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Review

Clinical implications of recent advances in proteogenomics

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Running title: Clinical proteogenomics

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Abstract

Proteogenomics, the alliance of proteomics, transcriptomics, genomics and bioinformatics, was first proposed for refining genome annotation using experimental data acquired on gene products. With high-throughput analysis of proteins made possible with next-generation tandem mass spectrometers, proteogenomics is greatly improving human genome annotation *per se*, and is helping to decrypt the numerous gene and protein modifications occurring during development, aging, illness and cancer progression. Further efforts are required to obtain a comprehensive picture of human genes, their products, functions, and drift over time or in reaction to microbiota and pathogen stimuli. This should be performed not only to obtain a general overview of the human population, but also to gain specific information at the individual level. This review focuses on the clinical implications of proteogenomics: novel biological insights into fundamental biology, better characterization of pathogens and parasites, discovery of novel diagnostic approaches for cancer, and personalized medicine.

Keywords:

Proteogenomics, Cancer, Onco-proteogenomics, Shotgun proteomics, Pathogens, Virulence factors, Personalized medicine

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1. Introduction.

The numerous applications of genomics in human health have led to considerable changes in clinical laboratories, where quantitative polymerase chain reaction and next-generation sequencing facilities are now widespread. Mass spectrometry-based analytical tools have been used in clinics for a long time, mainly for the identification and quantification of small molecules related to toxicology and endocrinology [1]. A novel increase in interest for such approaches can be explained by the unrivaled discriminative performance of mass spectrometry, granting the higher selectivity required for protein-based assays. While antibody-based detection of proteins remains widespread, this is restricted by the availability of antibodies and their limits in terms of specificity. Novel strategies for preparing medical samples and acquiring and interpreting mass spectrometry data are being developed leading to the identification and quantification of distinct proteoforms through specific and sensitive assays [2]. As an example, the evolution of diagnostics in microbiology over the past five years has been impressive, with the possibility to identify pathogenic bacteria within a few minutes using matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) mass spectrometry [3]. This approach is based on the measurement of the masses of low molecular weight, basic and abundant proteins from pathogens previously isolated on agar plates or grown in liquid blood cultures. These recorded mass profiles can then be matched against species-specific profiles present in curated databases. Such technical advances have contributed to the demonstration of the simplicity and advantages of using mass spectrometry-based approaches in the development of diagnostics, paving the way for more use of mass spectrometry in clinics.

With regard to diagnostics, omics approaches may be considered redundant in terms of information or even antagonist technologies, as they require specific instruments and

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expertise. However, concerning discovery and validation, omics are highly complementary [4]. Here, we describe how the alliance of proteomics, transcriptomics, genomics, and bioinformatics contributes to the elucidation of novel biological insights into fundamental biology, a better characterization of pathogens, and the discovery of novel diagnostic approaches for cancer. Although this alliance, named proteogenomics, was first devoted to improving genome annotation [5-9], novel applications have rapidly flourished. In the present review, we focus on the clinical implications of the most recent advances in proteogenomics.

2. Proteogenomics comes to the clinical scene.

Proteomics is heavily dependent on protein sequence databases. A cornerstone concept in biology is that genetic information together with its dynamic expression through transcription and protein regulation result in cellular behaviors and pathologies. Hence, human gene sequences and their environments have been studied for many years. The human genome was established as a result of worldwide collaborations [10,11]. Its analysis shed light on several genetic causes of disease and contributed to the improvement of our knowledge regarding cell defense systems and organismal responses to injury, toxic compounds and pathogens. Genome annotation is the result of sequencing data analysis, based on knowledge of proteins collected over time, and the basic transcription and translation rules established several decades ago. With the predicted protein coding sequences to hand, bottom-up proteomics can be applied on a genome-wide scale. This approach basically consists of digesting proteins into smaller peptides, measuring their precise masses together with the masses of their fragmented products by tandem mass spectrometry, and then matching these lists of parent and fragment peptide masses to theoretical protein sequences. Novel genomes can now be quickly

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sequenced and are automatically annotated through bioinformatic pipelines that apply basic translation rules, as well as the conservation of protein sequences in evolutionarily related organisms. However, genome annotation with such pipelines is far from perfect, and is still subject to debate [12].

The limits of genomics, transcriptomics and proteomics. Errors in genome annotation can result in overcalling or missing open reading frames, bad prediction of numerous translational starts, and splicing errors (Armengaud J, 2009). These errors can subsequently lead to wrong protein identifications in mass spectrometry analyses. Several studies have shown the incorrect annotation of a significant number (10–20%) of N-termini from annotated bacterial polypeptides [13]. As the gene-calling task in eukaryotes is more complex than in these microorganisms because of introns, an even higher rate of annotation errors can be predicted for complex biologic species. Indeed, in protozoan parasites such as *Toxoplasma gondii* and *Neospora caninum*, the assessment of splicing and alternative splicing of mRNA remains a daunting task [14]. Moreover, it has been shown experimentally that conservation of N-terminal protein sequences derived from comparative genomics does not always apply [15]. In fact, the specific traits of a given strain or subspecies should be explained at the molecular level by both protein sequence specificities and abundance changes. Sequence polymorphisms and mutations, gene duplications, acquisition of novel protein sequences, N-terminal polypeptide changes and splicing differences may all contribute to protein sequence differences between organisms. In addition, reversible variations in expression are also involved, such as those associated with chromosome spatial organization, which is currently being explored through methods derived from the chromosome conformation capture (3C) technique [16,17], and also post-translational modifications. All of these events are responsible for the presence of not yet described proteoforms that define subtle phenotypic differences between individuals [2]. As a result, direct derivation of gene structure from a

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closely related genome may be a source of mistakes. It thus becomes clear that DNA- and RNA-derived data should be combined with proteomic analysis in order to obtain a more comprehensive picture of molecular mechanisms.

