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Review

Toward an integrated pipeline for protein biomarker development ☆

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ABSTRACT

Protein biomarker development is a multidisciplinary task involving basic, translational and clinical research. Integration of multidisciplinary efforts in a single pipeline is challenging, but crucial to facilitate rational discovery of protein biomarkers and alleviate existing disappointments in the field. In this review, we discuss in detail individual phases of biomarker development pipeline, such as biomarker candidate identification, verification and validation. We focus on mass spectrometry as a principal technique for protein identification and quantification, and discuss complementary -omics approaches for selection of biomarker candidates. Proteomic samples, protein-based clinical laboratory tests and limitations of biomarker development are reviewed in detail, and critical assessment of all phases of biomarker development pipeline is provided. This article is part of a Special Issue entitled: Medical Proteomics.

1. Introduction

Biomedical and translational science literature widely claims that molecular markers will revolutionize diagnosis and prognosis of almost every disease, including cancer, neurodegeneration and cardiovascular diseases. Such expectations arise mainly from recent exciting developments in the high-throughput -omics technologies which are set to analyze expression of every human gene. Increased availability of -omics technologies makes them very attractive to search for biomarkers and, as a result, leads to a steadily increasing number of biomarker discovery studies. The number of publications which report on putative disease biomarkers is continuously increasing, while the number of novel Food and Drug Administration (FDA)-approved protein biomarkers remains very low [1–3]. Indeed, no major cancer biomarker for screening or early diagnosis has been approved in the last 25 years [4]. In fact, the lately approved ovarian cancer biomarker, human epididymis protein 4 (HE4), is intended for either monitoring cancer recurrence [5] or prediction of malignancy along with CA-125 [6], but not for early diagnosis. With the underestimated difficulties of biomarker discovery and development, many overstated expectations are followed by later disappointments in the actual progress in the field [4]. Rational design and implementation of individual phases as parts of an integrated pipeline should facilitate systematic development of protein biomarkers and may soon bring new successful stories to the field and alleviate existing disappointments.

2. Proteins as biomarkers

Various classes of molecules may be considered as potential disease biomarkers. Advantages of proteins as a class include their enormous diversity, dynamic turnover and secretion into blood and bodily fluids. There is an estimated number of 20,300 genes [7], 40,000 unique metabolites [8], ~100,000 mRNA transcripts, and up to 1.8 million of different proteoforms, if post-translational modifications are considered [9]. Such enormous diversity of proteoforms increases chances to identify a marker, or a panel of markers, for each disease state. Since protein sequences may also reflect some genomic variations, a single instrumentation platform of mass spectrometry can measure not only changes in protein abundance, but also genomic and transcriptomic variations, such as mutant proteins

Abbreviations: AQUA, absolute quantification; ELISA, enzyme-linked immunosorbent assay; FDA, (the US) Food and Drug Administration; iTRAQ, isobaric tags for relative and absolute quantification; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; m/z, mass-to-charge-ratio; PSA, prostate-specific antigen; PSAQ, Protein Standard Absolute Quantification; PTM, post-translational modification; QconCAT, quantification concatemer; SCX, strong cation exchange; SILAC, stable isotope labeling by amino acids in cell culture; SNP, single nucleotide polymorphism; SRM, selected reaction monitoring

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or alternatively spliced proteoforms. Finally, proteins secreted into blood and body fluids can be measured with the minimally invasive tests.

Enormous protein diversity, however, poses the analytical challenge of detecting a specific protein in complex biological matrices. For example, detection of a particular nucleotide in the genome of a human cell should meet the analytical challenge of searching through 3.2×10^9 nucleotides, while detection of a certain amino acid in interleukin 6 sequence in blood plasma has the challenge of searching through 10^{13} amino acids [10]. Use of post-translational modifications as biomarkers would be an even more challenging undertaking due to the even higher complexity and dynamic turnover. For those reasons, typical protein biomarker pipelines are still focused on discovery of consensus protein sequences with differential abundance in disease rather than on discovery of differences in proteoforms or post-translational modifications.

3. Addressing unmet clinical needs with protein biomarkers

Unmet clinical needs, the intended use of biomarkers and their potential to facilitate the medical decision making in combination with concurrent diagnostic procedures should be considered well in advance. Specific applications of biomarkers typically include diagnosis, screening, prognosis, disease monitoring, or prediction of the response to therapy [11].

