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REVIEWS

Tackling the human adipose tissue proteome to gain insight into obesity and related pathologies

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Obesity is becoming an important public health problem given its strong association with insulin resistance and Type 2 diabetes. Previously considered an inert depot, fat is now regarded as a highly metabolically active tissue in many pathophysiological processes. In humans, the accumulation of omental rather than subcutaneous adipose tissue appears to be tightly linked to cardiovascular disease and other important comorbidities. Proteomics has emerged as a method for the large-scale study of proteins in biological samples, for instance, fluids, cells or tissues, which encompasses not only the identities of the proteins present, but also quantification and post-translational modification events. Human adipose tissue proteome analysis, still in its early stages, may help understand the molecular mechanisms of obesity and the role of omental fat in the pathogenesis of obesity-associated diseases. This review covers recent advances in human adipose tissue proteomics, focusing on the analysis of the omental and the subcutaneous fat.

KEYWORDS: 2D fluorescence difference gel electrophoresis • adipose tissue • metabolic syndrome • mass spectrometry • obesity • omental fat • polycystic ovary syndrome • proteomics • secretome • subcutaneous fat

Obesity is recognized as one of the most important public health problems facing the world today. According to the WHO, more than one billion adults are overweight, and 300 million are clinically obese. Prevalence is growing exponentially worldwide, both in developed and developing countries [1]. Of particular concern, obesity has also increased dramatically over the last decades in children and adolescents [2].

Obese subjects have a decreased quality of life and life expectancy, as well as an increased risk of suffering insulin resistance, Type 2 diabetes, cardiovascular disease (CVD), hepatic steatosis, pulmonary and muscular pathologies, psychological disorders, and cancer, among others. The authors of a recent systematic review searched reports to identify prospective studies of incident cases of 20 cancer types, including 282,137 incident cases. Associations were generally similar in studies from North America, Europe and Australia, and the Asia-Pacific region. Increased BMI was associated with increased risk of common and less common malignancies [3].

The physiologic processes that regulate weight and metabolism, including peripheral hunger

and satiety signals, the central integration of this information, and the integrated gastrointestinal response to food intake, have received intense investigation, particularly during the past decade. A person's weight and body composition are likely determined by interaction between his/her genetic makeup and social, cultural, behavioral and environmental factors. An increased intake of energy-dense foods, especially when combined with reduced physical activity, surely contributes to the high prevalence of obesity; however, the existence of complex systems that regulate energy balance requires that this paradigm be considered in a larger context [4].

Prevention, treatment and understanding of obesity etiology should be a priority for both scientific and government communities in view that this disease and its associated comorbidities are a major economic and societal burden.

Human adipose tissue: omental & subcutaneous fat biology

In humans, the adipose tissue is dispersed throughout the body with major intra-abdominal depots around the omentum, intestines and

Figure 1. Representative proteomic strategies. Two major proteomic workflows are shown, based on electrophoretic and chromatographic separation. Proteins extracted from biological samples can be separated according to either their Mw (1DE) or both their Mw and pI (2D-DIGE). Proteins, and especially peptides thereof produced by proteolysis, can be complementarily separated by LC. After in-solution or in-gel enzymatic digestion of the proteins, the tryptic products are analyzed by MS. Protein identification is achieved by interrogating protein databases with MS data from peptide mass fingerprints and/or MS/MS data from peptide fragmentation fingerprints. As an alternative, protein extracts can be separated on solid-state chromatographic surfaces and the retained proteins can be mass analyzed *in situ* for protein profiling.

1DE: 1D gel electrophoresis; 2D-DIGE: 2D difference gel electrophoresis; ESI: Electrospray ionization; HPLC: High-performance liquid chromatography; LC: Liquid chromatography; LIT: Linear ion trap; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; Mw: Molecular weight; pI: Isoelectric point.

perirenal areas, as well as in subcutaneous depots in the buttocks, thighs and abdomen. Some of these depots, known as omental or visceral fat, drain directly into the portal circulation and have been linked to many of the morbidities associated with obesity, including Type 2 diabetes and CVD [5]. In addition, adipose tissue can be found in many other areas fulfilling a function of mechanical support, including the retro-orbital space, fat pads of the heels, fingers and toes, and the bone marrow. Fat distribution, even in thin individuals with steady bodyweight and stable BMI, changes with age, decreasing in retro-orbital fat and subcutaneous fat and increasing in intra-abdominal fat [6,7].