Combining omics strengths with proteogenomics. Two decades ago, a pioneering idea was proposed: mining proteome data in order to correct genome annotation [18,19]. Peptide sequences certified by mass spectrometry could be mapped back onto the genome sequence to correct translational starts, firmly establish splicing events, or highlight the presence of unannotated protein coding sequences. This proteogenomic mapping was introduced by Jaffe et al. [7]. With the amazing technical evolution of tandem mass spectrometry, the throughput of proteomics has become adequate for large genome-wide surveys [20], allowing the proteogenomic mapping of numerous bacteria, archaea, protozoan parasites, fungi, plants, and animals. This strategy has also proved useful to improve the annotation of the human genome. However, even with the newest mass spectrometry tools, only the most abundant components of the so-called "expressed proteome" are detected. Because of a proteome sequence coverage that remains low, the approach is not yet sufficiently comprehensive. Over time, proteogenomics has evolved, taking advantage of RNA sequencing (RNAseq) and advanced sample preparation [21,22]. The presence of introns in eukaryotic genomes introduces an additional level of difficulty when predicting protein sequences. This issue has partially been circumvented by mapping splicing events from mRNA. Indeed, sequencing cDNA obtained from mature mRNA transcripts is directly informative. This has become a routine procedure for non-model organisms for which the genome has not yet been established [23-25] or is extremely difficult to annotate [26]. Moreover, proteogenomics can also be applied to the interpretation of proteomic data from a draft genome or transcriptome, without the need for a time-consuming genome annotation. In this case, the proteogenomic approach allows for a quick focus on the key proteins of a given metabolic or physiologic trait highlighted by the

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comparison of samples [27]. As depicted in Figure 1, proteomics can now be intimately combined with genomics and transcriptomics, together with the help of advanced bioinformatics, in order to get a more precise picture of molecular processes and diseases. This approach allows for the analysis of protein dynamics, providing information about cellular or subcellular localization and post-translational modifications. Proteogenomics has now come on to the clinical scene. The human body can be analyzed at a more comprehensive cellular and molecular level. Pathogens can be better characterized. Novel candidate biomarkers corresponding to specific proteoforms can be proposed for diagnostics. In the following chapters, we will review the different applications of proteogenomics and their perspectives.

- Insert Figure 1 here -

An expanding proteogenomic toolbox. Numerous methodological improvements in proteogenomics have already been published. For example, specific labeling of the N-termini of proteins and detection of the products by high resolution tandem mass spectrometry has been successful for systematic N-terminomics [13,28-31]. In terms of annotating the N-termini of human proteomes, novel specific datasets have been recorded [32,33], showing alternative translation initiation sites, pervasive endoproteolytic processing, and stabilization of protein fragments *in vivo* by an extensive post-translational Nα-acetylation. The integration of RNAseq and proteomic data has confirmed human coding genes [34] and contributed to the detection of gene mutations [35]. This alliance of transcriptomics and proteomics is also encompassed within the boundary of proteogenomics [36]. Protocols to enrich specific subproteomes and to improve proteome coverage by chromatography and mass spectrometry are also being developed [33]. In order to facilitate data interpretation, ribosome-protected mRNA fragments may be extracted and sequenced. This so-called “ribosome profiling” has enabled an in-depth characterization of alternative translation start sites [22]. This

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methodology has been applied to the human HCT116 cell line, for which nine previously unannotated protein products could be identified, as well as several alternatively spliced isoforms and protein variants [37]. Most of the proteins for which no experimental evidence has been recorded are membrane proteins. Kitata et al. [38] specifically explored membrane-enriched fractions of eleven non-small cell lung cancer cell lines, thereby identifying 178 previously unrecorded membrane-associated proteins. This work exemplifies the advantages of mass spectrometry approaches to broaden proteome coverage through analysis of specific subproteomes. Further evidence for the existence of a given protein may be obtained using *in vitro* transcription/translation strategies developed to devise precise conditions and parameters that are then applied for the identification of missing proteins in human cells and tissues using targeted mass spectrometry [39]. Last but not least, numerous bioinformatic pipelines or tools have become available to assist this inventory of missing proteins or splice variants by proteogenomics [40-46], while metrics for high confidence identification of "missing" proteins are just emerging [47]. Besides the common core proteome, more specific sets of proteins with highly variable sequences require dedicated efforts. For example, immunoglobulin light chain amyloidosis caused by the accumulation of clonal proteins can be probed by proteomics if peptide sequence variability is taken into consideration, because each sequence is unique to each patient [48]. A traditional database search strategy is inefficient in such a quest because of the lack of peptide sequences in reference databases comprehensively covering the variable region of immunoglobulin light chains. However, the successful strategy consisted of peptide mapping using an augmented protein database constructed from mRNAs from a cohort of Alzheimer's disease patients [48].

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3. Boosting the hunt for human missing proteins and splice variants.

The "Human Proteome Project" (HPP) conducted under the auspices of the Human Proteome Organization aims to improve the annotation of the human genome and to generate a comprehensive map of human proteins, their interactions, and their regulation [49]. Proteogenomics embraces the validation of the existence of "missing proteins", which comprise as-yet undetected protein products of annotated gene sequences as well as the detection of hitherto unannotated proteins and the correction of gene structures. A pioneering study interrogating tandem mass spectrometry data against an exhaustive theoretical protein database generated from a 6-frame translation of the entire human genome allowed the identification of previously unexpected peptides corresponding to splice variants in blood samples [50]. Amid the different projects supported by HPP, tandem mass spectrometry data from numerous laboratories were gathered in a unique database in order to get a global picture of the human proteome, which includes the identification of expressed proteins, splice variants and post-translational modifications: the PeptideAtlas compendium. Using a normalized Trans Proteomic Pipeline, the consistent analysis of mass spectrometry data from different sources allowed the mapping of at least one specific peptide to each of 11,868 SwissProt entries in 2012 [51]; 12,934 in 2013 [52]; and 14,070 in July 2015 [53]. This database has proven to be a prime tool of choice for developing targeted mass spectrometry strategies, and further confirms previously unreported peptide sequences. It also provides insights derived from sampling origins, such as which proteins are abundant in a given tissue, and at which developmental stage. Another database, Proteomics DB, enables the retrieval of proteomic information for a large set of human tissues, cell lines, and body fluids [54]. The latest one-shot extensive draft map of the human proteome validated 17,294 genes, accounting for 84% of annotated protein-coding genes [55]. Further analysis of large shotgun