The discovery of *diagnostic* biomarkers would benefit those diseases for which the correct diagnosis is clinically challenging. For example, there is no single diagnostic test for Alzheimer's disease, so its current diagnosis is based on several criteria including medical history, mental status testing and physical and neurological examinations [12]. Histological examination of post-mortem brain regions still remains the gold standard for Alzheimer's disease diagnosis [13]. Although two cerebrospinal fluid biomarkers, amyloid β-protein fragments 1–42 and tau protein, have been included in the diagnostic criteria for the symptomatic pre-dementia phase of Alzheimer's disease [14], new biomarkers are needed for diagnosis of asymptomatic preclinical phase [15] and for diagnosis through a minimally-invasive blood test [16]. Reduction of numbers of invasive biopsies and diagnostic costs are additional values of diagnostic biomarkers. Some diagnostic procedures, such as computed tomography (CT) scan, may not be readily available in small medical centers or remote areas. Blood-based biomarker tests will thus facilitate quick decision-making and ultimately reduce diagnostic costs.

Early detection of rapidly progressing fatal diseases, such as cancer, is needed to provide a sufficient time window for treatment. Identification of *screening* biomarkers for early detection of rare diseases with low prevalence in the population is very challenging and may not even be feasible for some cancers [17]. For example, potential screening biomarker for ovarian cancer should have specificity of at least 99.5% at 80% sensitivity, to provide a positive predictive value of 10% [18]. Biomarkers with *prognostic* value are needed to predict the disease outcome and prescribe relevant therapies. Likewise, *monitoring* of disease progression and *prediction* of therapy efficiency are other specific applications of biomarkers.

4. Biomarker development pipeline

Similar to drug discovery and development, biomarker development should be designed as a multiple step process. The ultimate purpose of the biomarker development pipeline (Fig. 1) is to assess as many candidates as possible and exclude ineffective markers as early as possible. Upon presentation of the unmet clinical needs, biomarker development includes identification of proteins in the relevant biological sample, qualification of biomarker candidates, verification of candidates in the independent set of samples, development of a pre-clinical assay, clinical validation, and, finally, assay approval by health agencies, such as the FDA. The cost and duration of the whole biomarker development pipeline may be as high and as long as the cost and duration of a drug discovery project. For instance, it took nearly 8 years since the discovery of human epididymis protein 4 (HE4), an ovarian cancer biomarker, to conduct all essential validation studies and receive an FDA approval [5,19]. Similar to HE4 story, heart failure biomarker interleukin 1 receptor-like 1 protein (also known as ST2) was discovered in 2003, while its clinical assay was cleared by the FDA in 2011 [20]. And it took almost two decades for tryptase, a serum-based biomarker of mastocytosis, to reach the clinic [21].

The first phase of a biomarker development project often involves *identification* of proteins in clinical samples. Even though mass spectrometry is the most powerful technique for protein identification, it still suffers from relatively poor capabilities for protein quantification. Various label-free and label-based approaches were introduced to equip global protein identification with quantification capabilities and thus facilitate selection of biomarker candidates. Following protein identification, biomarker *qualification* provides an evidence of association between protein abundance and the clinical outcome. Certain filtering criteria are usually applied to select a manageable number of candidates and proceed to the verification phase [22].

The aim of biomarker *verification* is to measure the most promising candidates in a large set of samples and exclude false candidates. ELISA and mass spectrometry-based selected reaction monitoring (SRM) [23] are commonly used assays for biomarker verification. Advantages of ELISA include low cost, high sensitivity and high-throughput measurement of proteins in biological fluids of high complexity, such as blood serum. SRM assays facilitate multiplex verification of medium- and high-abundance proteins for which immunoassays are not available and provide attractive multiplexing capabilities. Combination of immunoaffinity enrichment of proteins or peptides with mass spectrometry measurements resulted in SISCAPA [24], MSIA [25], iMALDI [26], and immuno-SILAC approaches [27] which advanced verification of novel protein biomarkers.

Biomarker verification is followed by development of a pre-clinical assay and biomarker *validation*. Proper validation includes measurement of each biomarker in hundreds of samples from multiple centers, blinded analysis, establishment of reference values and selection of clinically meaningful or surrogate endpoints [28,29].