Adipose tissue, previously seen as an inert fat depot, is now recognized as a key, highly active and versatile organ with many important physiological and pathological roles. Besides adipocytes, adipose tissue contains connective tissue matrix, nerve tissue, stromal-vascular (SV) cells, and immune cells. Most of the adipose tissue functions are carried out via molecules secreted

by adipocytes, macrophages and other fat cells, capable of acting locally and in many nonadipose tissues, such as muscle, liver and stomach tissues, as well as on nervous, immune and vascular systems, and even on bone turnover [4]. Therefore, fat is an endocrine organ which cross-talks with other essential organs like the liver, the muscle, the pancreas and the brain, being a crucial regulator of whole-body homeostasis.

Proteomics technologies

Proteomics is the large-scale study of proteins, including their interactions, post-translational modifications (PTM), localization and functions [8]. A collection of proteomic approaches employing multiple methodological strategies and technological platforms are available for protein detection, identification and characterization in disease-related processes, at the whole cell or tissue level, and even in subcellular structures and in biological fluids (FIGURE 1).

Figure 2. 2D protein separation of human omental adipose tissue. Adipose tissue proteins were solubilized with the Partial Mammalian Proteome Extraction Kit (Calbiochem), and a total of 100 µg of protein were loaded onto 4–7 pH strips in the first dimension. For the second dimension, SDS-PAGE with 12% polyacrylamide was used, and proteins were visualized by silver staining. **(A)** Whole tissue extract. **(B)** Highly soluble proteins (fraction 1). **(C)** Proteins with intermediate solubility (fraction 2).

Protein separation

Most protein separation approaches are based on electrophoretic or chromatographic methods. The classical approach for the quantitative profiling of protein expression has relied on 2DE in combination with mass spectrometry (MS) [9]. An important advance in 2DE is the fluorescence difference gel electrophoresis (DIGE) system, in which two pools of samples are labeled with different fluorescent dyes, mixed and subjected to separation in the same 2DE gel [10]. This technology allows reliable quantification of differences in protein expression between two conditions with minimal technical variability.

Complementary gel-free schemas for proteomic analysis rely on liquid chromatography (LC) separation of proteins or peptides [11], with or without isotopic or isobaric labeling [12]. An important implementation of LC, the multidimensional protein identification technology, is based on the combination of two stationary phases for increased resolution in the separation of complex peptide mixtures [13]. Usually, these chromatographic technologies are online-coupled to mass spectrometry (LC-MS) for automated protein identification, characterization of PTMs, and protein quantification.

Protein identification & quantification

Owing to its superior sensitivity and selectivity, MS is the analytical technique of choice for protein identification. MS instruments ionize and vaporize the analytes using different ionization techniques, notably ESI and MALDI. These ions are then separated and detected in the gas phase within the mass analyzer, which is normally based on electric fields (e.g., TOF, ion traps or Orbitrap) or magnetic fields (e.g., FT-ICR). For protein identification and characterization, proteins are usually digested with trypsin. The masses of the resulting peptides, or fragments thereof, are accurately measured by MS, and these experimental values are matched against theoretical values calculated from sequence databases. Isotopic or isobaric labeling of peptides is the widest used methodology for quantification in gel-free approaches [12].

Proteomics of human adipose tissue

Despite that proteomic-based approaches are widely used to study a variety of human tissues relating to different disorders, the proteomic analysis of tissues presents some challenging hurdles. Tissues are heterogeneous as they are comprised of multiple cell types and are difficult to obtain in sufficient quantities. Moreover, to minimize technical variability for comparative studies, it is essential to train clinical staff to collect samples from similar areas, across all patients treated with identical surgery procedures, and rapidly freeze biopsies at -80 °C to avoid protein degradation.

Adipose tissue proteomic studies present additional difficulties due to its large content in triglycerides (often more than 50% of the tissue volume), which interferes with protein solubilization and separation, and albumin, both of which hamper subsequent 2DE analysis. To deplete albumin and other abundant proteins, we have assayed some ligand-affinity methods such as the albumin and IgG removal kit (GE Healthcare), the montage albumin deplete kit (Millipore), and the mimetic screening column kit (Prometic Biosciences). However, as albumin is a carrier/transport protein that binds other important molecules, many other proteins were removed upon treatment (PERAL *ET AL.*, UNPUBLISHED). This fact, together with the reduced 2DE reproducibility, discouraged us to follow this approach.