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proteomic datasets resulted in a better annotation of specific human subproteomes: the mitochondrial proteome [33], hippocampus and brain proteome [56,57], liver proteome [58], and spermatozoan proteome [59], as well as several human chromosomes: chromosome 1 [60], chromosomes 2 and 14 [61], chromosome 4 [62], chromosome 7 [63], chromosome 9 [64], chromosome 12 [65,66], chromosome 16 [67], chromosome 17 [68], chromosome 18 [69,70], and chromosome 22 [71]. **Table 1** lists the main outcomes of these studies. Such a quest for missing proteins demands regular updates taking advantage of novel shotgun proteomic data [12], but also novel genome and RNAseq data. A strong focus on the annotation of disease-related proteins is extremely fruitful, as shown recently with the analysis of clinical specimens from patients with gliomas, Alzheimer's disease, and Parkinson's disease [65]. Being more precise with single nucleotide polymorphisms and splice variants is also achievable by means of proteogenomics [43,72-74].

- Insert Table 1 here -

4. Recent advances in human fundamental biology.

From gene expression to protein quantities. A broader definition of proteogenomics has emerged. Proteogenomics is not restricted to genome reannotation but can be aimed at functional analysis [21]. Most of the recent insights have been derived from a strategy coupling RNAseq-inferred protein identification with mass spectrometry quantification. Among others, an ultra-large proteomic analysis of 86 human colon and rectum tumor samples was performed using customized sequence databases from patient-matched RNAseq data [75]. This work identified numerous previously unreported single amino acid variants and allowed proteogenomic subtyping of colorectal cancer specimens. Interestingly, the

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availability of quantitative information at the protein as well as RNA levels demonstrated a modest transcript-protein correlation, thereby confirming the distinct RNA and protein quantities observed in previous measurements [76,77]. Indeed, protein quantity is a result of the balance between gene expression and protein degradation, which can be modulated by RNA editing, post-translational modifications, specific protein cleavage or degradation. Figure 2 shows the advantages of integrating different omics data regarding the numerous proteoforms present in cells and throughout the development of an individual.

- Insert Figure 2 here -

Novel proteogenomic insights into the immunopeptidome and the surfaceome. The immunopeptidome comprises all the small fragments of proteins that are displayed by cells on their surface in order to be checked by immune cells. The immune cells are in charge of discarding cells that are damaged, diseased, or contaminated by intracellular pathogens, as well as pathogens themselves, which can all be distinguished by non-self presented peptides. A novel atlas has been proposed to improve our knowledge of the immunopeptidome [78], allowing the development of improved personalized strategies for immune-based therapies. Deciphering the full repertoires of peptides bound to HLA molecules, in both health and disease, may be a daunting task [79], but novel strategies for recording and extracting mass spectrometry data make this possible. Recent work on cancer cell lines and primary cells has yielded data for an impressive number of HLA peptides, validating protein degradation as a key factor for HLA presentation in addition to identifying specific peptide sequences from a human colon cancer cell line [80]. A wider concept, the cell surface protein repertoire (the so-called surfaceome) is of pharmacological interest for improving drug addressing or defining new cancer vaccine targets. Such an atlas is currently being nourished with proteogenomics-derived information [81].

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Regulatory networks for understanding cellular mechanisms and diseases. Proteogenomics can help to define novel therapeutic targets based on a comprehensive overview of the molecular players in cells (Armengaud, 2010). As an example, high-throughput phosphoproteomics allows the analysis of signal-induced phosphoregulations that can occur in perturbed contexts such as diseases. Recently, a global analysis of kinase signaling networks upon EGF and IGF1 stimulation in models of acquired drug resistance demonstrated how the analysis of global phosphorylation responses to stimuli could provide information on cancer resistance mechanisms. Cells chronically treated with PI3K or mTORC1/2 inhibitors present different kinase responses to stimulation [82]. Importantly, a substantial advantage of mass spectrometry is its possible combination with isotopomeric labeling [83,84]. This allows the analysis of co-cultured samples through deconvolution of signals resulting from direct cell-cell interaction and can be applied to study the impact of specific microenvironments in disease development. A study comparing contact-initiated phosphosignaling in interacting EphB2- and ephrin-B1-expressing cells allowed the definition of the complex asymmetric and nonautonomous processes regulating Eph-ephrin signaling. In this case, cells were labeled with stable-isotope amino acids to differentiate phosphopeptides regulated in two interacting cell populations. The phosphorylation-based signaling of both cell types was analyzed in a system including multiple membrane receptors (as opposed to working with a recombinant ligand for stimulation), exemplifying the study of directional cell signaling in dynamic multicell population contexts [85]. These studies increase in interest if all the cellular protein players and the different proteoforms are well defined, thus highlighting the interest of applying proteogenomics whenever possible.

