

## A proteomic approach to obesity and type 2 diabetes

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### Abstract

The incidence of obesity and type diabetes 2 has increased dramatically resulting in an increased interest in its biomedical relevance. However, the mechanisms that trigger the development of diabetes type 2 in obese patients remain largely unknown. Scientific, clinical and pharmaceutical communities are dedicating vast resources to unravel this issue by applying different omics tools. During the last decade, the advances in proteomic approaches and the Human Proteome Organization have opened and are opening a new door that may be helpful in the identification of patients at risk and to improve current therapies. Here, we briefly review some of the advances in our understanding of type 2 diabetes that have occurred through the application of proteomics. We also review, in detail, the current improvements in proteomic methodologies and new strategies that could be employed to further advance our understanding of this pathology. By applying these new proteomic advances, novel therapeutic and/or diagnostic protein targets will be discovered in the obesity/Type 2 diabetes area.

**Keywords:** diabetes mellitus type 2 • obesity • proteomics • biomarkers

### Introduction

The incidence of diabetes mellitus type 2 (DM2) is increasing at an alarming rate world-wide. This is due, in part, to the dramatic rise in

the obesity epidemic as DM2 is a comorbidity frequently seen in obese patients [1]. Because not all obese patients develop DM2 and

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not all patients suffering from DM2 are obese or overweight [2], it is of interest to understand the mechanisms underlying the association between these two entities to predict which patients are at higher risk for developing this disease, and possibly for the development of preventative therapies. DM2 appears as a result of insulin resistance. During the ontogeny of this disease, the pancreas begins to produce greater amounts of insulin to maintain euglycemia that collectively overcomes this resistance. But as time advances, sufficient insulin levels cannot be maintained to control normal levels of glycemia. The clinical characteristics of DM2 appear several years after the onset of this process during which time the person is asymptomatic. Once DM2 clearly appears, it must be attended immediately as it is very difficult and practically impossible to reverse the disease progression. In any event, factors such as exercise and diet can 'help', as it is of utmost importance to properly control DM2 through life style, and early intervention of the factors previously mentioned may result in normal glucose levels. Thus, early detection, or even more importantly, the possibility of avoiding DM2 onset would be of extreme benefit.

The alteration of protein structure, function, production and interactions, in part if not completely, contributes to the underlying mechanism of many diseases including diabetes [3]. Comprehension of pathological-mechanisms whereby molecular and environmental actions lead to the development and progress of diabetes is important for the prevention and treatment of this disease. In such an endeavour, approaches such as genomics, metabolomics and proteomics are being applied to identify more specific biomarkers for DM2 for its early detection, management and for devising new therapies. In this review, we will focus on the use of proteomics for the identification of novel biomarkers, and perhaps, therapeutic targets for the identification and subsequent treatment of DM2. As in any research study, *efficient and up-to-date methods must be established and used for selection and proper handling of the clinical samples and data* [4].

Simply defined, proteomics is the large-scale study of proteins, particularly of their structures and functions [5, 6]. Proteins are vital parts of living organisms, as they are the main components of all cellular processes. The term *proteomics* was first coined in 1997 [7] as an analogy with *genomics*, the study of an organism's genome. The word *proteome* is a blend of protein and genome and was coined by Marc Wilkins in 1994 while working on the concept as a PhD student [8]. The proteome is the entire set of proteins [8] produced or modified by an organism or system. This will vary with time as a result of distinct requirements or stresses that a cell or organism encounters. Proteomics allows the study of the entire set of proteins, produced or modified by an organism or system and it varies with time and a variety of environmental factors. Indeed, it is formed on the basis of research and development of the Human Genome Project [9, 10]. Thus, while the genome is static the proteome is dynamic. It is also a fundamental component of functional genomics. While *proteomics* generally refers to the large-scale experimental analysis of proteins in a given cell/tissue/organism, techniques used in the studies can also be applied to protein identification and purification. One of the key methodologies used in this area is mass spectrometry [11]. We will discuss in detail the sample preparation step as a key issue for clinical proteomic research in the following section.

## Sample preparation

The sample preparation of body fluids (blood, serum, urine and salivary) or tissues (*e.g.* adipose) is an extremely important pre-requisite for achieving robust and reproducible data *via* proteomics. Once the body fluids and/or tissues have been obtained for future proteomic analyses, they must be frozen rapidly (*i.e.* at  $-80^{\circ}\text{C}$  in a bio-banking system) usually in aliquots distributed in several vials to avoid sample deterioration from repeated freeze-thaw cycles. When working with adipose tissue samples, the selection of the representative areas from the human body is extremely important according to the clinical research goal. Tissue handling is also of paramount importance as different tissues will require different homogenization and processing protocols. Several examples are explained in the following sections and in Table 1. For clinical proteomic purposes, changes can take place in proteome assays that can easily modify experimental results. For example, contamination of protein-samples can cause results to be skewed, as artefacts and/or contaminants can mask low expressed proteins during mass spectrometry (MS) analysis, and they can also give rise to poor protein-separation during electrophoresis and chromatography, and in addition, artefacts can interact with the given protein, thus changing the 3D-structure [12].

There are many steps that can be taken in protein handling and storage processes, which may help to minimize any damage and, in turn, maximize accuracy of results. Crucial steps in the process are to use anti-proteases and anti-phosphatases, and to freeze the protein sample quickly after harvesting in several vials. A very common premise is that 'there are no routine sample preparation-protocols' for a given proteomic experiment; it is always necessary to optimize them according to the clinical goals and according to the designed proteomic strategies to be used. In any event, optimized protocols for a given sample or samples from the same clinical assays can be used routinely. Moreover, several international laboratories [from Human Proteome Organization (HUPO), EUPA and SeProt] are standardizing specific protocols for storing clinical samples apart from those for analysis *via* proteomics and MS using reproducible protocols from different laboratories. This is important when we carry out analyses and the data are coming from different experiments from different laboratories [13–15].

Although the amount of protein obtained from patient samples can be very low, clinical samples allow highly valuable data to be achieved even with a limited amount of samples. Once the type of sample to be used has been selected, it is necessary to optimize the protocol for tissue disruption/cell-lysis or for the isolation of the proteins from body fluids using commercial reagents including protease and phosphatase inhibitors. When analysing serum/plasma, it may be necessary to use an albumin/IgG depletion kit to identify proteins whose concentration is low [15].

We have found that many proteins do not exist as a single form but as isoforms probably generated by post-translational modifications (PTM) of a given protein. Thus, for any proteomic protocol used, one must be knowledgeable about protein isoforms. In certain instances, it is also recommended that the purified complex mixture of proteins be dilapidated. Additionally, one often analyses a given tis-

