to directly and reliably access low-abundance proteins of ng/ml concentration range in body fluids, unless sample pre-fractionation or enrichment steps are applied. Protein quantification directly in plasma digests has been shown by multiplexed S/MRM assays, covering a dynamic range of 4–5 orders of magnitude and reaching a LOQ of 1 µg/ml (Fig. 1) [59]. Therefore, the least abundant protein quantified by S/MRM in trypsin-digested plasma lies 1–2 orders of magnitude above the required levels as defined by the abundance range of approved plasma biomarkers. Combination with enrichment steps (e.g., Glyco S/MRM) leads to an increase in LOQ of more than 3 orders of magnitude. However, the need for enrichment before S/MRM analysis reduces the sample throughput, might affect the analytical precision and makes non-glycosylated proteins transparent to the method.

Nevertheless, a trend towards the application of S/MRM in clinical studies is now clearly evident. When it comes to the DM field, the S/MRM application is greatly delayed compared to the situation in other diseases (e.g., prostate cancer). S/MRM was first used for diabetic biomarker research in 2010 by Kim et al. to analyze large numbers of clinical vitreous and plasma samples for diabetic retinopathy biomarker verification [60]. At that time there were already more than 90 publications reported for plasma biomarker studies in that year (Fig. 4) [61]. This delay may be partially attributed to the limited resource of diabetes-related S/MRM assay library (discussed in Section 5). The Human Diabetes Proteome Project (HDPP) consortium, which was initiated at HUPO 2012, plays an important role in collecting and integrate diabetes-related proteomic knowledge. This would be helpful for S/MRM assay library generation. More diabetic clinicians would be encouraged to make their efforts to closely cooperate with proteomic researchers and apply this method into clinical studies.

4.2.4. SWATH based proteomics and its potential in diabetic biomarker discovery

Data-independent acquisition (DIA) is an emerged proteomic technology. Several implementations of the method have been developed, including MS^E [62], all-ion fragmentation (AIF) [63], Fourier transform-all reaction monitoring (FT-ARM) [64], SWATH (Sequential Window Acquisition of all Theoretical fragment-ion) Acquisition [40, 65,66], and multiplexed MS/MS (MSX) [67]. These new strategies have been developed to complement existing proteomic technologies. In particular, SWATH-MS combines DIA with a data analysis targeted strategy that is analogous to that of S/MRM. In SWATH-MS, data acquisition includes the sequential selection of sequential precursor ion mass windows, fragmentation of all precursor ions in each window, and recording of the combined fragment ion spectra, which allows us to

detect a comprehensive proteome coverage with the favorable accuracy, sensitivity, sample throughput and reproducibility (Fig. 3). Importantly, in contrast to S/MRM, this data acquisition mode converts the peptides in a biological sample into a high-resolution digital map recording information of all ionized precursor ions present in the sample. From this fragment ion map targeted peptides are identified by the extraction of signal groups corresponding to fragment ion signals of the targeted peptide and by computing the likelihood that a particular signal group represents the targeted peptide.

It can be expected that by virtue of its unique features SWATH-MS can accelerate and simplify the long, difficult, and uncertain path from initial biomarker discovery in biological samples, to a clinically approved biomarker. Traditionally, biomarkers have been screened between samples from pre-diabetes, newly onset diabetes to advanced stage with various diabetic complications. This process requires a long follow-up period. Clinical samples need to be stored appropriately (e.g., in -80 degree) over a long time. In contrast, if SWATH maps are immediately acquired after sample collection, we obtain a real, quantitative proteomic recording as personalized digital representation for each patient, which can be stored instead of real clinical samples. This feature will facilitate the longitudinal profiling of the proteome and the retrospective identification of diabetic biomarkers.

By now, although no study to date has used SWATH MS or for that matter any other DIA method for diabetic biomarker discovery, it is worthwhile to note that this technique has the potential to address many of the current limitations in clinical biomarker discovery.

5. Protein validation in diabetic biomarker research

The discovery of candidate biomarkers is the initial step toward the development of biomarkers for diabetes. The following step is to validate these biomarker candidates to select the few that merit the effort and expense of pre-clinical testing.