Omics-based personalized monitoring. Genomic and proteomic studies have traditionally relied on the use of reference databases built on the general population, thereby masking individual variations. A significant potential of proteogenomics is the ability to apply

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personalized analysis by combining DNA or RNA and proteomic data from a single patient. The first large personal omics profiling was performed on a single individual over a period of 14 months [86]. Blood samples were analyzed with a combination of genomics, transcriptomics, proteomics, metabolomics, and even an autoantibody profile was carried out, producing an invaluable dataset called the "Snyderome" [87]. Dynamic changes in molecular and biological pathways could be compared over this period, where the patient alternated between a healthy state, two viral infections and the onset of diabetes. The whole genome sequence of the subject was determined as a genomic baseline. By implementing this pilot study of personalized medicine, individual-specific single nucleotide variants and edited transcripts were shown to be translated into proteins. How gene and protein isoforms vary across different environmental conditions was described in this integrative personal omics profile, a concept that was abbreviated as "iPOP". This work highlights definitively the possible future use of proteogenomics for personalized medicine, and the benefit of complementary tools such as genomics, transcriptomics, proteomics, and metabolomics. To interpret proteomic data from a single patient without the need for deep sequencing of the genome, the use of databases compiling all polymorphism events occurring at the population level is an attractive alternative. Because proteomics brings information on the abundance of proteins, their polypeptide structure and post-translational modifications, focus on these molecular players should be a central pillar in personalized medicine.

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5. Onco-proteogenomics, the alliance of proteomics and genomics for a better understanding of cancer pathways and for novel diagnostic tools.

The application of proteogenomics in oncology began several years ago, and its success recently gave birth to a specific subfield dubbed "onco-proteogenomics" by Helmy and colleagues [88]. Numerous key studies fueled this important medical field [88-93]. The discovery of aberrant cancer-specific peptides, including products of gene fusion, odd splicing variants and novel expressed genes not present in the normal human proteome catalogue, can be performed by individual proteogenomic analysis where transcriptome and proteome data from each patient are combined. Initiatives such as the Clinical Proteomic Tumor Analysis Consortium (CPTAC, http://proteomics.cancer.gov) provide a fully integrated account of DNA, RNA, and protein abnormalities found in several tumor types [94]. The quest for biomarkers to detect early stage cancer has been a long journey, exemplified by a proteomic study of blood samples from patients with early stage ovarian cancer and of healthy women, which led to the proposal of three candidate biomarkers [95]. Of these, two were truncated forms of proteins that were only identified via the mass spectrometry approach: a truncated form of transthyretin and a cleavage fragment of the inter-α-trypsin inhibitor heavy chain H4. Proteins responsible for breast cancer susceptibility and dissemination have also been investigated with increasingly more powerful approaches, as exemplified by the latest study combining shotgun and targeted proteomics [96]. The advantages of proteogenomics to study the blood peptidome/degradome profile of cancer was illustrated with samples from breast cancer patients compared with healthy individuals [97]. A very similar set of plasma proteins was observed in the patient and control samples but strikingly different degradation patterns were observed, with 71 protein substrates degraded into 839 distinct peptides in the breast cancer patients while 50 proteins were degraded into 425 peptides in the healthy plasma

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samples. Although no major differences were observed in the cleavage specificities between the two conditions, the proteins cleaved were not the same. These changes, not identified by conventional bottom-up proteomics, potentially provided unique signatures that may be of diagnostic utility. Many studies have already demonstrated the importance of cancer-driving mutations of proteins such as RAS [98] and BRAF [99]. These have been the basis of diagnostic and cancer therapeutic developments for many years, paving the way towards targeted therapies where kinase inhibitors can reduce cancer burden. High-throughput technologies widen the window of analysis from a handful of proteins surrounding cancer drivers to large functional networks. Such studies shed light on the cellular consequences of oncogenic mutations and have shown that signaling pathways can be specifically dysregulated in defined pathological contexts. This has broadened the therapeutic options, since a pathway (or several pathways) can be targeted through many components in opposition to single mutated oncogenes or tumor suppressor genes that can be difficult to target [100]. Several proteogenomic approaches have been applied on cancer samples. Potential markers of colorectal cancer were proposed after the in-depth analysis of three human colon cancer cell lines [101]. Comparison of paired colorectal cancer and nontumorigenic tissues specified cancer-associated proteins and deregulated pathways such as focal adhesion, cytoskeleton or Rho signaling [102]. Human colon and rectum cancers were also categorized to delineate five proteomic subtypes and to prioritize candidate driver genes [75]. Advanced proteogenomics relying on proteomics and RNAseq data was able to identify multiple peptide mutations in colon cancer, as well as immunoglobulin gene variations/rearrangements, thus indicating a tumor immune response [103]. The genetic aberrations of 32 tumor antigens in invasive ductal carcinomas were documented by proteogenomic analyses, highlighting a quicker identification than with large-scale screening of genomic analyses [104]. A recent study explored the tumor microenvironment to assess exosome oncogenicity [105].

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A screen for alternative splicing via proteogenomics identified a novel protein isoform derived from cancer-related splicing variants in a highly metastatic gastric cancer cell line [106]. Proteogenomics is efficient for the identification of aberrant cancer peptides [107] and gene mutations [35] that could be neoantigens deserving further attention [108]. The system-wide approaches presented here illustrate the advantages of proteogenomics in the context of biomedical research by identifying potential biomarkers, together with providing mechanistic insights into cancer-regulated networks and cancer progression. The possible general strategies for personalized onco-proteogenomic diagnostics are illustrated in Figure 3.