**Table 1** Research examples of diabetes and obesity studies using proteomic tools

Tool	Sample	Resulting data	Ref.
2DE silver MALDI-TOF	Plasma proteins/blood-sera in ob/ob mice that are obese because of the lack of leptin	EPS is a potent gene expression regulator (in ob/ob mice) in obesity, insulin resistance and DM. Ferritin and adiponectin as important factors for future DM2. Expression level of Apo A-I, IV, C-III, E, retinol-binding protein 4 and transferrin were shown to be altered and their levels are normalized after EPS treatment. Resistin is up-regulated while adiponectin is down-regulated in diabetes and obesity.	[33]
2DE-DIGE MALDI-TOF	Adipose tissue	9 higher expressed proteins in the adipocytes from old compared to young obese patients: Prohibitin 1 Protein disulphide isomerase A3 Beta actin Profilin Aldo-ketoreductase 1 C2 Alpha crystallin B Anexins A1, A5, A6 4 lower expressed proteins in the adipocytes from old compared to young obese patients: Keratin type 2 cytoskeletal 1 Keratin type 2 cytoskeletal 10 Haemoglobins A, B Signal transducer and activator of transcription 3 as the central molecule in the connectivity map and the apoptosis pathway	[92]
iTRAQ LC-MS/MS	Heart tissue	29 proteins up-regulated from a total of 1.627, while 84 were down-regulated in the db/db mice compared with the control group Calnexin was found to be decreased whereas integrin-linked protein kinase was decreased in the phlorizin treated DM group compared with the DM group	[93]
SDS-PAGE LC-MS/MS/MS LTQ-FT ELISA	Subcellular fractionation of the mouse preadipocyte cell line 3T3-L1 with and without insulin treatment into cytosol, membrane, mitochondria and nuclear fractions and nuclear fractions Genetically modified animal models (bGH, GHA and GHR-/- mice and tissue-samples	3.287 identified proteins that form part of the adipocyte proteome Useful information to unravel the complexity of the adipocyte in obesity Addressed that adiponectin is generally negatively associated with GH activity, regardless of age Useful information about the associations of total and HMW adiponectin with insulin sensitivity and longevity Circulating adiponectin levels correlated strongly with inguinal fat mass, implying the effects of GH on adiponectin are depot-specific	[94, 95]
Phosphoproteomics SILAC anti-pY immunoprecipitation	Brown adipocytes	From the 40 insulin effectors identified, 7 (SDR, PKC binding protein, LRP-6 and PISP/PDZK11, a potential calcium ATPases binding protein	[96]

**Table 1.** Continued

Tool	Sample	Resulting data	Ref.
2DE-gels stained by Sypro-Ruby	Platelet-free plasma from the patients	53 differentially spot-proteins from which 51% were shown to be down-regulated comparing Vit D deficiency The HMW form of adiponectin is down-regulated in obese paediatric patients with Vit D deficiency Thrombospondin 1 (TSP1) is up-regulated while histone deacetylase 4 (HDAC4) is down-regulated	[97]
SCX MS/MS	Peripheral blood mononuclear cells	TSP1 and HDAC4 recover their normal expression level due to physical exercises	[98]
2DE-gels LC-MS/MS MALDI-TOF MS	Liver sample	A diet rich in n-3PUFA decreases the expression of regucalcin, aldehyde dehydrogenase A diet rich in n-3 PUFA increases the expression of a POLI protein-A-1, S-adenosylmethionine synthase, fructose 1,6 biphosphatase, ketohexokinase, malate dehydrogenase, GTP-specific succinyl CoASynthase, Ornithine aminotransferase, protein disulfide isomerase A3	[99]
2DE-gels MALDI-TOF MS and MS/MS	Human subcutaneous (SQ) and white adipose tissue (WAT)	The levels of several proteins in human SQ-WAT are not homogeneous between different WAT depots Twenty-one proteins showed differential intensities among the six defined anatomical locations, and 14 between the superficial and the deep layer (such as vimentin, heat-shock proteins, superoxide-dismutase, fatty acid-binding protein, alpha-enolase, ATP-synthase among others)	[100]
2DE-DIGE MALDI-TOF MS and MS/MS	Visceral adipose tissue (VAT) from pre-obese diabetic patients	The presence of diabetes influences the VAT abundance of several proteins Diabetic patients showed increased VAT abundance of glutathione S-transferase Mu 2, peroxiredoxin-2, antithrombin-III, apolipoprotein A-IV, Ig κ chain C region, mitochondrial aldehyde dehydrogenase and actin, and decreased abundance of annexin-A1, retinaldehyde dehydrogenase-1 and vinculin, compared with their non-diabetic counterparts.	[83]
Label-free quantitative proteomics	Salivary samples from patients with diabetes	This study demonstrates that differences exist between salivary proteomic profiles in patients with diabetes based on the A1C levels	[84]
2DE MALDI-TOF MS and MS/MS	Serum samples from obese children	This research study establishes the bases of the utility of proteomics to assess clinical improvements in obesity. Down-regulated proteins in obese patients: transthyretin apolipoprotein-A1, apo-J/clusterin and vitamin D binding protein. ApoA1 was further down-regulated under the presence of up-regulation insulin resistance, whereas weight reduction induced its up-regulation. Apolipoprotein-A1 and haptoglobin were validated via ELISA as true potential candidate biomarkers	[85]

Representative assays detailing in each column –the goals, technologies, type of sample to be analysed and the resulting data– are placed schematically in this table. 2DE-electrophoresis is one the most common tools used in diabetes and obesity research studies when using proteomics. Nevertheless, currently, more scientific articles are appearing and showing the advantages when applying HPLC or nano-HPLC coupled directly to mass spectrometry (LC-MS) to avoid losing low expressed proteins or putative biomarkers. Biomarkers, adipocyte and insulin proteomes have been the most common goals followed by scientists to unravel diabetes and obesity pathologies. All of them –and many others– allowed us to advance and establish the right platforms and current technology-innovations will permit improve diagnoses and refine therapies via identifying new biomarkers by proteomics.

sue along with serum or urine. This type of complementary data may or may not be easily interpreted but many clinical research studies show that analysing both types of samples offers the possibility to explain linked biological processes. Additionally, the selection of a given proteomic platform must be made according to the goal of the experiment [16, 17]. For example, high-throughput-proteomic strategies may be useful for discovery of biomarkers specifically related to various stages of obesity and DM2, and they may help to improve current treatments and innovative therapies. On the other hand, once the target proteins are selected by the discovery phase of the research, analysis of those proteins in the complex mixture of biological samples can be conducted by selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) assays. The sample-preparation of body fluids is a tedious but critical step in obtaining reliable results [16–19]. For example, urine contains low levels of proteins compared to blood and a large amount of sample and/or a concentration procedure may be required for analyses. The timing of the sample collection may influence the results because the protein composition of body fluids may be different according to the different stages of the treatment for each patient. Indeed, we hypothesize that the serum from a patient suffering DM2 may contain different protein patterns at diagnoses (t0) during the treatment (t1) and also at the end of the therapy (t2). Nevertheless, there is no reference protein-map for DM2 or for obesity; thus, if we are able to 'build' the protein-pattern reference map of obesity and DM2 compared to healthy controls, we should improve our understanding of these pathologies.

Clinical application of proteomics, applied according to space (serum, urine, blood) and time (diagnoses, treatment and/or cured states), can be used to study the evolution of patients suffering obesity and DM2, to unravel the mechanisms involved in disease progression. In the end, we hope that the patients will benefit.

In the following sections, we discuss specific details useful for carrying out obesity and DM2 proteomic research.

## Special issues concerning tissue/blood sampling

As stated above, sample integrity and storage are key factors for obtaining efficient and reproducible data. Within the last decade, the quantity and quality of stored samples are high because of the creation of biobanks including sample collection procedures. This implies that the samples are kept intact, preserving their chemical and physical characteristics that may ultimately result in data indicative of their functions and/or roles within the cell. Thus, different types of samples (blood, urine, tissues) from the patients with different pathologies are available for study [18, 19] or can be collected and properly stored in an individual laboratory's biobank.

Another important step is related to the standardization of protocols for data acquisition from different biobanks so that different disease states and/or changes throughout time can be compared [20]. If one of the main goals is to establish a long-term repository of biological/clinical samples and to make these samples available to multiple scientific studies, then specific protocols must be used.

For example, if we have a particular interest in diabetes, fasting samples should be collected and stored. Additionally, a patient's phenotypic data must be recorded including age, sex, weight, BMI, fasting glucose and other clinically important parameters. These data should be obtained at diagnoses and at different states throughout the disease. Protocols to store clinical samples should provide clear information on how and when the samples were collected, processed and organized to ensure their long-term integrity for the study of a given disease [21].