Finally, a clinical-grade assay is developed and subjected for the approval by the FDA. The list of FDA-approved protein biomarker assays currently includes more than 200 proteins [30]. The majority of FDA-approved protein assays utilizes ELISA, and not a single mass spectrometry-based protein assay has been approved for clinical use yet [31]. In addition, there is no yet a single FDA-approved protein biomarker that has been discovered by mass spectrometry and proteomics. Recently approved cancer biomarkers, HE4 protein and PCA3 mRNA, were discovered by microarray-based differential transcriptomic approaches. Due to the long duration of biomarker development projects, we may soon witness the approval of protein biomarkers which were discovered by mass spectrometry and proteomics in the 2000s.

5. Biological samples for protein biomarker discovery

A variety of biological samples such as blood, proximal fluids, tissue samples, cell lines and laboratory animals (Fig. 2) are suitable for protein identification. Blood serum or blood plasma is routinely analyzed in the clinical laboratory due to their minimally invasive collection and systemic circulation. Being the fluid of choice from the clinician's and patient's perspectives, blood plasma, however, is the most challenging sample to analyze by proteomic techniques. Blood plasma proteins have a dynamic range of concentrations of more than ten orders of magnitude, with albumin and cytokines being the most and the least abundant proteins, respectively [3]. Such a wide dynamic range allows for identification of high- and medium-abundance proteins while low-

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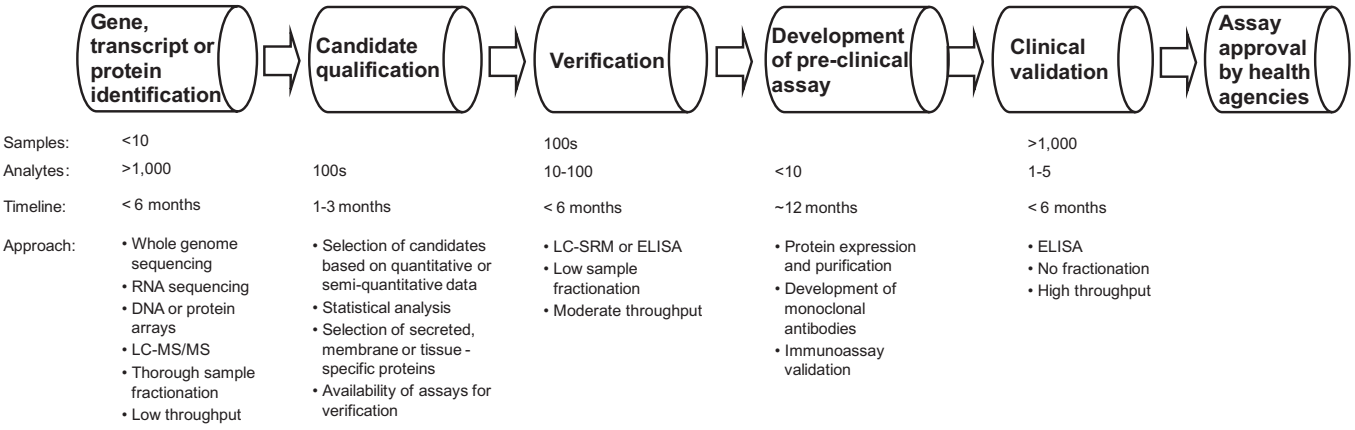


Fig. 1. Integrated protein biomarker development pipeline. Proteomics in combination with other -omics approaches provides dozens of biomarker candidates which are assessed at the verification phase in the independent cohort of samples. Upon verification and development of pre-clinical assays, the most promising biomarker candidates are validated in thousands of clinical samples. Finally, the diagnostic assay is subjected to a rigorous evaluation and approval by health regulatory agencies. As biomarker candidates proceed through the pipeline, the number of clinical samples increases and low-throughput mass spectrometry-based methods are replaced with high-throughput immunoassays.

abundance proteins, the most promising candidate biomarkers, are often overlooked. Urine, a liquid by-product of the body, is a great fluid for non-invasive diagnosis and a good source of biomarkers of renal and urological diseases. However, low amounts of urine proteins excreted under normal physiologic conditions (less than 150 mg per day) and the wide dynamic range of protein concentrations make analysis of low-abundance proteins in urine a challenging undertaking [32].