- Insert Figure 3 here -

6. Revisiting human pathogens, parasites, and toxins with comprehensive proteogenomic approaches.

A better knowledge of the behavior of our age-old enemies – bacterial pathogens, viruses, and parasites – is vital to counteract them. Systems biology relies on the most comprehensive description of all parts of the system and their interactions. Proteogenomics is helpful in defining the ultimate missing pieces in terms of coding genes and proteoforms [6]. Although most of the methodological advances in proteogenomics have been proposed and optimized with nonpathogenic models [30,109-111], numerous proteogenomic studies have been performed on pathogens in order to better annotate their genomes and describe their cellular mechanisms. As a result, virulence factors and their modulation are now better understood [112,113], and a more comprehensive view of the interactions between a given pathogen and its host can be drawn [114]. The proteogenomic analysis of 15 million MS/MS spectra

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recorded from the bacterium *Yersinia pestis KIM* proteome allowed six genes to be discovered, numerous translational starts to be corrected, and an ultra-rare start codon, AUU, to be documented [115]. A key enzyme for virulence was shown to be erroneously annotated in terms of the translational start. This protein, Yersiniabactin thioesterase, participates in the biosynthesis of a siderophore, an important virulence factor involved in iron acquisition from the host. Such work was further complemented with novel data acquired on other *Y. pestis* strains [116]. The *Bacillus anthracis* and *Streptococcus pyogenes* proteogenomes were also probed through a large survey [117], as well as those of *Shigella flexneri* [118], *Helicobacter pylori* [119], enterotoxigenic *Escherichia coli* [120] and the mycoplasma, *Spiroplasma melliferum* [121]. The genomes of a pathogenic yeast, *Cryptococcus neoformans* [122], and a highly opportunistic pathogen, *Candida glabrata* [123], have also been reannotated based on proteogenomic results. Because these eukaryotic pathogen genomes are rich in introns, the validation of gene models by identification of the corresponding proteins is of great interest. Proteogenomics has proven its efficiency in such tasks by validating 83% of the predicted protein-coding genes in the latest organism. Proteogenomics was also found to be useful for defining biomarkers for whole-cell MALDI-TOF identification of pathogens such as *Neisseria meningitidis* [124] and *Francisella tularensis* [125].

Genome annotation of complex protozoan parasites can also be improved by proteogenomics. This has been exemplified with *Leishmania donovani*, which is responsible for the most severe form of leishmaniasis [126-129]. This analysis resulted in a solid background for conducting further experimental studies on this highly relevant parasite model [130]. Two other protozoan parasites, *Toxoplasma gondii* and *Neospora caninum,* have been analyzed with the help of RNAseq and proteomics data [14]. These studies highlighted the importance of maintaining active curation efforts to improve genome resources. A proteogenomics

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strategy has also been used to discover and characterize a novel bioactive peptide from the venom of a marine cone snail [131], and to assess susceptibility to endotoxin in animals [132].

Expert commentary

In its *sensu stricto* definition, proteogenomics combines genomics and proteomics to improve genome annotation and discover novel proteoforms. From a wider viewpoint, proteogenomics integrates different omics-related data in order to consolidate the gene structure and function of key proteins in a given biological system, and to add new information that cannot be derived directly from DNA/RNA-based technologies, such as abundance, post-translational modifications, and protein localization. For instance, the composition of the extracellular matrix, which is a crucial factor in the behavior of cancers, should be analyzed from a proteome perspective rather than through gene expression methodologies. Proteogenomics was first successfully applied to better annotate the genomes of numerous bacterial pathogens. Over the last decade, the proteogenomics toolbox has been enriched with methods for sample preparation, as well as numerous bioinformatic algorithms to facilitate data interpretation. It has also gained throughput with the technical advances of tandem mass spectrometry and RNAseq technology. The most recent results were obtained for the human genome annotation, which has been refined by means of large-scale studies in the framework of the Human Proteome Project, as well as for the genome annotation of several important human pathogens and parasites. Better multi-omics personalized monitoring is expected with these results in hand, together with higher-level integration of data acquired from the same individual that can be obtained by means of proteogenomics. Characterization of novel proteoforms produced during cancer progression through alternative splicing or mutations has

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given rise to a new specific field, called onco-proteogenomics. Developments in this area should lead to interesting novel concepts in terms of clinical diagnosis and prognosis. These clinical implications require large investments in proteogenomics-inspired multi-omics monitoring of large cohorts.

Five-year view

The analytical potential of the current generation of mass spectrometers is considerable. In the next five years, major improvements should be proposed in tandem mass spectrometry, leading to instruments with higher sensitivity, higher dynamic range, and higher *m/z* scan speed. For example, a new fragmentation mode, EThcD, has recently been introduced, combining electron transfer dissociation (ETD) and beam-type collision-induced dissociation (HCD). Such novelty holds great promise for the sequencing of large proteins with post-translational modifications and for *de novo* sequencing [133]. Moreover, the development of new mass spectrometry acquisition modes, especially data-independent acquisition [134,135], should increase the coverage of low abundance proteoforms. A result of this technological trend will be more comprehensive proteogenomic studies, and this should reinforce scientific outcomes and medical implications. Developments in bioinformatics and statistics should take into account the giant size of datasets that will need to be analyzed, as well as the gigantic size of databases. The best experts in the new "big data" field may be attracted by such a challenge, where most concepts are still to be developed in order to allow and speed up data interpretation. To this aim, removing the noisiest or least informative data from the datasets and databases is probably a winning strategy. Tremendous efforts have been already made to better annotate the human genome, but to date almost two proteins out of ten predicted remain

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missing. Exploiting the current tandem mass spectrometry datasets has already provided some interesting refinements in terms of missing proteins and splice variants. However, the current version of the human proteome is far from perfect, with numerous protein-coding genes still hypothetical. Apart from the identification of crystal-clear evidence of their existence, the documentation and cataloguing of functional and quantitative data for the corresponding gene products will bring some clues about function. The next challenge is to integrate in proteogenomics a new stage of molecular information such as metabolomics [136] or subcellular microscopic localization.

Novel, challenging proteogenomics projects for the personalized monitoring of individuals throughout life could be proposed. In this case, the main challenge will be that of informatics for the storage and treatment of data throughout an individual's lifespan, acquired with heterogeneous methodologies, given that technological improvements will occur frequently over time, with important breakthroughs allowing important scale changes. Long-term illness, exposure to toxicants, and aging could be monitored with such a multi-omics integrated approach. Onco-proteogenomics should rapidly become mature in terms of methodology and should lead to a better knowledge of cancer progression. Onco-proteogenomics could give rise to a paradigm shift in terms of cancer-related biomarkers, which could most probably be a set of proteoforms determined for each individual and each cancer type that could appear or change in abundance under specific conditions, rather than a given biomarker for the whole population. In such a case, the validation of the discovery approach would be more important than the biomarkers themselves. Of note, such proteogenomic biomarkers may not be amenable to targeted antibody- or PCR-based detection. Rather, nucleic acid sequencers and tandem mass spectrometers might be common in the near future in clinical and oncological diagnostic laboratories. Better knowledge of pathogens and parasites is also likely to be obtained by proteogenomics, leading to novel fundamental advances in fighting these agents.