Traditionally, antibody-based assays have been the major methods used for biomarker verification. Western blot and ELISA were the only choices for diabetic biomarker validation till the year 2010 when Kim et al. applied S/MRM for diabetic retinopathy biomarker research [60]. In the cases where specific antibodies are already available, the validation of a biomarker candidate can be relatively simple. However, for most novel protein candidates discovered in recent proteomics studies, the Western blot/ELISA approach is limited mainly by the lack of high quality antibodies. Therefore, a high sample throughput analysis of target proteins is necessary.

MRM or S/MRM, the Nature Methods “Method of the Year” for 2012, has emerged as the method of choice for biomarker verification [68]. It is practically workable that more than 100 candidates can be detected with absolute quantification in a single MS measurement. A recent study reported this S/MRM method enable to quantitate 312 peptides simultaneously in 45 min (9 s per assay) [69]. Additionally, the cost of a mass spectrometer for S/MRM-MS is in the range of \$500,000, approximately the equivalent of the development of five ELISA assays. Moreover, Kuzyk et al. tested S/MRM reproducibility by simultaneous quantification of 45 plasma proteins [59] and they achieved a CV below 20% for 94% of the measured analytes. These features make S/MRM well-suited for verification of large numbers of proposed clinical biomarker proteins in efficient, economical, and accurate mode.

However, prior information is required for S/MRM analysis: (1) the specific proteins of interest must be identified; (2) for each protein of interest, multiple tryptic peptides, which can uniquely define the protein, must be identified; and (3) the fragment ions of these tryptic peptides must have good and unique signal intensities. This implies that each protein of interest needs to be well-defined (S/MRM assay generation) before S/MRM analysis. The S/MRM assay (reference map) is defined as the collection of fragment ion spectra of peptides corresponding to predicted protein sequences based on the genome [70], containing all essential coordinates of informative peptides. The well-established S/MRM assay libraries can be readily used for peptide detection in complex proteomes similar to the use of antibodies for protein detection.

There are two different ways to generate spectral libraries for S/MRM assays. One is based on deep shotgun sequencing of natural proteins in biological samples, which will directly generate suitable and relevant spectral database to serve as assay libraries. By combining 91 high-confidence shotgun proteomic experiments, Farrah et al. assembled a high-confidence human plasma proteome reference set possessing 1929 non-redundant protein sequences [71], which represents a valuable reference resource for S/MRM assay generation. The second method for assay library is based on sequencing libraries of synthetic peptides. The use of synthetic peptides eliminates sampling bias of the shotgun measurements and sample preparation steps. Currently, several spectral libraries have been generated including *Saccharomyces cerevisiae* proteome [70], *Streptococcus pyogenes* proteome [72], and *Mycobacterium tuberculosis* proteome [73].

The process of assay library generation is easier, time-saving, and cost-effective in comparison with immunoassay development. Take advantage of this S/MRM assay database, researchers can directly perform