Due to the lower complexity of their proteome and elevated levels of disease-relevant proteins, proximal fluids in contact with the affected tissue can be a better alternative for biomarker identification. Low-abundance blood and urine proteins are present at much higher concentrations in proximal fluids and may indicate disease at early stages, thus providing early diagnosis [33,34]. Available examples include cerebrospinal fluid for the study of neurodegenerative diseases, amniotic fluid for fetomaternal screening and seminal plasma and expressed prostatic secretions for male infertility, prostate inflammation and prostate cancer. Limitations of biological fluids include invasive collection procedures, small volumes, frequent contamination by high-abundance blood proteins and low availability of samples from healthy individuals [35].

Tissue samples collected by biopsy or surgery are another source enriched with potential biomarkers; however, low availability of normal tissues, cell heterogeneity and protein cross-linking in the archived formalin-fixed, paraffin-embedded (FFPE) tissues are clear limitations. Laser capture micro-dissection [36] and novel protocols for FFPE tissues [37] are set to alleviate these limitations of tissue proteomics.

A cell culture-based approach for biomarker discovery relies on identification of disease-associated proteins in the cell lysate or cell secretome. The main advantages of cell lines include their availability, cost-effective analysis and detection of low-abundance secreted and membrane-bound proteins [35]. Due to their regulatory and signal transduction roles, secreted and membrane-bound proteins often enter systemic circulation and are thus promising molecules for the blood-based diagnostics. An array of cell lines can recapitulate different stages of disease progression. A single cell line, however, will not represent the whole spectrum of disease heterogeneity. In addition, since immortalized cell lines bear certain mutations or viral genes and are cultured outside of their normal tissue microenvironment, cell lines may be very simplified or even irrelevant disease models [38].

Biomarker discovery using laboratory animal models, such as mouse xenografts, accounts for *in vivo* interaction between the host mouse tissues and human cancer cells. Genetically identical animal models also minimize genetic and environmental variabilities. Collection of samples from animal models is simple and can be performed at any stage of disease development. In some cases, such as mice xenograft models of human cancers, the size of the tumor in proportion to the body weight is large, thus resulting in the increased levels of potential biomarkers in circulation and the higher likelihood of their identification. Animal

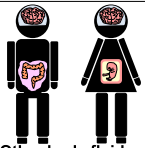
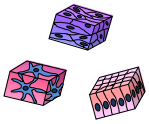
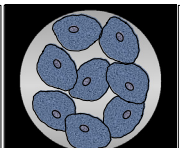
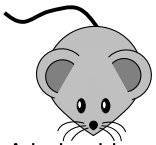
Characteristics/ Biological sample	 Blood	 Urine	 Other body fluids	 Tissues	 Cell lines	 Animal models
Study design	Any diseases	Some diseases	Some diseases	Some diseases	Single disease	Single disease
Availability	+++	+++	++	+	+++	+++
Non-invasive sampling	+	++	–*	–	++	–
Detection of low-abundance proteins	–	–	+	++	+++	+
Potential as a clinical sample	+	+	–*	–†	–	–

Fig. 2. Clinical samples suitable for biomarker discovery. Samples vary in their availability and difficulty of collection, potential for identification of low-abundance protein biomarkers and suitability for development of clinical assays. *Some body fluids, such as saliva, sweat, tears and seminal plasma are collected non-invasively and also can be used as diagnostic samples. †Except when protein levels in tissues are measured by immunohistochemistry.

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model data, however, may not be easily translatable into models of human diseases [39].

6. Identification of protein biomarker candidates by mass spectrometry

Mass spectrometry has been extensively used to identify thousands of proteins in various biological samples. Even though there were recent significant achievements in top-down proteomics [40], the vast majority of proteomic data has been generated by bottom-up proteomics and shotgun mass spectrometry. Bottom-up proteomic approaches provide unsurpassed capabilities in terms of the number of protein identifications. Identification of near-complete proteomes of 59 human cell lines of NCI-60 cell line collection (>10,000 proteins) has been recently demonstrated [41]. Quantification of near-complete proteomes of human cell lines (>8000 proteins) in just a few hours using high-resolution quadrupole-Orbitrap mass spectrometer has been claimed [42].