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Key issues

● Proteogenomics combines the strengths of different omics approaches. Based on the six-frame translation of genome or RNAseq transcriptome sequences, proteogenomics allows the quick identification of key proteins from a shotgun proteomic dataset. Better annotation of translation starts and splicing events can also be obtained from the peptides recorded by tandem mass spectrometry.

● Proteogenomics is today the customary approach for discovering novel human genes and documenting the different proteoforms that could be produced from each of the ~20,000 human protein-coding genes. To date, approximately 18% of human genes have not been characterized through the detection by mass spectrometry of any peptide sequence evidence. Advances in covering the whole human proteome are being achieved through a multinational proteogenomics consortium.

● Omics-based personalized monitoring is starting to be implemented, representing an important breakthrough in personalized medicine. Proteogenomics allows individual polymorphisms to be taken into account when analyzing proteomic data. Although the cost of long-term studies on large cohorts restrains its generalization, proteogenomics approaches show attractive perspectives.

● Onco-proteogenomic approaches have been implemented for a better understanding of cancer pathways. Because of the important genomic drift of cancer tissues, novel proteoforms not yet present in human protein sequence databases can be detected by tandem mass spectrometry when searching unassigned MS/MS spectra. These unexpected peptides or proteoforms could be used as novel diagnostic biomarkers, or at least for personalized monitoring.

● Numerous human pathogens, largely bacteria, have been better characterized in terms of protein sequence patrimony by proteogenomics. Complex protozoan parasites are also under

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study to improve their genome annotation and to better describe their cellular mechanisms. New knowledge could emerge from proteogenomics-inspired characterization of microbe-host interactions, and consequently novel drugs targeting the most sensitive molecular players could be proposed.

Financial & competing interests disclosure

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Table 1. Main features of proteogenomic annotation of the human proteome.

Subproteome	Description of the study	Scale of the study	Significant output	References
Mitochondrial proteome	N-terminome analysis of human mitochondria-enriched samples from U937 human monocytic cells.	Identification of 2,714 unique proteins, including 810 mitochondrial.	Identification of the N-termini of 693 unique proteins (283 mitochondrial); confirmation of 120 already annotated processing cleavage sites; characterization of 302 new cleavage sites.	[33]
Hippocampus and brain proteome	Proteomic analysis of 2 subcortical brain areas and correlation of their proteomic expression profiles with human brain transcriptome data available at Allen Brain Atlas.	Identification of 4,235 unique proteins.	Identification of 894 proteins with brain-wide expression not previously identified by MS; Identification of 112 proteins with region-specific expression.	[56]
Liver proteome	MRM screen of proteins identified in the liver with low protein evidence (one peptide only) in peptide atlas.	Monitoring of 1,110 transitions corresponding to 185 proteins.	Presence of 57 proteins confirmed with one peptide; identification of 7 proteins with no previous MS information.	[58]
Spermatozoa proteome	Catalogue of missing proteins in total protein extracts from isolated human spermatozoa.	Identification of 11,281 peptides corresponding to 1,547 proteins.	Identification of 89 missing proteins; confirmation of the presence of 3 uncertain proteins.	[59]
From the chromosome-centric human proteome project				
Chromosome 1	OMICs-integrated analysis of three human liver cell lines presenting different lung metastatic potentials.	Identification of 1,308 (out of 1,719) proteins.	Mass spectrometric evidence for 60 additional chromosome 1 gene products.	[60]
Chromosomes 2 and 14	Analysis of 40 human samples in order to identify missing proteins encoded by chromosomes 2 and 14.	85,326 dat files searched against a database containing 30,952 unique peptide sequences	Detection of 83 unique peptides from 58 proteins that were not previously validated.	[61]
Chromosome 4	Exploration of proteomics data on chromosome 4 for cancer biomarker identification.	Analysis of 757 protein-coding genes and their experimental evidence at the protein level.	Identification of 141 chromosome 4-encoded proteins as cancer cell-secretable/shedable proteins and 54 chromosome 4-encoded proteins that have been classified as cancer-associated proteins.	[62]
Chromosome 7	Functional annotation of the proteins of chromosome 7 that do not have evidence at the proteomic, antibody, or structural levels.	Bioinformatics analysis to gain insights into the 170 "missing" proteins of chromosome 7.	Identification of 90 “missing” proteins; putative functional annotations for 27 proteins.	[63]
Chromosome 9	Bioinformatic and proteogenomic analysis to catalogue chromosome 9-encoded proteins from normal tissues, lung cancer cell lines and lung cancer tissues.	Identification of 75% of the human chromosome 9 genes.	Identification of 46 missing proteins, 15 being detected only in lung cancer tissues; identification of 21 SNPs and 4 mutations containing peptides from normal human cells/tissues and lung cancer cell lines, respectively.	[64]