## Serum samples

One of the major difficulties in studying the serum proteome is that two major groups of serum proteins, albumin and immunoglobulins, comprise approximately 95% of the total, while the remaining 5% belongs to a variety of other protein types including cytokines, hormones, enzymes and cytoplasmic and nuclear proteins. Those highly abundant proteins may generate substantial background in any proteomic analysis and may mask the significant changes in proteins of interest. Albumin and IgG can be removed from serum using affinity chromatography to refine the identification of biomarkers (low expressed proteins) for a given pathology [22]. However, since albumin interacts with many blood components, it is possible that by removing albumin, one may inadvertently remove important proteins that are important to a given pathology, in this case DM2.

The lipids contained in serum can have an important role in specific pathologies, such as hyperlipidemia, cardiovascular diseases, *etc.*, as they can interact with serum proteins. In some cases, it is necessary to remove these lipids to identify the required proteins. Fractionating the serum *via* centrifugation allows one to then separate chylomicrons, very low density lipoproteins (such as VLDLPs) from the total protein sample [23–25].

Results of proteomic analyses comparing a diseased *versus* healthy state and the different stages of the disease can ultimately identify putative therapeutics and/or therapeutic targets as well as diagnostic biomarkers. It has been suggested that the concentration of specific proteins in serum can be useful for the early detection of insulin resistance and that this may even be possible before the appearance of the symptoms [23, 24, 26]. Thus, identification of differentially expressed serum proteins could be potentially important for the prevention and treatment of DM2, even in obese children [26–28].

## Proteomic methodologies

The most significant breakthrough in proteomics has been the use of MS for the identification of proteins directly obtained from clinical samples or from samples in which the proteins were previously separated by two dimensional (2DE) gel electrophoresis or chromatography. Typical MS based identification of a protein utilizes tryptic digestion [29] prior to MS analysis to improve the accuracy or mass measurements. Mass spectrometry ionizes protein or peptide sam-



ples and measures their mass-to-charge ratios. Two ionization methods often employed are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). The time-of-flight (TOF) analyzer is often used with MALDI. The TOF analyzer accelerates the ions in an electric field and measures the time they take to reach the detector. If the sample's charge is the same, velocity is inversely related to their masses. The quadrupole mass filters are used with ESI. It consists of four parallel metal rods. By controlling electric field within the four rods by a radio frequency voltage, only ions with a certain mass-to-charge ratio can be selected and reach the detector. The quadrupole ion trap works similar to the quadrupole filter, but instead of passing a single ion species through, it traps ions and releases them sequentially.

Tandem MS is an array of MS that enables peptide sequencing. The first MS isolates a peptide to be analysed and the second MS fragments the peptide by collision-induced dissociation (CID) by a neutral molecule such as nitrogen. Finally a third MS measures mass-to-charge ratio of the resulting fragments [30]. A series of three quadrupoles can be used in the same manner. Since CID fragments a peptide randomly at the peptide bonds, sequence information can be obtained by analysing the resulting mass spectrum.

Mass spectrometry can be coupled to chromatography. For selected proteomic analyses, liquid chromatography (LC) is often used to separate proteins or peptides, then masses of the separated peptides are determined by MS. For LC-MS analyses only microgram quantities of a sample are necessary for its characterization [31, 32].

The 2DE gel-based technique is a very useful tool for protein separation [33], where during the first dimension proteins are separated according to their net charge by isoelectric focusing *via* immobilized pH gradient gel-strips. Subsequently, during the second dimension, proteins from the gel-strips are resolved according to their molecular weight using SDS-PAGE. 2DE can be very useful as post-translationally modified protein isoforms are typically revealed. These protein isoforms are not easily resolved by the other methods. A drawback of 2DE is related to gel-to-gel variations and the necessity of a large amount of samples (usually from 300 µg to 1 mg) for analysis. Moreover, the detection limit of proteins in 2DE is in the microgram range. In spite of this, interesting 2DE studies have resulted in important data pertaining to DM [34].

In addition, 2DE-gels have been coupled to differential in-gel electrophoresis (DIGE). In 2DE-DIGE, the protein samples are labelled with fluorescent dyes and then separated by 2D-PAGE. Different biological samples are labelled with different fluorescent dyes, mixed together and separated in the same gel. The gels are scanned by laser and the images of 2D-PAGE gels for multiple samples are obtained from the single gel. 2D-DIGE overcomes gel-to-gel variations and enables comparisons of multiple samples directly. Although 2DE-DIGE is a more refined strategy than 2DE, the detection limit remains at 0.5 µg.

In contrast to 2DE, LC-MS is a gel-free tool where digested proteins or tryptic peptides can be separated according to their physical and chemical properties (*i.e.* hydrophobicity, charges and pH). LC-MS has a higher sensitivity than 2DE-gels allowing the detection of proteins present at low levels and at the same time highly expressed proteins. Nevertheless, high abundant proteins

such as some serum proteins (albumin) must be depleted to refine the resulting data [34].

Ion exchange chromatography is a popular methodology that allows the purification of proteins as well as other charged molecules. In strong cation exchange chromatography (SCX) positively charged molecules are attracted to a negatively charged solid support. Conversely, in strong anion exchange chromatography (SAX), negatively charged molecules are attracted to a positively charged solid support. Ion exchange chromatography consists of a methodology to separate molecules based on differences related to their accessible surface charges. This technique is extensively applied in the pre-fractionation and/or purification of target protein(s) from crude biological samples. It involves the reversible adsorption of charged molecules to immobilized ion groups on a matrix of an opposite charge, thus, interactions among molecules and active-sites on the membrane support, occur in a convective manner *via* pores. Once the sample is loaded and equilibrium is reached, the molecules reach the adsorption step because of appropriate charge and displace the counter ions. Finally, they bind reversibly to the matrix. Generally, the unbound materials will pass by the column with the void volume. In the third stage, *via* increasing the ionic strength of the eluting buffer, substances are removed from the column. Also, high protein recovery rates with intact biological activity are produced by the relatively mild binding and eluting conditions of this separation method. Furthermore, SCX and SAX can be easily coupled to MS [35, 36].

## Analysis of protein phosphorylation

It is very important to analyse phosphorylated proteins in clinical research, as they may imply new targets for drug/therapy innovations. We will detail it in a simple manner *via* the most currently useful tools.

### Sequential elution of (IMAC) followed by TiO<sub>2</sub>

Immobilized metal affinity chromatography (IMAC) and titanium dioxide (TiO<sub>2</sub>) are metal-beads that can be packed to construct manual metal-affinity chromatography-tips to isolate primarily multi-phosphopeptides *via* IMAC and mono-phosphopeptides *via* TiO<sub>2</sub> [35]. Thus, this tool is useful for the isolation of mono and multi-phosphorylated protein/peptides from a biological complex-analyte in a single experiment. The basic principle relies on the fact that metals contained in IMAC and TiO<sub>2</sub> carry positive charges and link the negatively charged-phosphopeptides. TiO<sub>2</sub>, IMAC and ZrO<sub>2</sub> are all useful for phospho-enrichments, but Thingholm *et al.* [37] demonstrated a higher yield from SIMAC when purifying mono and multi-phosphopeptides in a single experiment; thus we recommend SIMAC as it works well for clinical samples. The most common techniques for enrichment for individual and/or global phosphorylation are IMAC and TiO<sub>2</sub> [38], which are based on the high affinity of positively charged metal ions. However, conversion of carboxylate groups to esters effectively eliminates non-specific retention of non-phosphorylated peptides, although this constitutes a drawback because of the increased complexity in the subsequent MS analysis.