In a typical bottom-up proteomics experiment, proteins are denatured, and cysteine residues are chemically modified to prevent reformation of disulfide bonds. Following that, proteins are cleaved by proteolytic enzymes, such as trypsin, into relatively short peptides, and peptides are often subjected to multi-dimensional chromatography separations. Fractionated peptides are then desolvated by electrospray ionization (ESI) and transferred to the mass spectrometer for gas-phase separation, isolation and collision-induced fragmentation. Upon detection of fragment ions, the resulting MS/MS spectra are assigned to peptide sequences using bioinformatics search algorithms such as database matching, *de novo* sequencing or hybrid approaches [43]. For the large-scale proteomics studies, database matching remains the method of choice for peptide and protein identification. Each acquired fragment ion spectrum is matched to the theoretical spectrum predicted for each peptide in a protein sequence database. The best scoring peptide match is selected for a subsequent statistical analysis, which estimates the false-positive rate of peptide and protein identifications. Conventional software tools for peptide and protein identification include MASCOT [44], X!Tandem [45], SEQUEST [46], ProteinProspector [47] and Andromeda [48].

Proteome composition of many biological fluids has been extensively studied for the purpose of identification of protein biomarkers [49–52]. For example, extensive analysis of seminal plasma proteome was performed in order to develop diagnostics of urogenital diseases such as male infertility, prostatic inflammation, and prostate cancer [53–55]. As a follow-up of those studies, several biomarkers for the non-invasive differential diagnosis of male infertility were recently verified [56,57]. Likewise, extensive analysis of the amniotic fluid proteome was completed in order to identify biomarkers for diagnosis of Down syndrome and fetal diseases [58]. More than a thousand proteins were identified in the amniotic fluid of pregnant women carrying chromosomally normal fetuses and fetuses with Down syndrome at different gestational ages, and the most promising candidates were verified [52,59,60].

Since each type of biological sample has its own advantages and disadvantages, combination of complementary samples increases the likelihood to discover true positive markers. As an example, recent extensive search for pancreatic cancer biomarkers employed proteomic analyses of secretomes of seven pancreatic cell lines, pancreatic juice samples, ascites fluid and tissues from patients with pancreatic ductal adenocarcinoma [22,61,62].

7. Protein quantification by mass spectrometry

Methods of quantitative proteomics are rapidly evolving to provide better sensitivity and reproducibility with better capabilities for multiplexing and throughput. Common methods rely on label-free quantification or quantification of proteins or peptides labeled by stable isotopes of carbon, nitrogen or oxygen.

With label-free quantification approaches, the number of MS/MS spectral counts or integrated signal intensities (MS1 or MS/MS) is measured and correlated to the protein amount. Advanced spectral counting techniques, such as Protein Abundance Index (PAI/emPAI) [63,64], Absolute Protein Expression (APEX) [65], Normalized Spectral Abundance Factor (NSAF) [66] and Normalized Spectral Index (SI_N) [67] account for additional parameters, such as number of theoretical tryptic peptides per protein. The use of label-free quantification methods based on MS1 peptide ion intensities, such as MaxQuant LFQ [68] and iBAQ [69] or MS/MS fragment ion intensities, such as data independent acquisition (DIA)-based methods [70–72], is steadily increasing due to recent advances in high-resolution mass spectrometry [73–75]. Label-free quantification methods offer straightforward, fast and affordable options for protein quantification [73,76].

Label-based quantitative techniques, such as SILAC and iTRAQ (isobaric tags for relative and absolute quantification), are widely used together with protein identification approaches to prioritize biomarker candidates. SILAC relies on the metabolic labeling of the cell proteome using a mixture of ^{13}C- and ^{15}N-labeled arginine and lysine [77]. Since protein digestion by trypsin results in peptides with C-terminal arginine and lysine residues, all tryptic peptides except the C-terminal peptide will include ^{13}C- and ^{15}N-isotope labels. “Heavy” cells are subjected to drug treatment or environmental perturbations (for example, induction of hormone insensitivity) while “light cells” grown with non-labeled amino acids are used as a control. Following that, an equimolar mixture of lysates of “heavy” and “light” cells is analyzed by LC–MS and the heavy-to-light ratio of peptide intensities is used to calculate the relative protein abundances (Fig. 3a). Even though SILAC is not applicable to primary cells, tissues or biological fluids, it is easily implemented and provides accurate relative quantification. SILAC labeling is also used to prepare mixtures of heavy isotope-labeled cellular proteomes for their use as spiked-in internal standards for accurate relative quantification of proteins in biological fluids and tissues. Such approaches are implemented in the SILAP [78], super-SILAC [79] and SILAC mouse approaches [80].