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Chromosome 12	Mass spectrometry profiling of 30 different histologically normal human tissues and cell types in order to refine chromosome 12 genomic annotation.	Identification of 1,535 proteins encoded by 836 genes on human chromosome 12.	Identification of 89 proteins for which prior experimental evidence was missing; confirmation of the start sites of ~200 proteins by identifying protein N-terminal acetylated peptides; identification of alternative start sites for 11 proteins that were not annotated in public databases; identification of 12 novel protein coding sequences.	[66]
Chromosome 12	Functional analysis of the proteome encoded by chromosome 12 from databases and patient samples in order to detect proteins potentially involved in gliomas and major neurological conditions.	Identification of 1,066 protein coding genes.	Identification of 171 proteins defined as "missing"; first mass spectrometric evidence for two "missing" proteins; identification of 103 differentially expressed proteins with secretory potential.	[65]
Chromosome 16	Proteogenomic analysis of chromosome 16 and development of SRM methods for quantification of chromosome 16-encoded proteins.	Coverage of 41% of chromosome 16 proteins.	SRM identification of 49 known proteins and recombinant forms of 24 "missing" proteins.	[67]
Chromosome 17	Proteogenomic analysis of chromosome 18.	Identification of 1,169 protein-coding genes.	List of 59 "missing" proteins as well as 201 proteins that have inconclusive mass spectrometric identifications.	[68]
Chromosome 18	SRM and RNAseq profiling of proteins encoded by chromosome 18 in plasma, liver and HepG2 cells, followed by copy number estimation.	277 proteins targeted by SRM.	Identification of 209 proteins in the plasma, 168 in the liver, with over 50% overlap; 27 proteins remained undetected; estimation of protein copy numbers for 228 master proteins, including quantitative data on 164 proteins in plasma, 171 in the HepG2 cell line, and 186 in liver tissue.	[69,70]
Chromosome 22	Proteogenomic analysis of chromosome 22.	Proteomic profiling specifically focused on the 442 RefSeq gene entries corresponding to chromosome 22-encoded genes.	Protein evidence for 367 genes including 47 proteins that are currently annotated as "missing" proteins; confirmation of the translation start sites of 120 chromosome 22-encoded proteins; evidence of novel coding regions.	[71]

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Figure 1. Overview of the proteogenomics pipeline. A database of theoretical protein sequences can be created based on genome sequencing data (6-frame translation of all sequenced contigs), transcriptomic data (6-frame translation, alternative splicing events, etc.), and genomic data (single nucleotide variations and more complex polymorphisms). This database allows the interpretation of the mass spectrometry data acquired with standard proteomic approaches. The proteogenomic integration of these experimental data leads to an improved genome annotation, and an improved protein sequence database.

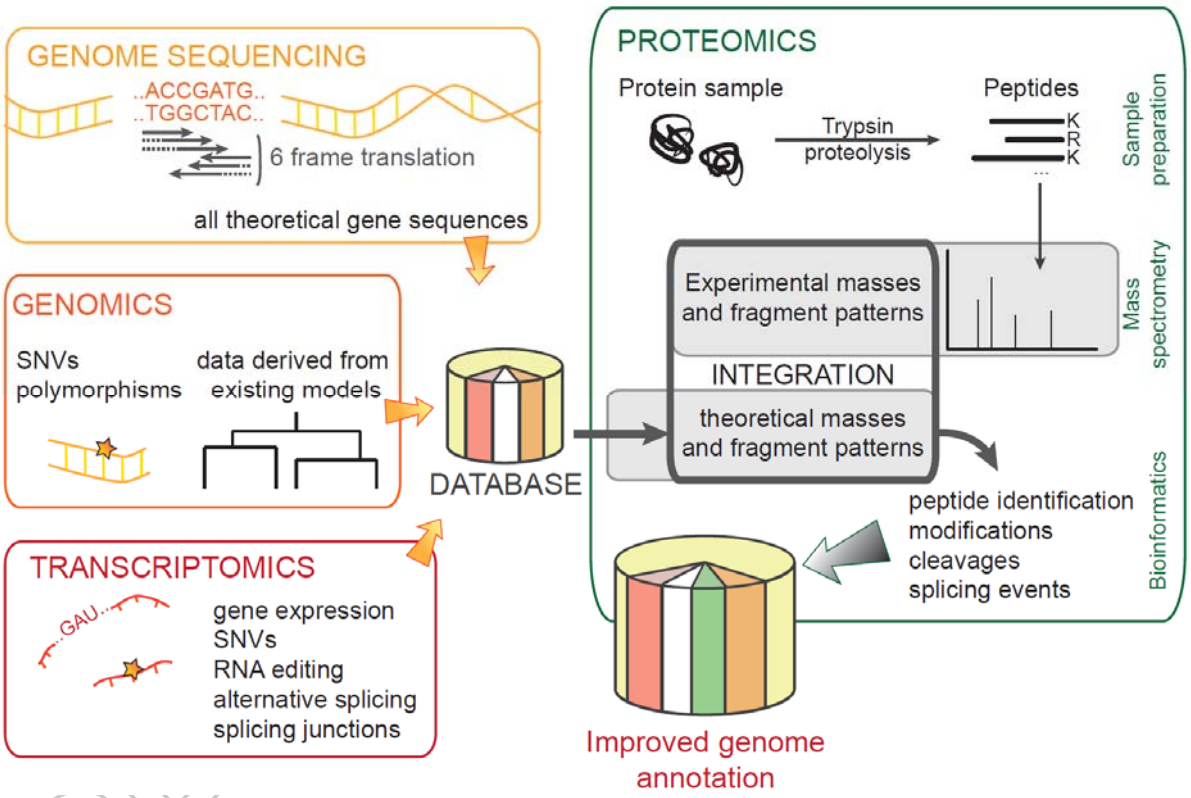

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Figure 2. Combining omics strengths. The advantages of genomics, transcriptomics and proteomics can be combined in proteogenomics, both in qualitative and quantitative terms.

