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Minerva Endocrinology 2024 September;49(3):293-9
DOI: 10.23736/S2724-6507.23.04075-7

REVIEW

THE MULTIDISCIPLINARY MANAGEMENT OF PITNETS: FROM PATHOPHYSIOLOGY TO INNOVATIVE THERAPIES

Proteomics for the identification of peripheral markers in pituitary disease

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ABSTRACT

Although precision medicine moved its first steps from genomic medicine, it has gone far beyond genomics, considering the full complexity of cellular physiology. Therefore, the present time might be considered as the "post-genomic era." In detail, proteomics captures the overall protein profile of an analyzed sample. The goals of proteomic analysis are to perform a global analysis of protein expression and function, to systematically define the role proteins in physiological and pathological condition, to increase mechanistic understanding of the biological processes and to discover new biomarkers and therapeutic targets. In this narrative mini-review, the role of proteomics is discussed with a particular focus on the few attempts of the application of proteomic platforms for the identification of new biomarkers in pituitary diseases, namely in acromegaly, GH deficiency and male secondary hypogonadism.

(*Cite this article as:* Grande G, Graziani A, De Toni L, Ferlin A. Proteomics for the identification of peripheral markers in pituitary disease. Minerva Endocrinol 2024;49:293-9. DOI: 10.23736/S2724-6507.23.04075-7)

KEY WORDS: Proteomics; Pituitary diseases; Biomarkers.

The term "-omics" refers to the study of biological systems on a large scale.

The conclusion of the Human Genome Project, in 2003, represented an important milestone that led to the age of genomic medicine[1] Subsequent technological advances have reduced the cost of sequencing the human genome from the original $3 billion to $1500.[2]

Notwithstanding the quantity of information provided by genome sequencing, it has to be underlined that genome sequence defines just one among the factors involved in multifactorial diseases. Indeed, the Human Genome Project discovered about ~21,000 protein coding genes, representing only 3% of the genome. However, further mechanistic studies into non-coding DNA uncovered a plethora of regulatory mechanisms through interactions with both protein and RNA.[3] These studies revealed four million locations within our genome that might serve to control the transcriptional activity of the 21,000 genes. Genetics cannot predict therefore the diversity of protein expression patterns, post-translational modifications (PTMs), or protein-protein interactions that control an individual's response to disease or treatment.

So, albeit precision medicine moved its first steps from genomic medicine, it has gone far beyond genomics, considering the complexity of cellular physiology.[1] Therefore, the present time can be considered as the "post-genomic era."

The "proteome" might be defined, indeed, as the overall protein content of a cell that is characterized by their localization, interactions, post-

translational modifications and turnover, at a particular time. The term "proteomics" was first used by Wilkins in 1996 to denote the "PROTein complement of a genOME."[4] Proteomics captures the overall protein profile rather than the expression of individual genes. Proteomics might help to resolve the biological complexity of precision medicine. The study of proteins themselves might in fact help to study their characteristics, functions, and changes in diseases. Because of the nature of the proteome, proteomic- and interactomic- (the study of protein interactions) based personalized medicine is fluid, adapting to individuals and individual situations. Mass spectrometry (MS) based-proteomics offers today the most holistic, integrated system for clinical analysis of patient samples, seeking both known and unknown biomolecules. Over the last decade innovation in technology and bioinformatics development have allowed for the identification of molecular signatures of diseases according to proteomic profiles, and thus may become standard practice in the clinical laboratory.[5] Recent advances in proteomic techniques, proteomic data analysis, and application of proteomic techniques in clinical settings undoubtedly represent a real promise for early disease diagnosis, prognosis, and theragnosis on an individual basis. In the area of male reproduction, it seems the most promising and powerful platform which has been recently applied in order to widely study the physiology and pathophysiology of male reproduction and to identify novel markers of disease.[6, 7]

According to our knowledge and to our literature review, there are no available data regarding new biomarkers and proteomics in pituitary diseases such as partial and total hypopituitarism, Cushing disease, hyperprolactinemia and particular forms of central hormone production (such as TSH-omas or GnRH-omas).