iTRAQ labeling allows for relative protein quantification by using MS/MS peptide fragments and low mass reporter ions [81]. Amine groups at peptide N-terminus and lysine side chain are labeled with isobaric tags which include a reporter group, a mass balance group, and a peptide-reactive group. The reporter groups range from 113 to 121 m/z, the balance groups range from 32 to 24 m/z, while the combined m/z ratio is kept constant. Following fragmentation of the tag in the mass spectrometer, the balance groups are lost as neutral fragments and reporter groups acquire different m/z ratios. All other sequence-informative fragment ions remain isobaric, and their signal intensities are additive. The relative abundance of the peptides is thus deduced from the relative intensities of the corresponding reporter ions (Fig. 3b). While often used in biomarker discovery studies, iTRAQ has some limitations, such as underestimation of the protein fold changes and interference by cross-label isotopic impurities [82]. Alternative quantitative approaches based on chemical labeling of intact proteins include isotope coded affinity tagging (iCAT) [83] and tandem mass tags (TMT) [84].

To enable intra-assay and intra-laboratory comparisons, biomarker verification approaches often require measurements of absolute protein concentrations. In general, stable isotope dilution-based quantification provides excellent reproducibility, linear response and precision regardless of the quantification standards used [85]. Protein Standard Absolute Quantification (PSAQ) standards, concatemers (QconCAT) and proteotypic heavy isotope-labeled peptides with a trypsin-cleaved quantification tag facilitate absolute quantification of proteins. Full length isotope-labeled proteins are the ideal internal standards for absolute quantification [86]. Such standards retain most physical and chemical properties of the intact protein during pre-analytical sample

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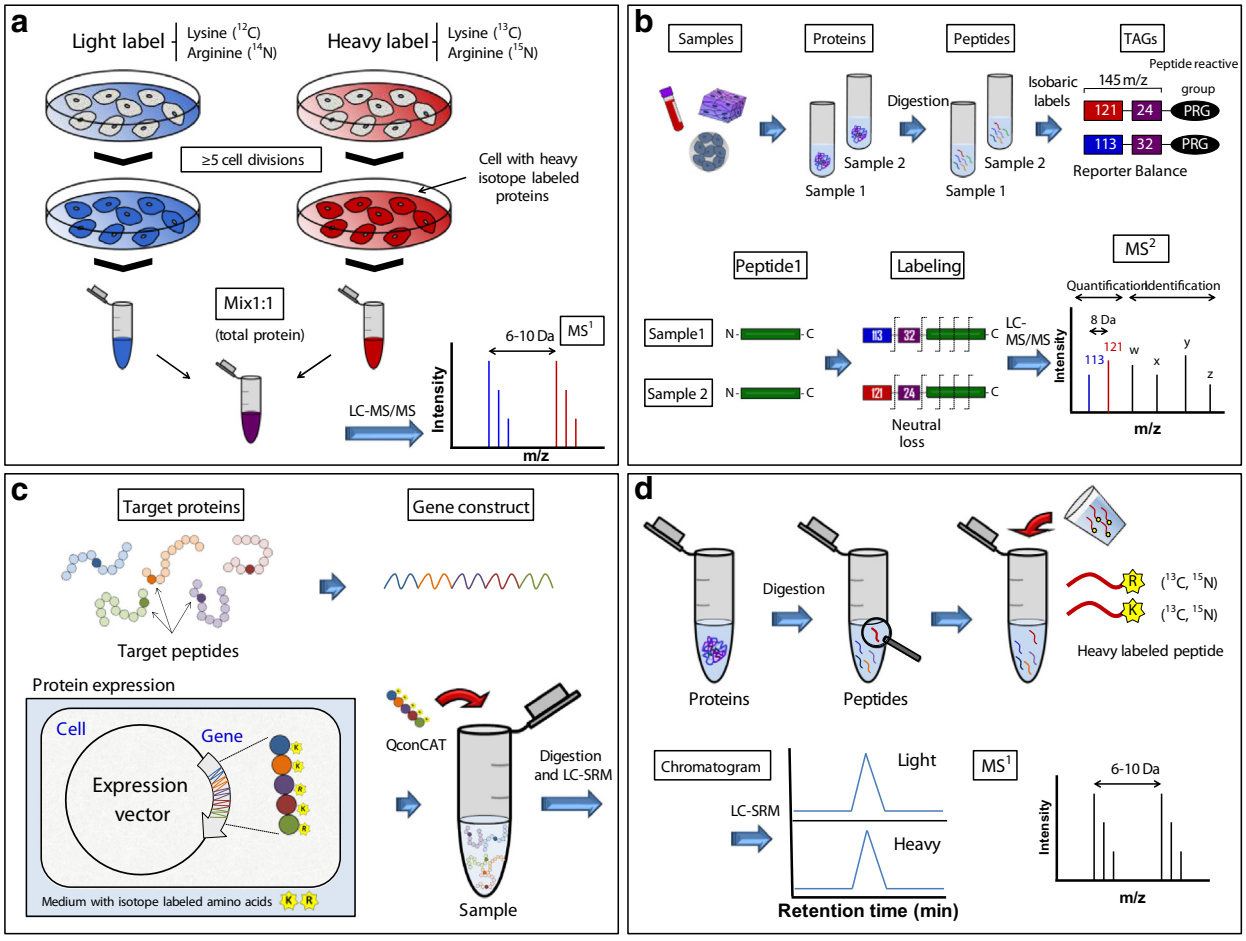