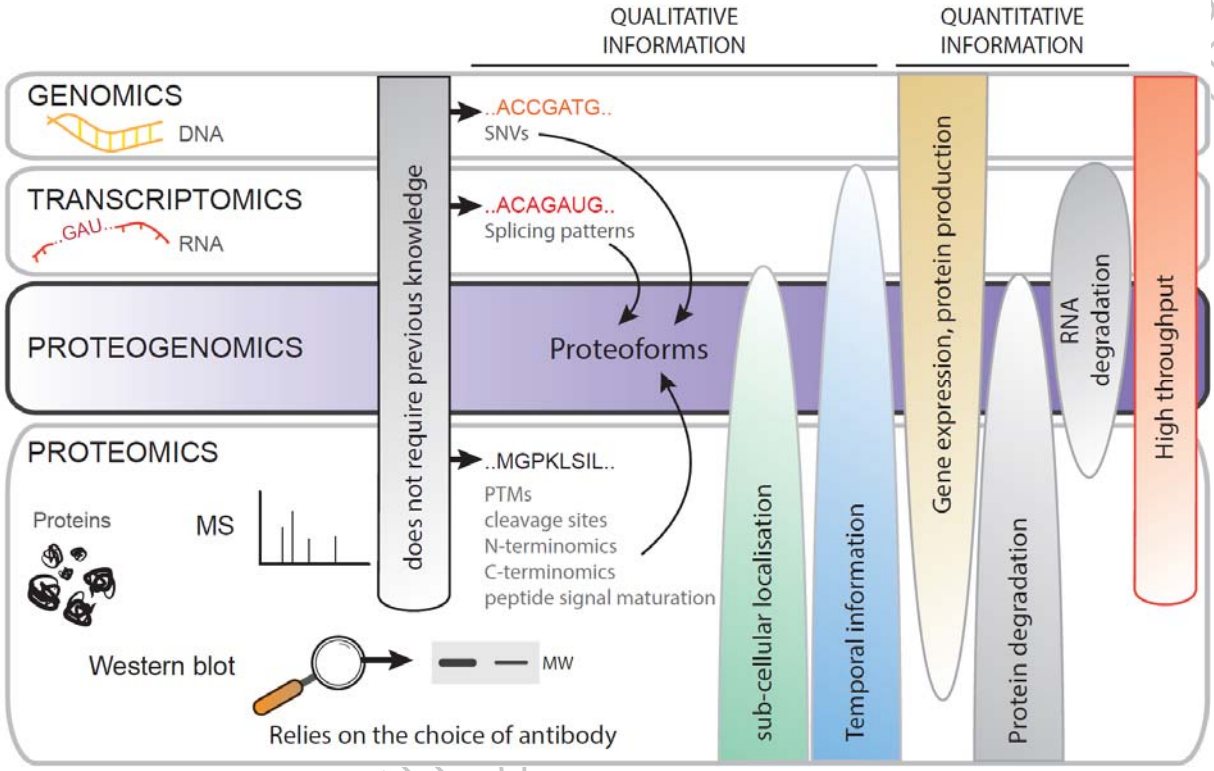

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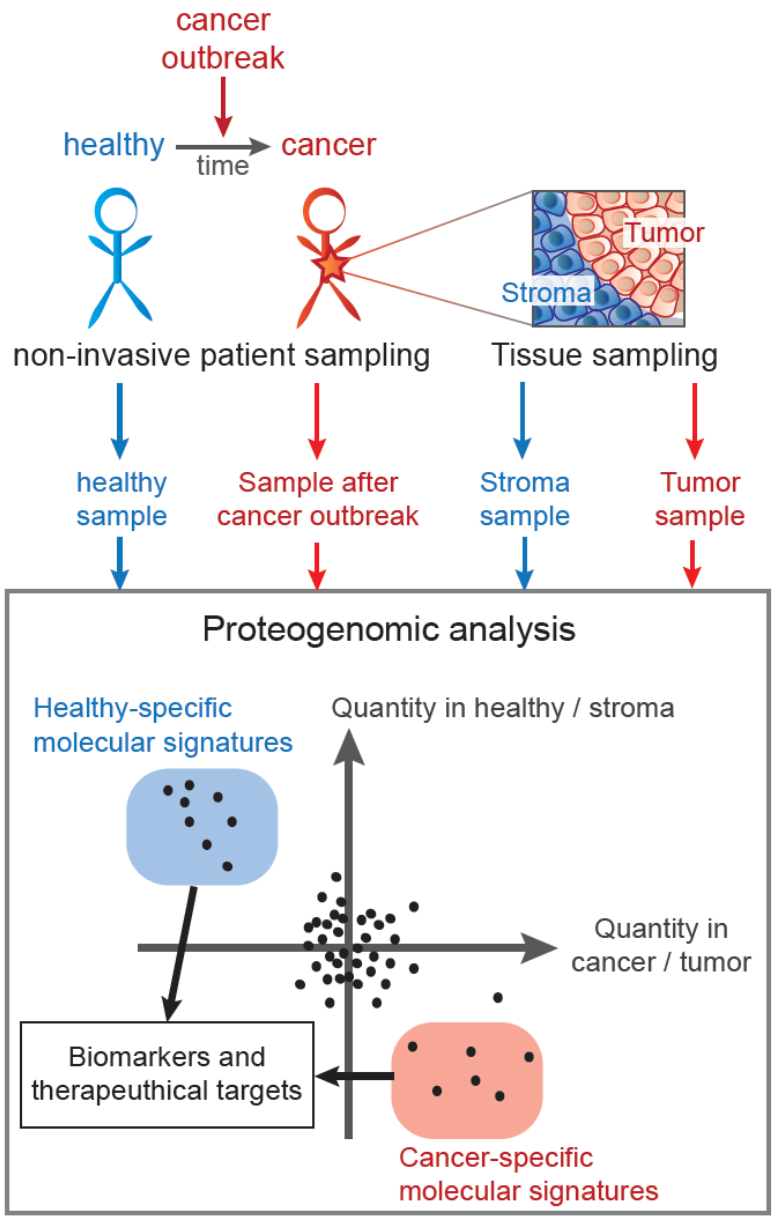
Figure 3. General strategy in personalized onco-proteogenomics. Potential markers and therapeutic targets are obtained by identifying novel proteoforms by proteogenomics and their quantitation in healthy and non-healthy samples.



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