During the last 10 years,  $\text{TiO}_2$  has emerged as the most common of the metal oxide affinity chromatography based phosphopeptide enrichment methods. This technique offers increased capacity compared to IMAC resins to bind and elute mono-phosphorylated peptides.  $\text{TiO}_2$  exploits the same principle as IMAC, and is similarly prone to non-specific retention of acidic non-phosphorylated peptides. However, when loading peptides in 2,5-dihydroxybenzoic acid [39], glycolic and phthalic acids, non-specific binding to  $\text{TiO}_2$  is reduced, thereby improving phosphopeptide enrichment without a chemical modification of the sample.  $\text{TiO}_2$  is often considered to be interchangeable with IMAC. It works on similar levels of sample quantity (e.g. micrograms of protein) for the identification of phosphorylation-sites by MS analysis. Recently, SIMAC [39–41] appeared as a phosphopeptide enrichment tool that exploits the properties of IMAC coupled to  $\text{TiO}_2$ , making it possible to carry out more refined studies.

Another phosphopeptide enrichment procedure prior to MS analysis is  $\text{ZrO}_2$  [42] and its principle is based on metal affinity chromatography like IMAC and  $\text{TiO}_2$ .  $\text{ZrO}_2$  permits the isolation of single phosphorylated peptides in a more selective manner than  $\text{TiO}_2$ . It has, in fact, been successfully used in the large-scale characterization of phosphoproteins [43–46]. Furthermore, strategies that consist of fractionating and subsequently enriching phosphopeptides are based on strong cation/anion exchange (SCX and SAX) chromatography and HILIC interaction chromatography. Calcium phosphate precipitation is also a useful pre-fractionation step to simplify and enrich phosphopeptides from complex samples which can be coupled to IMAC [46].

### Isobaric tag for relative and absolute quantitation

This technique allows the relative and absolute quantification of 2 to 8 complex-samples at the same time [47]. It uses a multiplexed isobaric chemical tagging-reagent. This tag permits multiplexing of 2 to 8 complex-protein samples and produces identical MS/MS sequencing ions for all 8 versions of the same derived tryptic peptide. Quantification and analyses are performed *via* comparing the peak-areas and peak-ratios in the MS/MS mode. The reporter-ions used are from 114 to 117 Da and from 113 to 119 and 121 Da. To carry out protein expression level studies, it can be a good option to apply isobaric tag for relative and absolute quantitation (iTRAQ), even though stable isotope labelling by amino acids in cell culture (SILAC, when using cell lines) and/or MRM or SRM can also be successfully employed (see below) [48, 49].

SILAC is another approach for *in vivo* incorporation of a label into proteins for subsequent MS-quantitative analysis. The identification step is currently carried out in MS/MS mode. Its principle is based on metabolic incorporation of a given 'light' or 'heavy' form of the amino acid into the proteins. Amino acids are incorporated *via* substituted stable isotopic forms such as deuterium,  $^{13}\text{C}$  and  $^{15}\text{N}$ . Basically, during SILAC experiments, two cell-populations are grown in a culture media that is identical except that one contains the 'light' label and the second one the 'heavy' label. These labels allow the comparison of two physiological conditions (e.g. healthy *versus* ill). Two conditions are distinguished *via* the labelled analogue amino acid that was

added to the culture media. The newly synthesized proteins within cells have the same physical and chemical properties except for the mass differences as a result of the isotope [50].

### Selected reaction monitoring or multiple reaction monitoring

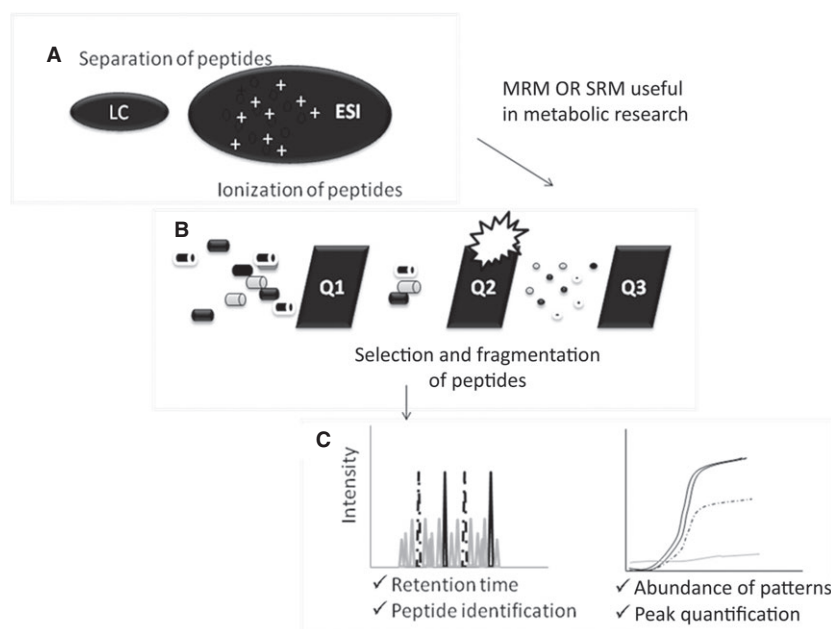
This is a quantitative proteomics MS/MS-based tool. When using ESI, first, a peptide precursor is selected and isolated to obtain an ion population that represents 'the precursor'. Next, the ion population is fragmented to produce product ions or 'daughter ions'. The signal-intensity of the fragmented ions represents the abundance of peptides and/or proteins for a given sample. Indeed, this method allows for absolute quantitative data. Selected reaction monitoring or MRM is carried out in specific mass spectrometers called 'triple quadrupole' and 'ion traps'. The sensitivity and specificity of such strategy is extremely high. In SRM assays, two mass analyzers are used as static mass-filters to monitor a particular fragment-ion from a selected precursor-ion. The resulting selectivity from the two filtering stages coupled to the high-duty cycle data in quantitative assays allows for extremely high sensitivity [51–60] (Fig. 1 shows in a simple scheme of SRM/MRM useful for metabolic research).

### Label-free quantification

This is a MS-based method that allows the determination of the relative amount of protein from 2 or more complex samples [61]. Label-free quantification does not use a stable isotope containing compound to chemically bind to the proteins. In a label free quantitative proteomic analysis, protein mixtures are analysed directly and samples are compared to each other after independent analyses. As a result, there is no mixing of samples, so that higher proteome coverage can be achieved and there is no limit to the number of experiments that can be compared [62]. Moreover, label-free approaches may be divided into two main groups by the way that the abundance of a peptide is measured. The first group comprises methods that are based on the ion count and compare either maximum abundance or volume of ion count for peptide peaks at specific retention times between different samples. Since ionized peptides elute from a reversed-phase column into the mass spectrometer, their ion intensities can be measured within the given detection limits of the experimental setup [43–45, 63–68] (Fig. 2 shows in a simple manner the scheme of Label-free useful for metabolic research).