Fig. 3. Approaches for quantitative proteomics. Commonly used approaches include (a) Stable Isotope Labeling by Amino acids in Cell culture (SILAC). Cells are cultured in "light" or "heavy" media supplemented with lysine and arginine amino acids labeled with stable isotopes of ^{12}C/^{14}N or ^{13}C/^{15}N, respectively. Upon six divisions, cells cultured in the "heavy" media contain mostly heavy isotope-labeled proteins (>98%). Following that, "heavy" and "light" cells are lysed, and cell lysates are mixed in equimolar amounts prior to mass spectrometry analysis. SILAC labeling results in a mass shift of 6–10 Da for each tryptic peptide of the "heavy" protein, and the heavy-to-light ratio of intensities is used for the accurate relative quantification. (b) Isobaric Tags for Relative and Absolute Quantification (iTRAQ). Amine groups are labeled with isobaric tags which include a reporter group (121 and 113 m/z), a mass balance group (24 and 32 m/z), and a peptide-reactive group, while the combined m/z ratio is kept constant (145 m/z). Following fragmentation of the tag, the balance groups are lost as neutral fragments, and reporter groups acquire different m/z ratios. All other sequence-informative fragment ions remain isobaric, and their signal intensities are additive. (c) Quantification concatemers (QconCAT). A gene encoding multiple proteotypic peptides is constructed, inserted into a plasmid vector and expressed in the medium containing heavy isotope-labeled arginine and lysine. The concatemer is expressed, purified and used as a spike-in standard. (d) Stable-isotope dilution or AQUA approach. Known amounts of synthetic heavy isotope-labeled peptides are spiked into the sample and used for absolute quantification (provided complete protein digestion or the use of trypsin-cleavable tags) or accurate relative quantification (provided incomplete digestion).

preparation, protein and peptide separation, and peptide ionization prior to quantification by mass spectrometry. PSAQ standards, however, do not have post-translational modifications and their production is laborious [85]. QconCAT strategy utilizes concatemers: artificial protein constructs which include multiple trypsin-cleavable proteotypic peptides [87]. A gene of such construct is expressed in cells grown in a medium with heavy isotope-labeled arginine and lysine. Concatemers, similar to intact protein standards, are spiked into the sample prior to trypsin digestion (Fig. 3c). This is a cost-effective approach in which as many as 50 tryptic peptides can be expressed within a single QconCAT construct [88]. Even with the use of QconCAT standards, the completeness of tryptic digestion has to be assessed for each proteotypic peptide [85]. Finally, stable-isotope dilution strategy, or AQUA [89], is used to quantify proteins through spiking synthetic heavy isotope-labeled peptides which have identical physical and chemical properties as endogenous proteotypic peptides (Fig. 3d). Although being fast and straightforward, the AQUA approach does not account for the variability of trypsin digestion [85]. Quantification of proteins using PSAQ, QconCAT or AQUA standards is typically implemented by SRM assays [56,90]. With state-of-the-art SRM assays, as many several hundred peptides can be measured simultaneously in the unfractionated digest of biological fluid, while achieving coefficients of variation under 15% [91]. Addition of stable-isotope labeled standards enables accurate relative or absolute quantification and determination of the correct analyte in the presence of co-eluting peptides. Combination of SRM assays with SISCAPA (Stable Isotope Standards and Capture by Anti-Peptide Antibodies) facilitates verification of low-abundance biomarker candidates in clinical samples, including urine and blood plasma [24,92]. Accurate relative or absolute quantification is pivotal for prioritization of the most promising candidates and exclusion of false discoveries early in the biomarker development pipeline.