Mass spectrometry (MS) based proteomics

Mass spectrometry is based on the ionization of a molecule. The sample is introduced in a high vacuum chamber. Inside the ion source the organic sample, transformed into gaseous form is bombarded by a stream of electrons emitted by the instrument. The electron beam is made perpendicularly to the influence of the gas propagation direction. The product ions, which are in the gaseous phase, are separated in the analyzer on the basis of their mass/charge ratio (m/z). The data processing system records these electrical signals as a function of the ratio m/z and converts them into a mass spectrum. The mass spectrum is unique for each compound and can be used as chemical "fingerprint" to characterize the sample or for identification in complex mixtures.[8]

MS-based techniques are applicable to the study of complex protein mixtures. This technology yields precise mass values through the measurement of the mass-to charge ratio (m/z) of ions generated from peptides and proteins within the sample.

Recent years have witnessed a significant improvement of mass spectrometers that provide accurate masses of analytes, such as time of flight (TOF), Fourier transform ion cyclotron resonance (FT-ICR), and Orbitrap detectors.[9]

Proteomic platforms can be classified in quantitative and qualitative[10] as well as in top-down and bottom-up on the base of different strategy utilized in the sample treatment.[11] The main point of qualitative platforms is to define the complete set of proteins present in a certain sample, post-translational modifications (PTMs) comprised, the typical set of proteins specifically expressed in cellular sub-compartments, without considering their abundance. However, qualitative proteomics must face the unequal distribution of the concentration of distinct proteins present in the biological sample, because the highly abundant proteins can prevent the detection of that ones at low concentration.[12] As reported in Figure 1, top-down and bottom-up approaches are two popular approaches that differ in the protocol applied for the sample treatment. Top-down platforms analyze proteins and peptides in their naturally occurring form, giving particular attention to avoid, as much as possible, any sample alteration. Conversely, the bottom-up approach consists in the analysis of the sample digested by specific enzymes, generally trypsin, which cleave proteins in correspondence of defined amino acidic residues. The presence of a protein in the sample is

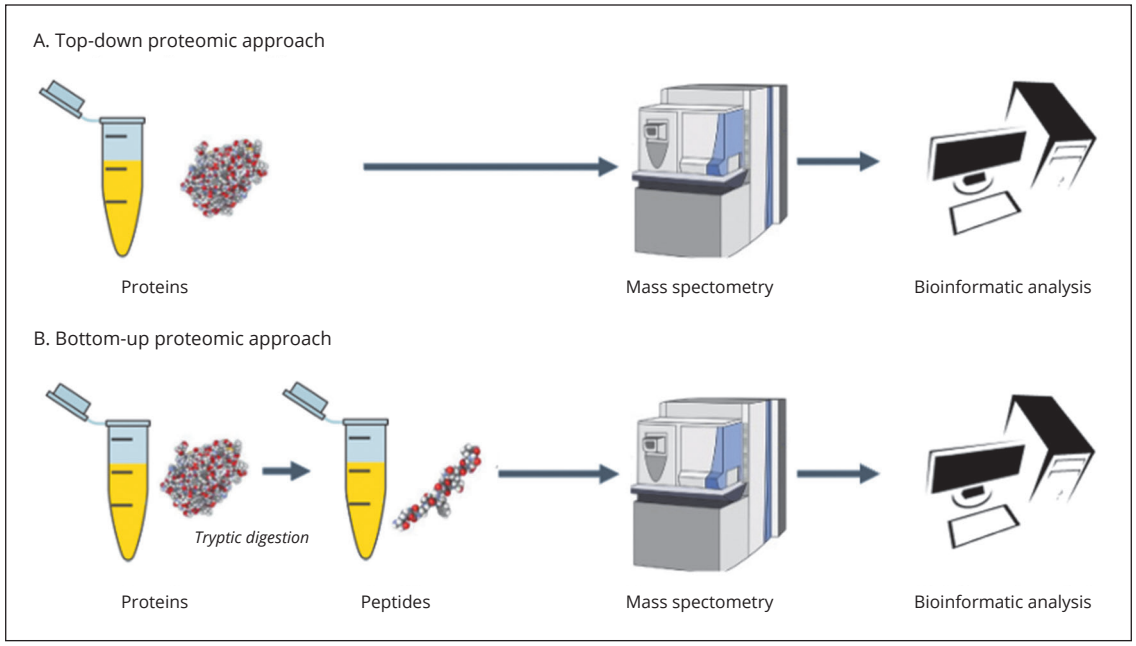

Figure 1.— Top-down and bottom-up approaches.