We have applied fractionation tools to reduce the complexity of adipose tissue protein mixtures based on reagents with differential solubilization properties (partial mammalian proteome extraction kit, Calbiochem). We analyzed two fractions: one containing very soluble proteins (fraction 1) and another containing proteins with intermediate solubility (fraction 2). Serum albumin was kept in fraction 1, as shown in FIGURE 2. This approach increases the number of spots detected, at the expense of doubling the amount of biopsy required, as well as the number of gels to be analyzed.

Due to the aforementioned limitations, only a few reports have addressed the proteomic investigation of human adipose tissue. A pioneer study carried out in 2004 by our group [14], described the

Figure 3. Expression of FABP5 protein in the omental adipose tissue.

Immunohistochemical detection (brown) of (A) FABP5 and (B) macrophage-specific antigen CD68, in omental fat from a morbidly obese patient. Images are representative of adipose tissue sections collected from five subjects. a, adipocyte; ma, macrophage.

optimization of a protocol for protein extraction, and improved focusing in the alkaline region of 2DE maps based on cup-loading and DTT replacement by hydroxyethyl disulfide [15] in the strip rehydration solution. This led to the first description of the human adipose tissue proteome [14].

Differential expression of proteins in omental & subcutaneous adipose tissue

The size of the omental, more than the subcutaneous, fat is strongly related to a higher risk of suffering insulin resistance, Type 2 diabetes, dyslipidemia and CVD, among other diseases and comorbidities [5,16]; this is probably due to their dissimilar gene expression patterns and different biochemical, cellular and metabolic properties [17–23]. Recently, our group has investigated the difference between the omental and subcutaneous human adipose tissue proteome from healthy obese patients ($30 > \text{BMI} < 40 \text{ kg/m}^2$) by 2D-DIGE and MS, together with immunoblotting and immunolocalization analysis [24]. This study revealed 43 differentially expressed proteins and underlined the high metabolic activity of the omental compared with the subcutaneous adipose tissue, since a set of proteins which were upregulated in the omental fat were engaged in such metabolic pathways as carbohydrate and lipid metabolism, oxidation–reduction, lipid transport, protein synthesis, protein folding, cellular stress and inflammation. Despite that some of these deregulated proteins had been previously identified in studies based on animal models or *in vitro* adipocytes [24], most of these proteins represented newly described molecules involved in the pathophysiology of obesity and/or associated disorders, thus opening up new possibilities in the study of these diseases. Given the novelty of some of these proteins, they have not undergone appropriate biological follow-up yet and the study of their functional role is underway in our laboratory.

Some of the dysregulated proteins identified were confirmed by immunoblotting and immunolocalization assays. In spite of the high fat content of adipose tissue, immunohistochemical and immunofluorescence analyses could be successfully performed to find out the distribution of specific proteins in the adipose tissue. One of the proteins studied has been the fatty acid-binding protein, epidermal FABP5, which plays an important role in the adipose tissue for transporting not only fatty acids resulting from adipocyte lipolysis, but also retinoic acid into the nucleus favoring survival pathways [25]. Immunohistochemical analysis revealed FABP5 expression in adipocytes as well as in nonfat cells issuing from the SV fraction. Some of these SV cells were found to be macrophages, as shown by the CD68 labeling (FIGURE 3). Recently a few reports have shown an increased infiltration of macrophages in the omental fat, compared with the subcutaneous fat [26,27], which could account for the overexpression of FABP5 in

the omental fat from obese subjects. The infiltrated adipose tissue macrophages localize to ‘crown-like structures’ around individual adipocytes and are widespread in the omental fat of morbidly obese individuals [28]. FIGURE 4 shows the co-localization of FABP5 and CD68 in an omental fat cryosection from a morbidly obese patient.

In addition, we have used these strategies to confirm the presence of epithelial cytokeratins (CK-8, CK-7, CK-18 and CK-19) in the omental adipose tissue [24], the only fat tissue presenting mesothelium sheets surrounding the fat lobules (FIGURE 5) [29,30]. In our laboratory these cytokeratins are routinely employed to differentiate omental from subcutaneous fat samples. We have hypothesized that omental mesothelial cells might increase the mechanical stress of hypertrophied adipocytes during obesity development, producing cellular stress, which, in turn, would activate the unfolded protein response (UPR) signaling pathway. Of note, mouse model studies have suggested that UPR not only interferes with the insulin signaling pathway, but is also involved in initiating inflammation [31].