### Electrospray ionization and tandem mass spectrometry MS-n (Nano-ESI-MSn)

Tandem MS [69], ESI coupled with CID and MS/MS potentially represents one of the most sensitive, discriminating and direct methods for the qualitative and quantitative high-throughput analysis of sub-picomole amounts of protein. Nano-ESI-MS/MS allows identification

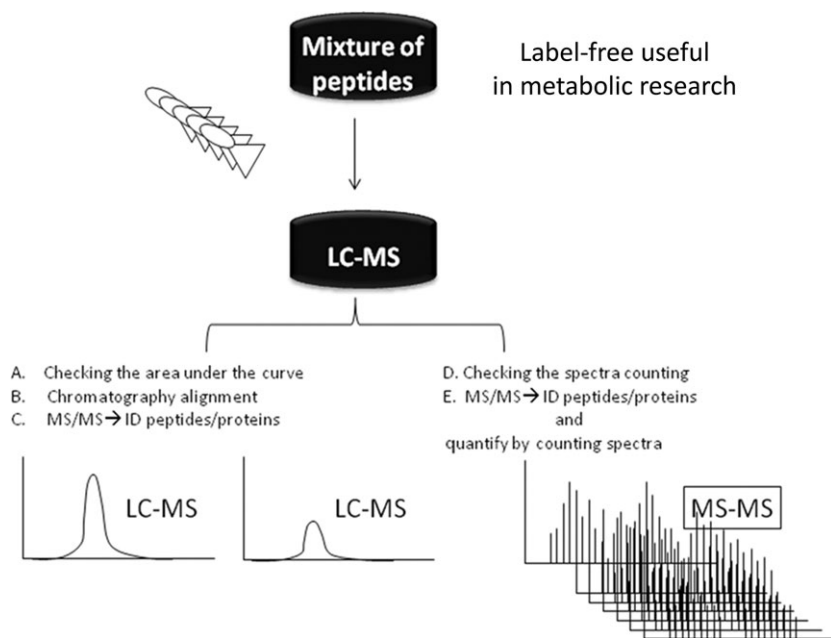


**Fig. 1** Scheme of SRM/MRM useful for metabolic research. SRM or MRM for quantitative assays consists of: **(A)** following, for example, ionization ESI type, **(B)** a peptide precursor is first isolated to obtain a substantial ion population of mostly the intended species. This population is then fragmented to yield product ions **(C)** whose signal abundances are indicative of the abundance of the peptide in the sample. SRM can be carried out on a triple quadrupole, where mass-resolving  $Q_1$  isolates the precursor,  $Q_2$  acts as a collision cell and mass-resolving  $Q_3$  is cycled through the product ions which are detected upon exiting the last quadrupole. A precursor/product pair is often referred to as a transition.

of the previously labelled and isolated proteins/peptides (and phospho-proteins/peptides) coming from complex samples (*i.e.* blood and/or CSF) and subsequently, data analyses of the quantified (from iTRAQ label) and modified residues (from isolation SIMAC) must be conducted *via*, for example, Matrix-Science Mascot searching ([http://www.matrixscience.com/cgi/search\\_form.pl?FORM-VER=2&SEARCH=MIS](http://www.matrixscience.com/cgi/search_form.pl?FORM-VER=2&SEARCH=MIS)).

Through the previously mentioned tools, we can achieve: (i) identification (ID) of protein candidate biomarkers, (ii) analyse how the

intracellular signalling -networks are activated/deactivated *via* phosphorylation during disease progression and (iii) determine which proteins and phosphoproteins or other PTMs are up and/or down-regulated in several clinical states (healthy or ill). The resulting data is specific for a given time and/or disease state (*i.e.* after diagnosis, during treatment) of a specific patient/s and according to each different type of sample (blood, sera, urine) as the proteome is dynamic (space and time). Finally, the resulting identified protein-biomarkers can be validated by ELISA and/or western blotting and *via* SRM/MRM



**Fig. 2** We suggest to perform this scheme of Label-free quantification useful for metabolic research. **(A)** Peptide signals are detected at the MS1 level and are distinguished from chemical noise/background by their characteristic isotopic pattern. **(B)** These patterns are then followed *via* the retention time dimension and are used to rebuild a chromatographic elution-profile of the mono-isotopic peptide mass. **(C–E)** The total ion current of the peptide signal is then integrated and used as a quantitative measurement of the original peptide concentration. For each detected peptide, all isotopic peaks are first found and the charge state is then assigned. Label-free can be carried out *via* Fourier Transform Ion Cyclotron Resonance (FTICR) or Orbitrap.



assuming that an antibody is available for a given protein [70–72]. To summarize, to obtain reliable and reproducible biomarker data, it is always necessary to standardize protocols for the collection, handling, storage and processing of samples for subsequent proteomic analyses using any of the procedures described above. These tools allow the analysis of hundreds of proteins at a given time in a very small sample size with high sensitivity. The main difference among these current techniques is related to the sensitivity, the detection-level of the selected method [73–80].

## Identification of proteins

Mascot Server (<http://www.matrixscience.com/>) is commonly used for identification, characterization and quantification of the resulting proteins after MS results have been collected. It allows free searches and contains protein sequence databases online from all organisms analysed to date. Through Mascot it is also possible to validate the identified protein-biomarkers from the clinical proteomic research study *via* manual inspection of all the spectra. Manual validation of spectra is required by the best proteomic journals to ensure the high quality of data. New and efficient bioinformatic software is routinely appearing on the market to carry out statistics and validation, especially for high-throughput analysis; thus, the validation-step can be developed more efficiently. Interesting and useful bioinformatic tools for proteomics research are well-established in other reviews [80, 81].

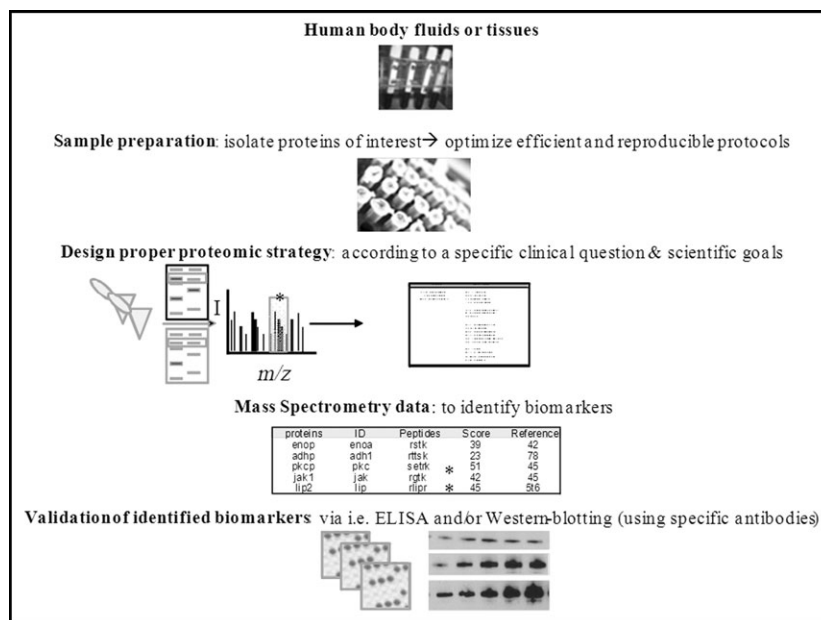
Thus, it is obvious that the methodology available to perform proteomics and phosphoproteomic studies has advanced dramatically in recent years, to improve our understanding of intracellular signalling networks. These methodological advances are now beginning to be

applied to studies of DM2 and the identification of new biomarkers. Figure 3 shows, in a simple manner, the basic work-flow useful for metabolic research (Fig. 3). Below we briefly review some of the most relevant studies in diabetes using proteomic approaches.