inferred by the detection of one or more of its specific fragments, implying bi-univocal correspondence between the intact protein and the tryptic fragments.[13] Both techniques carried out by tandem mass spectrometry require previous separation steps, to reduce the high complexity of the mixture. The different separation methods can be classified in: gel-based approaches, which can be applied for bottom-up analysis, such as the two-dimensional gel electrophoresis (2-DE); or gel-free-based approaches, employed for top-down experiments, for example liquid chromatography.

Serum proteomics for the identification of peripheral markers of acromegaly and GH deficiency

Acromegaly is a condition defined by the presence of GH and insulin-like growth factor 1 (IGF-1) hypersecretion. Over 95% of patients with acromegaly harbor a GH-secreting pituitary adenoma arising from somatotroph cells, whilst in less than 5% of cases the cause is found in an excess GH-releasing hormone secretion from a hypothalamic tumor, a neuroendocrine tumor or an ectopic GH production.[14]

The study of proteomics in patients with acromegaly, as well as the study of biochemical markers of the disease, is mandatory. In fact, persistent excess of both GH and its target IGF-1 results in a wide array of comorbidities that might not be reversible with disease control.[15] Moreover, normalization of IGF-1 and GH concentrations are the primary therapeutic aims in patients affected by acromegaly.

Only one study has been published aimed to identify serum proteomic changes in patients affected by acromegaly.[16] In this study, Cruz-Topete *et al.* analyzed serum samples of eight newly diagnosed acromegaly patients before and after transsphenoidal surgery for proteomic changes by two-dimensional gel electrophoresis. Protein spots displaying statistically significant changes, pre- *versus* post-surgery, were identified by mass spectrometry (MS), tandem MS (MS/MS), and western blot analysis. Six protein spots displaying decreased intensities after surgery were identified as: transthyretin (two isoforms), haptoglobin α2, β-hemoglobin, and apolipoprotein A-1 (two isoforms). One protein spot, identified as complement C4B precursor, was increased after the surgery.

However, some concerns may be expressed

about the real usefulness of these proteins as markers. In fact, although two isoforms of transthyretin are reduced, they might represent the effect of post-translational modifications, without any known clinical significance. Furthermore, the increase in haptoglobin α2 has not been associated with an increase in haptoglobin levels when assayed, so that its clinical significance is limited. Similarly, the decrease in 2 isoforms of apolipoprotein A-1 is not associated with an increase in apoA levels, thus suggesting that GH might interfere with apoA isoforms. However, the clinical relevance of these observation seems to be limited. The same group performed a serum proteomic study in eight patients with post-surgical GH deficiency, after surgery for pituitary adenoma. The study was performed before and after 3 months of GH treatment. Albumin depletion of the samples was performed using a ProteoPrep Blue Albumin and IgG Depletion kit (Sigma) following the manufacturer's instructions. Serum proteomics, after only albumin depletion, was performed by 2-D electrophoresis and mass spectrometry by a MALDI-TOF-TOF equipment. The levels of six serum protein spots were significantly altered after GH substitution. These proteins were identified as five different isoforms of haptoglobin (decreased in posttreatment samples) and one isoform of apolipoprotein A-I (increased in posttreatment samples). These results were independent of serum IGF-I levels.