Proteomics of omental adipose tissue in other obesity-related diseases

As mentioned previously, the omental fat has been associated with a higher risk for suffering abdominal obesity-related comorbidities. Polycystic ovary syndrome (PCOS) is a complex endocrine disorder with a high prevalence (6–7%) in premenopausal women [32]. Androgen excess is the central defect in PCOS, leading to hyperandrogenism, oligoovulation and/or polycystic ovarian morphology [33]. Obesity plays a relevant role in the development of PCOS. More than 40% of PCOS patients are overweight or obese in an American population [34]. On the other hand, PCOS is probably the most frequent association of obesity in premenopausal women [35]. This relation may be explained by adipose tissue

secretion of several molecules that favor the insulin resistant and low-grade chronic inflammatory state characteristic of PCOS [36,37]. It has been reported that weight loss in morbidly obese PCOS patients, after bariatric surgery, resolves PCOS signs and symptoms, and improves insulin sensitivity and other metabolic comorbidities, underlining the importance of abdominal adiposity in PCOS [38]. Considering the relevant function that adipose tissue plays in PCOS pathogenesis, our group has focused not only on proteomic, but also on transcriptomic analysis to study omental fat from morbidly obese women presenting with or without PCOS [39,40]. The transcriptomic studies, based on the microarray technology (GeneChips from Affymetrix), revealed changes in the expression profiles of 63 genes, related mainly with insulin- and Wnt-signaling, lipid metabolism, oxidative stress, inflammation and immune response, along with other genes previously related to obesity and related comorbidities [39].

The comparative proteomic study utilized 2D-DIGE and MALDI-MS [40], to uncover differences in the expression of proteins involved mainly in lipid and glucose metabolism, oxidative stress and adipocyte differentiation. Interestingly, both the transcriptomic and proteomic studies revealed the overexpression of glutathione S-transferase Mu-3 in the omental fat of PCOS women, illustrating the complementarity of these approaches. It is noteworthy that both works have not only confirmed the well-known alteration of the insulin-signaling pathway, but also revealed novel functional pathways related to inflammation and oxidative stress, among others, in agreement with previous studies on PCOS [41–43].

Another fat depot, the mammary adipose tissue, has been analyzed by 2DE, MS, antibody arrays and immunoblotting [44]. The authors identified 359 proteins from breast cancer patients involved in many biological processes including signal transduction, cell communication, energy metabolism, immune response, transport and apoptosis. This report offered for the first time an overview of the mammary fat proteome and its interstitial fluid, emphasizing the significant role of the adipocyte in the breast tumor microenvironment.

Proteomics of human adipogenesis

Two works have resorted to 2DE and MS to study human adipogenesis *in vitro*. Human adipose-derived adult stem (ADAS) cells were isolated by collagenase digestion of subcutaneous fat aspirates from lean subjects [45]. Protein extracts were compared before and after adipocyte differentiation from ADAS cells and

Figure 4. Immunofluorescence detection of FABP5 and CD68 in human omental adipose tissue cryosections. Co-staining of adipose tissue macrophages in 'crown-like structures' surrounding an individual adipocyte demonstrates co-localization of both proteins FABP5 (green) and CD68 (red). The counterstaining of nuclei (DAPI) is shown in blue. Images are representative of adipose tissue preparations collected from five subjects.

a set of proteins were found modulated, showing the relevance of the heat-shock protein (HSP) family in the adipogenesis process. Noteworthy, our comparative proteomic study in omental and subcutaneous fat also revealed the differential expression of three HSPs (HSP90, HSP70 and HSP27) [24]. HSPs have been involved in obesity, insulin resistance and diabetes [31,46,47], while HSP70 has been genetically associated with obesity and Type 2 diabetes in case–control studies [48,49]. Lee *et al.* focused on proteins that were differentially expressed by the differentiation of human mesenchymal stem cells into adipocytes, revealing four proteins associated with adipogenesis [50].

Proteomics of human adipose tissue secretome

Since most of the functions exerted by the adipose tissue are mediated by secreted proteins, the study of the secretome is of particular interest, and has been approached by a variety of proteomic methods. In primary human adipocytes isolated from omental fat tissue, the combined use of 2DE and MS allowed the identification of several serine protease inhibitors (serpins) [51]. Omental adipose tissue explants were cultured in media

Figure 5. Immunohistochemical detection of CK-18 in omental adipose tissue with anti-CK-18 antibody. Only the mesothelial cells enveloping fat lobules are specifically stained with anti-CK-18 antibody in brown (A). The immunostaining with the secondary antibody alone is also shown (B). Images are representative of adipose tissue preparations collected from five subjects. a: Adipocyte; me: Mesothelium; v: Vessel.