## Current and relevant studies of DM and obesity using proteomic approaches

Yang *et al.* [82], carried out 2DE of peritoneal dialyzed samples. They were able to visualize more than 300 protein-spots, from which 13 protein spots were selected to be excised and the corresponding proteins were digested and analysed by reverse-phase nano-ultra performance LC-ESI-MS/MS. The resulting data confirmed 10 spots/proteins with significant differential expression between the DM and chronic glomerulonephritis peritoneal dialyses samples. These authors state that the differentially expressed proteins identified in their study may not be new biomarkers. However, they also indicate possible targets for drug treatment and protein profiles that could be predictors of peritonitis, indicating the necessity for validation studies in the future. A possible way to validate the resulting identified protein-biomarkers from obesity and DM2 samples during clinical proteomic research studies is *via*: (i) ELISA and/or western blotting (using specific antibodies (Abs) for the identified potential biomarkers-) and (ii) *via* SRM/MRM [4]. Selected reaction monitoring or MRM is a method of MS/MS that allows one to monitor target peptides within a complex mixture of tryptic peptides. For example, proteins A1 and B2 are discovered as biomarkers for DM2 at diagnosis in obese patients, while proteins C3 and D4 are biomarkers for DM2 during treatment

**Fig. 3** General scheme of current proteomic-flow trough, for clinical metabolic research. Human body fluids (*i.e.* sera, urine and blood) have to be properly stored and prepared with optimised protocols. Subsequently, the proteins should be purified and/or isolated to get digested peptides (*i.e.* using trypsin). The adequate proteomic-MS based strategy is applied, and once we get the data (potential biomarkers), validation assays (*i.e.* ELISA and/or western blotting) can be carried out choosing specific antibodies to identify real protein-biomarkers. Currently, clinical proteomics research involves high-performance chromatography coupled to mass spectrometry avoiding 2DE-gels to identify high and low abundant proteins in a given clinical sample.



with complications and proteins E5, E6 and E7 are indicators of good prognosis. These candidate biomarkers can be routinely monitored in each patient to get more information about how to improve the treatment, regime and their prognosis.

In 2009 Kim *et al.* [83] carried out proteomic analysis in ob/ob mice, which lack leptin, before and after hypoglycaemic polysaccharide treatments. Their goal was to identify biomarkers of DM2 prognosis using 2DE-gel electrophoresis (2-DE). Specifically, these authors studied the influence of hypoglycaemic extracellular polysaccharides (EPS) coming from the macrofungus *Tremella fuciformis* on the differential levels of plasma proteins in ob/ob mice via 2-DE. They were able to visualize 900 spots of which 92 were differentially regulated in ob/ob mice. From these 92 spots, 40 were identified to be relevant diabetes-associated proteins. Furthermore, they were able to corroborate that high serum level of ferritin, which acts as an antioxidant via binding the iron-excess, is a risk factor for future-DM2, and that adiponectin plays an important role in the metabolic syndrome and inflammation, as low serum levels of adiponectin are a risk element for DM2. They also demonstrated that Apo A-I, IV, C-III, E, retinol-binding protein 4 and transferrin proteins were significantly altered in ob/ob mice, and their levels were normalized after EPS treatment. Through western blot, they observed that while resistin is up-regulated, adiponectin is down-regulated in diabetes and obesity and this was normalized with EPS. Moreover, to complement the resulting proteomics data, Kim *et al.* [83] investigated the differential gene expression patterns in different tissues, such as liver, adipose tissue and muscle of ob/ob mice in response to EPS treatment, by use of PCR arrays. The resulting data demonstrated that the expression level of many genes related to the onset, development and progression of diabetes was significantly down-regulated by EPS therefore suggesting that EPS might act as a potent regulator of gene expression for a wide variety of genes in ob/ob mice, particularly in obesity, insulin resistance and complications from diabetes mellitus [83]. Thus, Kim *et al.* [83] successfully coupled different OMIC tools to improve and corroborate the resulting data.

It is well-known that obesity and ageing affect adipocyte metabolism and the distribution of fat in subcutaneous and visceral depots. Moreover, weight gain and ageing can lead to similar clinical outcomes as, for example, insulin resistance, cardiovascular disease and atherosclerosis. Alfadda *et al.* [84] studied the expression level of proteins in obese patients in relation to their subcutaneous adipose tissues and age. Through 2DE-DIGE coupled to MALDI-TOF these authors were able to identify 9 highly expressed proteins and 4 lower expressed proteins in adipocytes from old compared to young obese patients. Some of the more highly expressed proteins include: (A1) prohibitin 1, (A2) protein disulphide isomerase A3, (A3) beta actin, (A4) profilin, (A5) aldo-ketoreductase 1 C2, (A6) alpha crystallin B and (A7) the annexins A1, A5 and A6. The 4 less abundant proteins are: (B1) keratin type 2 cytoskeletal 1, (B2) keratin type 2 cytoskeletal 10 and (B3) haemoglobins A and B. These are involved in regulation of apoptosis, cellular senescence and inflammatory responses, all of which are common pathologic events in obesity and ageing. In addition, signal transducer and activator of transcription (STAT) 3 was identified as the central molecule in the connectivity map and the apoptotic pathway as the pathway with the highest Mascot score:

(Mascot is a search engine which uses MS data to identify proteins from primary sequence databases, as the resulting digest peptide mixture is analysed by the sequence from MS as described above) ([http://www.matrixscience.com/search\\_form\\_select.html](http://www.matrixscience.com/search_form_select.html)). Differences in the abundances of prohibitin 1, protein disulphide isomerase A3, beta actin, profilin and STAT3 proteins were validated via immunoblotting. The data resulting from the studies by Alfadda *et al.* demonstrated that the proteins identified to be differentially expressed in aged obese versus young obese patients suggest an increase in resistance to apoptosis as a defence mechanism against a state of chronic low-grade inflammation. They also state that their data provide clues for unravelling the biochemical mechanisms underpinning obesity and obesity-related ageing [84].

Recently many researchers have begun to use MS-based quantitative tools (*i.e.* iTRAQ) instead of 2DE or 2DE-DIGE. The main advantage is that MS-based quantification allows measurements via adding isotopes labels; thus more proteins, including low expressed proteins, can be quantified. Mass spectrometry-quantitative studies using iTRAQ in DM2 represent a good possibility for meeting the challenge of improving the data collected to date, as it belongs to a very refined proteomic strategy.

A relevant example is the study of Cai *et al.* [85] who analysed cardiomyopathy and DM2 with the iTRAQ proteomic approach. Diabetic cardiomyopathy accompanying T2DM is a complicated disorder caused by a multifactorial pathology including altered cardiac energy metabolism and increased oxidative stress. Obesity is associated with high levels of circulating fatty acids, which can result in increased fatty acid uptake and TG accumulation in the myocardium. Furthermore, increased oxygen damage and generation of reactive oxygen species (ROS) augment cardiac damage. Thus, normalization of cardiac energy metabolism and reduction in oxidative stress may be important factors in the treatment of diabetic cardiomyopathy. Phlorizin has been reported to have an anti-diabetic effect because of its antioxidant properties. Although phlorizin is an antioxidant used in treating DM, its cardio-protective effects on diabetic cardiomyopathy are not well established. Through iTRAQ high throughput proteomics Cai *et al.* studied the function of phlorizin in preventing diabetic cardiomyopathy in db/db mice, which are obese and diabetic because of a defect in the leptin receptor. They coupled LC-MS/MS to iTRAQ to identify and characterize the protein profiles of phlorizin-treated and untreated db/db mice. Heart tissue from treated and untreated mice was prepared for iTRAQ analysis to obtain the protein-profile pattern of phlorizin's effect on myocardial proteins. A total of 1627 proteins were identified. Of the 113 differentially expressed proteins, 29 were elevated in the db/db group compared with the control group, but were decreased by phlorizin treatment. An additional 84 proteins were decreased in the db/db mice compared with the control group. The authors selected 2 proteins (calnexin and integrin-linked protein kinase) for western blotting analysis to validate the iTRAQ data. Calnexin was found to be decreased, whereas integrin-linked protein kinase was increased in the phlorizin treated DM group compared with the DM group. Quantification of the band intensity showed that the results were consistent with the iTRAQ data.