As a consequence, once again, proteomic data seems to suggest that GH function (deficiency or increase) might be associated with specific patterns of PTM in haptoglobin and apolipoprotein A-1. However, although the possible speculations about these PTMs, no clear putative markers have been identified by these studies. More recently, Ortea *et al*.[17] performed a more sensitive study, on serum samples of 15 pre-pubertal boys with GH deficiency and compared their proteomes with 15 age-matched controls. Serum proteomics has been performed after depletion of the seven most abundant proteins (not only albumin as previously performed) such as albumin, IgG, IgA, transferrin, haptoglobin, antitrypsin and fibrinogen. The seven most abundant proteins in the blood were depleted using the Hu-7 Multiple Affinity Removal System kit (Agilent Technologies, Wilmington, DE, USA) following the manufacturer's instructions.

Sample procedures included precipitation, tryptic digestion, sample pooling and proteomic analysis using a modern HPLC/nano-LC-MS/MS (hybrid quadrupole-TOF mass spectrometer Triple TOF 5600+). Bioinformatic analysis has been performed with more stringent criteria. Starting from these methodological premises, 263 proteins could be confidently detected and quantified on each sample. Twenty-five differentially expressed proteins have been identified and specifically 21 over-expressed and 14 reduced proteins. The top 10 serum protein biomarker candidates could be identified after applying a feature selection data analysis. Gene Ontology analysis pointed to the apolipoproteins (namely ApoA4, ApoB, ApoC1, ApoC2, ApoC3, ApoC4, ApoD, ApoE, ApoF, and Apo H) as over-represented highlighting this protein group as a promising biomarker panel for the diagnosis of GHD and for treatment evaluation.

Three different bioinformatic tools have been used to analyze the cumulative predictive cross-predictivity among the identified proteins. To obtain a final biomarker set with the minimum number of features able to obtain the best classifying performance, in fact, the combination of three machine-learning algorithms was applied, using quantitative data from the 121 proteins with P values <0.05. This feature-selection analysis produced a ranking of the proteins according to how well each classified a sample in the correct group. The output of the biomarker-selection process was visualized as an importance graph, where the abundance and co-occurrence of selected features are visualized graphically as a network. In order to assess the robustness of the selection process, the analysis was repeated 20 times; candidate biomarker proteins were ranked according to their selection frequency.

In addition, the predictive accuracy (*i.e.* the ability to differentiate between both groups) of the top-ranked proteins and their combinations was assessed by ROC analysis.

The combination of three proteins, apolipoprotein A-IV, complement factor H-related protein 4 and platelet basic protein, showed the best classification performance (AUC of 1.0; 100%

sensitivity and specificity), thus representing a putative diagnostic panel for GH deficiency.

Taken together, these data underline the importance of study design and of a sensitive methodological proteomic protocol which is essential in the aim of obtaining putative clinical markers for clinical conditions.

This is particularly important for the diagnosis of GH deficiency, since diagnosis includes nowadays a combination of direct and indirect measurements, such as testing GH levels in multiple blood samples after provocative tests, and IGF-I testing, as indirect measurement of GH action. Starting from these premises, the need undoubtedly exists to develop accurate systemic non-invasive panels of biomarkers of GH excess or deficiency, that could easily be implemented within a clinical analytical assay and used in clinical management of the patients.

Seminal proteomics in hypogonadism

Male hypogonadism (MH) is defined as "a clinical syndrome that results from failure of the testes to produce physiological concentrations of testosterone (T) (T deficiency) and/or a normal number of spermatozoa due to pathology at one or more concentrations of the hypothalamic-pituitary-testicular axis."[18] Secondary hypogonadism (or hypogonadotropic hypogonadism (HH)) is caused by a disease of the pituitary gland. It is characterized by low T concentrations in association with normal or low FSH and LH concentrations.