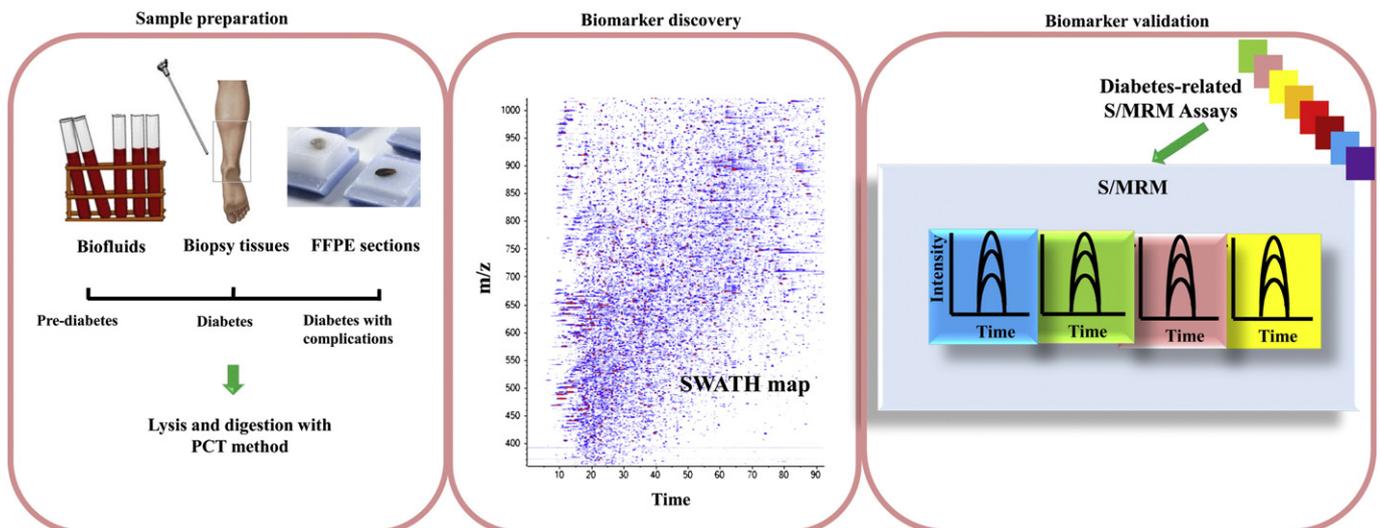


Fig. 5. Model of PCT-SWATH MS pipeline for DM biomarker research. Plasma, biopsy tissue samples, or FFPE sections can be processed by PCT-based lysis and digestion. The obtained peptides are analyzed by SWATH MS to identify protein candidates, which are, in succession, validated by S/MRM.

targeted navigation of *a priori* selected plasma proteins. HDPP consortium has already made publicly available (www.hdpp.info) resources for 1000 diabetes-related protein (the 1000-HDPP) database with links to their neXtProt, Peptide Atlas and Human Protein Atlas references. These resources will be useful to generate a collection of diabetes-related S/MRM assay library for worldwide resource-sharing, which, in turn, may accelerate the real clinical use of those MS-identified diabetic markers.

Therefore, we can collect plasma, biopsy tissue samples, or FFPE sections (PCT-based preparation) for SWATH analysis from pre-diabetic status to advanced stages of this disease, so as to get digital maps for permanent storage without information loss. With the diabetes-related mass spectrometric reference maps established, researchers can directly perform targeted navigation of *a priori* selected clinically relevant proteins by S/MRM (Fig. 5). It is worth mentioning SWATH-MS, owing to its high reproducibility comparable to S/MRM, has the potential to be used as validation approach as well although it requires rigorous cross-laboratory examination.

6. Conclusions

The incidence of DM is markedly increasing worldwide, which attracts more and more efforts on improving the clinical outcome for diabetic patients. Several clinical challenges remained to be overcome. The existing clinical markers fail to offer early diagnosis or predict diabetes and its complications. MS-based proteomics stands out as a promising approach to explore and validate new diabetic protein biomarkers.

In order to apply MS-based proteomic methods, proteins from biospecimens must be efficiently extracted and digested into peptides which are subsequently ionizable in the mass spectrometer. Plasma samples are the most favorite specimen for diabetic biomarker studies; however, efficient proteomic analysis of plasma is difficult due to the extra-high dynamic range of protein expression. Alternative specimens including tears, saliva, and fresh frozen/FFPE tissues also offer unique insight to the biomarker studies of diabetes and its complications. PCT is a promising sample preparation technology, which permits fast and robust proteomic sample preparation from minute amount of specimen with minimal technical variation.

MS-based proteomics is evolving rapidly and has penetrated into many branches of biomedical research in recent years. Obsolete methods still frequently appear in literature of DM research. The targeted proteomics (SRM) and next generation proteomics (SWATH) offer transformed reproducibility, sensitivity, and proteome coverage. However, they have not been properly introduced into DM research although initiative attempt like HUPO B/D-HPP has started to change the situation.

Conflict of interest

The authors declare that there is no duality of interest associated with this manuscript.

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