containing L- $[^{13}\text{C}_6, ^{15}\text{N}_2]$ lysine, followed by SDS-PAGE fractionation and protein identification based on LC-MS [52]. A total of 70 of the proteins identified incorporated the label and were considered true adipose tissue-derived components related with signaling, extracellular matrix, immune function and degradation functional categories. Moreover, Klimcakova *et al.* made use of a human cytokine antibody array to provide an overview of the proteins secreted by human subcutaneous adipose tissue, in particular to investigate the effect of PPAR agonists on the production of adipokines [53]. Results showed that PPAR ligands regulate adipokine secretion. The same antibody array was utilized to search for novel adipokines abnormally secreted by omental adipose tissue in obesity [54]. The analysis of factors from isolated adipocytes and SV fraction, from obese and nonobese men, identified six new adipokines increased in obesity. These molecules were associated with several features of the metabolic syndrome, supporting the link between obesity and its cardiovascular or metabolic comorbidities.

Expert commentary

Human obesity is a very complex dysfunction, which results from the interaction between the individual's genetic background and his or her environmental, social and cultural factors. Fat, formerly considered an inert depot, is now recognized as a highly active organ, orchestrating vital physiological roles by secreting a wide variety of molecules that influence many body tissues. Numerous proteomics approaches have been applied with different animal models and cultured cells (see an excellent review by Barceló-Batllori *et al.* [55]), but only a few works have examined the protein content of the human adipose tissue, or the identification of the adipose tissue secretome. These techniques have been also employed to unveil protein expression differences between omental and subcutaneous fat depots, as the omental tissue is tightly linked to obesity-related disorders. The information attained from

proteomic studies on human adipose tissue, the topic of this special report, is providing crucial information to understand in-depth the pathophysiology associated with enlarged adipose tissue.

Five-year view

These are exciting times for all those working in obesity and proteomics. The next 5 years will be a crucial time for applying proteomics capacities to gain insight into obesity and related pathologies, as well as for translating this knowledge to limit spreading of these disturbing disorders.

The mechanistic links between obesity and its comorbidities will be a fertile area to study by proteomics approaches. Fat is now recognized as a highly active cross-talking tissue with important physiological and pathological roles. Numerous adipose tissue proteins have been discovered associated with obesity, but probably other molecules are still awaiting discovery. Characterizing these products, studying their function and interaction with other tissues, and further exploring their effects will increase our knowledge about how fat excess is contributing to the pathogenesis of both established metabolic syndrome abnormalities and unknown obesity-related diseases. The comprehensive study by proteomics technologies of the fat cells and their secreted products, including the precursor cells, will help us to understand phenotypic changes of the adipose tissue induced by obesity. Moreover, these studies will shed light on how adipose tissue responds to changes in energy balance, and most important, how to devise anti-obesity therapies. Most of this work will require the identification of low-abundance proteins and detection of very subtle expression changes under obesity conditions, a difficult task with currently available proteomics tools. A combination of multiple approaches, including new instrumentation, such as the Orbitrap, to increase sensitivity and proteome coverage will be needed. Improved fractionation methods and the analysis of specific subproteomes, for example, the phosphoproteome, will

render a deeper view of the fat cells proteome. However, the central step will be the leap from a static proteome analysis to quantitative proteomics experiments, focusing on the dynamics of each protein and their closest interacting partners, which will provide unprecedented dynamic views of the entire cell proteome.

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Key issues

- Obesity predisposes to Type 2 diabetes, cardiovascular disease, hepatic steatosis, hypertension, pulmonary and muscular pathologies, cancer, and psychological disorders among other diseases.
- The adipose tissue is an important endocrine organ which cross-talks with other central organs like the liver, the muscle, the pancreas, and the brain, acting as a crucial regulator of whole-body homeostasis.
- Omental adipose tissue is more tightly linked to obesity-associated comorbidities, like cardiovascular disease and metabolic syndrome, than the subcutaneous fat. This is likely due to differences in the production of adipokines and fatty acids released from omental fat into the portal circulation.
- The adipose tissue, due to its high fat and albumin content, exhibits particular difficulties for proteome analysis.
- Proteomics characterization of signaling pathways in adipose tissue cells is pivotal for understanding the biological mechanism underlying obesity and its comorbidities.
- Prevention of childhood obesity by shifting obesogenic lifestyle habits from the infant age must be an important public health priority.

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