Cai *et al.* [85] were able to identify thousands of proteins of which 12–15% were differentially expressed. These proteins are involved in

cardiac lipid metabolism, mitochondrial function and cardiomyopathy. The resulting data suggests that phlorizin may prevent the development of diabetic cardiomyopathy by regulating the expression of key proteins in these processes. They also showed that phlorizin significantly decreases body weight-gain and circulating levels of glucose, triglycerides, total cholesterol and glycosylated-end products. Their findings suggest that normal myocardial structure was better preserved after phlorizin treatment and that this drug can be a novel therapeutic protocol for the treatment of diabetic cardiomyopathy, with the proteomic data helping to identify the possible mechanism [85].

Adipocytes play a central role in energy metabolism and in the obesity epidemic. Therefore, determining the protein composition of adipocytes should help to unravel important biological questions. In such an endeavour, Mann *et al.* [86] studied the adipocyte-proteome by proteomics plus mass spectrometry and bioinformatic tools. They first carried out subcellular fractionation of the mouse preadipocyte cell line 3T3-L1 with and without insulin treatment, into cytosol, membrane, mitochondria and nuclear fractions. They used SDS-PAGE gels from which the protein-bands were digested and the resulting peptides were identified *via* LC-MS/MS/MS (LTQ-FT). They were able to identify 3287 proteins that form part of the adipocyte proteome. Moreover, each fraction was analysed by western blotting and using specific antibodies for each fraction. In addition, they validated all resulting data by bioinformatics, obtaining one of the largest high confidence proteomes reported. The adipocyte-proteome is available in the Max-Planck Unified Proteome database [86]. This article contains very useful information to unravel the complexity of the adipocyte in obesity and other pathologies [86].

Another example from the group of Mann *et al.* [87] is related to unravelling the insulin induced intracellular signalling pathway *via* phosphoproteomics. It is well-known that the insulin signalling pathway is very important in metabolic diseases and cellular processes of ageing. The insulin receptor and its substrates are fundamental in the insulin signalling-network, with insulin binding to its receptor to trigger tyrosine phosphorylation cascades that subsequently activate other connected cascade networks. Understanding the activation of connected signalling networks is the key to unravelling diverse biological processes related to DM and obesity. To begin to understand the network of the tyrosine phosphorylation cascade, Mann *et al.* identified the tyrosine-phosphoproteome of the insulin signalling pathway by applying MS coupled to phosphotyrosine immunoprecipitation and SILAC in differentiated brown adipocytes. They also quantified the temporal dynamics of tyrosine phosphorylation events upon insulin stimulation in differentiated brown adipocytes. The authors applied high resolution quantitative MS-based proteomic tools (SILAC and anti-pY immunoprecipitation) to identify and quantify 40 protein effectors from the insulin pathway in differentiated brown adipocytes. To know the temporal dynamics of phosphorylation of proteins on tyrosine, the authors applied a triple label of SILAC with three differentially labelled cell populations being stimulated for different times. Data were corroborated by western blotting. From the 40 insulin effectors identified, 7 (SDR, PKC binding protein, LRP-6 and PISP/PDZK11, a potential calcium ATPase binding protein) were described for the first time to be involved in insulin signalling. In addition, Mann *et al.* indicate that this approach is capable of detecting the specific

pY containing proteins/peptides and quantify the level of their phosphorylation according to different stress and stimuli conditions over insulin network [87].

Walker *et al.* [88] have recently suggested that in obesity the 'networks' of metabolic signalling pathways are related to vitamin D status and that vitamin D regulation of adiponectin involves post-translational-events. They coupled 2DE-gels plus MS to identify relevant molecules in obese children dichotomized according to 25OH vitamin D (25OHD) levels. Platelet-free plasma from 42 obese children (M/F = 18/24) classified according to their 25OHD3 levels (<15 ng/ml = deficient and >30 ng/ml = non-deficient) was analysed. Image analysis (ChemiDoc Imager and PD Quest –software to analyse the resulting proteomic data-) was able to identify the protein-spots from 2DE-Sypro-gels that were differentially expressed according to each individual spot 'volume' *via* density/area integrating Sypro-Ruby staining to the Gaussian model. Their data showed 53 differentially expressed protein-spots, from which 51% were down-regulated (comparing VD deficiency and no deficiency of VD). One interesting identified biomarker is the HMW form of adiponectin, which was observed to be down-regulated in obese paediatric patients with vitamin D deficiency. Additionally, the identified biomarkers are able to be modulated *in vivo* with vitamin D supplementation. This proteomic approach represents a very interesting strategy to differentiate phenotypes of diseases and also to study specific therapy targets.

An important contributing factor for obesity and its associated comorbidities is a sedentary lifestyle, in addition to excessive food intake. Abu-Farha *et al.* [89] recently published an interesting study applying high-through-put proteomic analyses of peripheral blood mononuclear cells (PBMCs). The PBMCs samples were purified from lean and obese human males to identify and quantify differentially expressed proteins between both groups. In this study the mechanisms underlying obesity progression and the possibility of managing this progression with physical exercises were analysed. Using SCX plus MS/MS, 47 proteins differentially expressed between lean and obese patients were identified [89]. Thrombospondin 1 (TSP1) was up-regulated while histone deacetylase 4 (HDAC4) was down-regulated and after 3 months of physical exercises, TSP1 and HDAC4 returned to control levels.

Omega-3 polyunsaturated fatty acids (n-3 PUFA) are reported to decrease the symptoms of diabetes, obesity and insulin resistance related to the metabolic disorders. The proteins and pathways involved in the regulation of n-3 PUFA are unknown, especially those which produce beneficial health effects. Ahmed *et al.* [90] studied the effect of diets with high or low levels of n-3 PUFA on hepatic proteomic profile in mice. They applied 2DE-gels coupled to LC-MS/MS analysis plus specific software (ImageScanner III; Progenesis Same-spots, version 3.1). It is important to note that 800 µg of total protein needed to be loaded into 2DE-gels (it is now possible to use other proteomic tools, mentioned above, to avoid the necessity of using such a large amount of sample). The resulting protein-spots visualized *via* 2DE were digested with trypsin and analysed by MALDI-TOF (MS). They also carried out analysis by tandem mass spectrometry *via* LC-MS/MS to identify protein-spots from the 2DE-gels. MALDI-TOF MS is useful to study specific or a reduced number of protein-

spots, while when using LC-MS/MS, a greater number of protein-spots can be more rapidly identified. They found that a diet rich in n-3 PUFA decreases the expression of regucalcin, adenosine kinase and aldehyde dehydrogenase. In addition, diets rich in n-3 PUFA increase the expression of apolipoprotein A-I, S-adenosylmethionine synthase, fructose-1, 6-bisphosphatase, ketohexokinase, malate dehydrogenase, GTP-specific succinyl CoA synthase, ornithine aminotransferase and protein disulfide isomerase-A3. Thus, these authors showed that n-3 PUFA produces modifications in many proteins related to regulation of lipids, carbohydrates, the citric acid cycle and protein metabolism and promoting the hypothesis that there is a network of metabolic pathways affected by n-3 PUFA [90] (Table 1 shows examples of diabetes and obesity studies using proteomic tools: the type of sample and the clinical and proteomic goals are detailed, in addition the identified proteins/biomarkers, including the proteomic approach, are mentioned [91–102]). Martos-Moreno *et al.* [103] evaluated the ability of serum proteomic analysis to detect the metabolic alterations compared to standard clinical assays, and also to identify potential new candidate biomarkers from metabolic impairment in very young obese children. They discovered that isoforms of apolipoprotein-A1, apo-J/clusterin, vitamin D binding protein and transthyretin were down-regulated *via* 2DE coupled to MALDI-TOF (MS, MS/MS) in young obese patients with some changes in these proteins being enhanced by insulin resistant and partially reversed after weight loss.