Total T levels <8 nmol/L are deeply associated with the diagnosis of MH. A condition of hypogonadism is otherwise possible if the total T concentrations are 8-12 nmol/L. In this "grey zone" the diagnosis of MH is not immediate and the opportuneness for treating these patients with T should be guided by signs/symptoms of hypogonadism, albeit often those are non-specific.[19] In all patients, but especially in men with testosterone levels in the "grey zone" and signs/symptoms of MH, calculated free testosterone (cFT) determination – obtained by dosing testosterone, albumin and SHBG concentrations – allows a more accurate diagnosis of hypogonadism, since total testosterone determination alone misdiagnoses hypogonadism in about 8% of men with sexual symptoms.[20] However, these data underline the need of new peripheral markers of androgenic status, reflecting T action, especially in patients with T level in this so-called "grey zone."

Since prostate, epididymis and seminal vesicles are widely known to be androgen-dependent organs, seminal plasma might represent a valuable source of markers reflecting T action.[19]

In 2014, we successfully studied, for the first time, by using high-resolution mass spectrometry, the seminal proteome of patients affected by HH, evaluating them before and after 6 months of testosterone replacement treatment (TRT).[21] We studied 20 male patients with HH before TRT, whilst 10 patients were re-evaluated after 6 months of TRT. Ten normogonadic subjects were studied as a control group. Seminal plasma was analyzed by an Ultimate 3000 Nano/Micro-HPLC apparatus equipped with an FLM-3000-Flow manager module and coupled with an LTQ Orbitrap XL hybrid mass spectrometer. Sixty-one proteins were identified in the group of fertile men. Thirty-three of these identified proteins were absent in all the seminal samples by patients with HH. The expression of fourteen out of these 33 absent proteins was reverted in patients after TRT and 7 out of 14 differentially expressed proteins (prolactin-inducible proteins, lactoferrin, prostatic acid phosphatase, myeloperoxidase; zinc-alpha-2-glycoprotein, lactotransferrin, cystatin C) fell into a functional interaction network, centered on AR.

We furtherly repeated the study, by using a more modern technological platform and a quantitative bioinformatic strategy[22] and studying 10 male patients with postsurgical HH. 5 patients were studied after a brief course of 3-month of therapy with transdermal T to analyze the early-onset consequences of T therapy on seminal proteome. Eleven proteins were found to be lowered in the patients' group, compared with controls. In the population of five patients who received T therapy, the pre-treatment:post-treatment ratio was calculated for each identified protein, obtaining a list of under-expressed (ratio >1.5) or overexpressed (ratio <0.67) proteins after that therapy. Five proteins (semenogelin 1, semenogelin 2, prolactin-inducible protein, prostatic acid phosphatase, lactotransferrin) in-

creased after TRT. Finally, Western blot analysis confirmed proteomic data.

Further studies might, indeed, be necessary so as to understand if the assay of this panel of proteins in seminal plasma, derived by proteomic analysis, might be useful in the diagnosis and follow-up of patients with HH, and namely in patients with mild symptoms and T concentrations in the "grey zone." It is probably time, in fact, to pass to a "molecular androtest", representing a seminal fingerprint of male T peripheral action. In this perspective, seminal proteomics provided us the most promising platform at this aim.[19]

Conclusions

High-throughput -omics technologies let us study a large number of omics markers at the same time. Proteomics have been widely used to solve some of the pathophysiological questions related with several clinical conditions and identify new peripheral markers of disease. Although the cost of performing this technique remains a hurdle to its use in clinical laboratories, needing specific technical resources and professional expertise for technical analysis and data interpretation, proteomics represents today the best platform to identify putative markers of disease *in vivo*, to be confirmed by other methods and transferred into clinical workflows.

However, only few attempts have been still performed, in order to apply proteomic platform in the study of pituitary disease and identify peripheral markers of pituitary disfunction. However, preliminary data permitted to suggest two specific panels of peripheral proteins, namely serum proteins for GH deficiency and seminal proteins for HH, which might represent specific putative panels.

The identification of these non-invasive markers of hormonal activity should therefore be translated in clinical practice and might open new perspectives for the clinical workout of patients with pituitary disease.

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Conflicts of interest
The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Authors' contributions
All authors read and approved the final version of the manuscript.

History
Article first published online: April 9, 2024. - Manuscript accepted: November 15, 2023. - Manuscript revised: October 3, 2023. - Manuscript received: June 30, 2023.