Interestingly, they observed that low molecular weight isoforms of haptoglobin were increased in obese patients, enhanced in insulin resistant obese children and again decreased after weight loss, being positively correlated with serum interleukin-6 and NAMPT/visfatin levels. The significance for a potential candidate biomarker was confirmed statistically for low molecular-weight isoform haptoglobin (obese *versus* control and insulin-resistant *versus* non-insulin-resistant) and Apo A1 (IR *versus* non-IR). Indeed, apolipoprotein-A1 and haptoglobin were validated *via* ELISA to confirm the clinical significance of those potential biomarkers related to metabolic complications in young obese children.

This research study establishes the base or substructure of proteomic utilities for obesity clinical improvements, to identify, in the near future, true candidate biomarkers may help to improve therapies at the early onset of obesity and insulin resistance in childhood [104].

List *et al.* [105] carried out proteomic analysis on the skin of C57BL/6J mice with type 2 diabetes using non-diabetic mice as controls. To induce obesity and diabetes, authors applied high fat diet to mice during 16 weeks. They applied 2DE and PDQuest software for the analysis getting around 1000 distinct protein spots. From those 1000 spots, 6 were shown to be significantly decreased while 22 were over-expressed in the diabetic state compared to controls. These analyses were carried in a MALDI-TOF in MS and MS/MS mode. List *et al.*, observed that around 60% of the proteins that were up and/or down-regulated, belong to energy metabolism. The sample analysed in this study was diabetic skin, and it provides the identification of proteins from mouse-skin samples related to changes in obesity and subsequent diabetes. In addition, the authors remark on the relevance of skin biopsies coupled to proteomic

assays as a very useful tool non-invasive for diagnoses of hyperinsulinemia and diabetes [105].

Kopchick *et al.* [104] remarked in at the beginning of 2009 on the use of proteomics in general for blood, urine and tissue analyses for the discovery of new growth hormone (GH) induced serum biomarkers. They discovered that isoforms of transthyretin, clusterin, ApoE and ApoA1 are differentially expressed *via* MS and MS/MS coupled to 2DE-gels, thus these isoforms may be potential biomarkers for initiating studies using recombinant human growth hormone (rhGH). In addition, they suggest that with more biomarkers, it is possible to develop a robust, sensitive and specific test system for rhGH using a combination of multiple markers, thus achieving new targets and therapy improvements.

The detection of rhGH is difficult as it has a short half-life, thus, Kopchick *et al.* [106] indicate that novel and robust biomarkers of to detect rhGH abuse are needed. Through 2DE and MS, specific isoforms of alpha-1 antitrypsin and transthyretin were observed to be increased; while inter-alpha-trypsin inhibitor heavy chain H4, apolipoprotein A-1 and haemoglobin beta chain were observed to be decreased. The resulting data shows that high dose rhGH administration significantly reduced total serum protein concentrations and up- or down-regulated specific isoforms of five serum proteins. These protein isoforms may serve as potential biomarkers of rhGH treatment including when rhGH is misused and abused. For this study, serum samples- derived from the patients treated with rhGH in a randomized, double-blind, placebo-controlled-design were analysed. In addition, the biological meaning resulting from MS data was validated by western-blotting assays. Table 1 summarizes useful metabolic research examples carried out *via* different proteomics strategies (Table 1).

It is obvious that the current international human proteome sequencing programme (<http://www.thehpp.org/human> proteome project HUPO) will have an important impact on the diagnoses of diseases and the innovation of therapies [107–109]. It has been predicted that the complete human proteome will be fully sequenced in less than 2–3 years; thus several chronic diseases, such as diabetes, obesity and/or ageing, will benefit from proteomics research [110]. Therefore, reference map of the human proteome useful for many pathologies will be available, thus facilitating the use of proper controls for each clinical study [110], as it is very important to choose proper patients and good controls for independent verification, especially when looking for real protein biomarkers for improvements in diagnoses and therapies [111].

## Concluding remarks

The molecular mechanisms underlying obesity and its progression to DM2 are not completely known. Proteomics and other OMICS tools are helping to advance our understanding of the origin, onset, development, prevention and treatment of complex diseases including obesity and DM2. Moreover, as proteomics-MS-based technologies are becoming more sensitive and specific, employment of these tools is an important opportunity to augment our



knowledge of DM2 and obesity and identify new targets for diagnosis and treatment.

Liquid chromatography-mass spectrometry results in increased accuracy compared to 2DE-gels, especially because of the fact that LC-MS allows identification of low expressed proteins (good protein candidate biomarkers). Thus, although important information has been achieved *via* 2DE (especially concerning isoforms studies), new concepts can be reached when analysing clinical samples *via* proteomic approaches that directly apply LC-MS avoiding 2DE.

The new possibilities of proteomic strategies, which include SILAC, iTRAQ, label-free and nano-ESI-LC-MSn, could result in the identification of new phosphorylated biomarkers in the intracellular signalling networks involved in DM and obesity. It should be emphasized once more that correct clinical sample preparation is an essential pre-requisite and this should be standardized in biobanks.

To finalize, the advances in proteomic approaches and the complete sequence of the human proteome, will allow us to unravel changes in the proteomic profile of clinical samples from obese patients with and without diabetes, as well as DM2 with and without

obesity (Fig. 4 summarizes the pros and cons (tips) of several proteomic methodologies useful for metabolic research). Thus, it will benefit the patients and help to advance specific therapies. As a delegate of HUPO (<http://www.hupo.org/>; *Fostering international proteomic initiatives to better understand human disease*), for human proteome on children assays and studies at Hospital Universitario Niño Jesús (Madrid, Spain), we are seeking to support the human proteome in this context. We envision this will further benefit the understanding of the pathology of the diseases and ultimately improve the diagnoses and personalized treatments in the near future; thus, patients will undoubtedly be benefited.

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Methodologies	Pros	Cons	References
<b>Sample preparation</b>	Use anti-proteases and anti-phosphatases Use replicates stored at –80°C	Avoid if possible detergents: they can artefact low expressed proteins Avoid contaminants such as queratins	[12–15]
<b>2DE</b>	Isoform-protein studies	Visualisation of high abundant proteins	[33, 34, 102]
<b>SCX/SAX</b>	pre-fraction step to get accurate data	Need to optimise an efficient protocol for your clinical sample	[35, 36]
<b>IMAC</b>	Enrichment mainly in multi-phosphorylated peptides	Easy to loose mono-phosphorylated peptides	[38, 39]
<b>TiO2</b>	Enrichment mainly in mono-phosphorylated peptides	Easy to loose multi-phosphorylated peptides	[40]
<b>SILAC</b>	Enrichment in mono and multi-phosphorylated peptides	None has been described yet	[37]
<b>iTRAQ</b>	Label at peptides-level and up to 8 conditions can be compared and useful for tissues	Quantitation at MS2 level, needing specific softwares	[47]
<b>SILAC</b>	Label at amino-acids level and up to 8 conditions can be compared	Mainly useful for living cells	[49]
<b>SRM/MRM</b>	No label, highly sensitive, for all kind of samples	Needing very specialised personal, needing most cases to know the specific target-protein	[50–56]
<b>Label-free</b>	No label, highly sensitive, for all kind of samples	Needing very specialised personal, be careful with XIC normalization	[61]
<b>Nano-LC ESI</b>	Highly sensitive, today is the substitute of 2DE, high accuracy	Very clean samples must be prepared	[69]

**Fig. 4** Pros and cons of several proteomic tools useful in metabolic research. In this figure, a summary of pros and cons (tips) of several proteomic methodologies, has been detailed, from sample preparation to get MS data. We aim to place useful tools for proteomic metabolic research.



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## Conflicts of interest

The authors confirm that there are no conflicts of interest.

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