8. Biomarker verification

Protein identification and qualification phases of the biomarker development pipeline supply long lists of putative biomarkers, many of which are found differentially abundant due to the systematic biases. Common systematic biases include analytical bias resulting from variation in the sample preparation protocols, protein degradation and precipitation as well as biological bias, such as high inter- and intra-individual biological variability of protein concentrations. Use of low quality clinical samples, data over-fitting and inappropriate statistical

analysis result in the additional bias. To avoid over-fitting, any multiparametric pattern discovered in the training set of samples requires verification in the independent set of samples [93]. Since multiple proteins are measured at the identification and verification phases, multiple hypotheses testing should be performed, and the false discovery rate-adjusted *P*-values should be reported [94,95].

Regarding the number of samples, statistical estimations show that there should be at least 10 events, or independent samples, per each variable, or protein, measured [96]. According to our estimations, biomarker development project which includes identification of several thousand proteins in only one normal and one disease samples followed by verification of 10–20 of those candidates has a very low chance of success due to very high false-positive rates. Indeed, our simulations show that if we assume a 10-fold intra-individual variation of a protein concentration in 95% of samples, a log-normal distribution, and a 2-fold change cut-off, measurement of differential expression of proteins in one normal and one disease samples will result in a false-positive rate of 21%. Assuming the presence of 10 true biomarkers (1% true positive rate) and measuring 1000 proteins, 220 candidate proteins will be selected (210 false positives and 10 true positives). Therefore, verification of just 20 of these candidates (selected randomly from 220 candidates based simply on availability of ELISA or immunohistochemistry assays) will include mostly false positives (around 19 false-positives and 1 true positive). However, if we measure at the identification phase 1000 proteins in 10 normal and 10 disease samples, false-positive rate will drop to 1.2%. As a result, there will be 22 candidate biomarkers (12 false positives and 10 true positives) and the verification of 20 of these candidates will provide a substantial number of true positives. For the same reason, numbers of independent samples for verification and validation phases should be always supported by relevant sample size and power calculations. Note that even though pooling strategies reduce the impact of intra-individual variation, analysis of pools of samples still has clear disadvantages, such as measurements of mean *versus* median values and reduced statistical power.

Even though biomarker identification is often completed in tissues, cell lines or proximal fluids, verification phase would require biomarker measurement in the biological sample suitable for the use in the clinic [97]. Clinical and demographic parameters should be collected and matched for control and disease groups to exclude the impact of any confounding factors.

9. Biomarker validation

Biomarker validation is set to assess only the most promising candidates which were previously verified and for which accurate and precise analytical assays were developed. The validation phase must acknowledge the final clinical use of biomarker and include prospective and retrospective validation or validation for general population screening. While validation protocols are well developed and strictly regulated in the pharmaceutical industry, protein biomarker development pipelines still suffer from the lack of standardized validation protocols [28,98].

Clinical parameters of samples in the validation cohort should be well-defined to exclude biases when disease parameters are associated not with the primary cause of disease but with confounding factors, such as age, sex, race, medications, lifestyle habits, smoking, concomitant inflammation and stress. Bias in the sample collection presents one of the major threats which may compromise biomarker validation. It is not uncommon for healthy control samples to be collected in the low-stress environment of the general clinic, while disease samples are obtained in specialized clinics prior to surgery, which adds additional stress to patients and may result in discovery of biomarkers of stress. Clinical validation cohorts should also include samples with different subtypes and disease stages.

Even though a variety of analytical techniques is available for protein quantification, immunoassays still dominate in the field of clinical proteomics. Due to their relatively low sensitivity and throughput, mass spectrometry-based assays cannot as yet compete with ELISA. To be competitive, mass spectrometry-based assays should be able to analyze thousands of clinical samples with an inter-day CV < 20% in a week. Similar to drug clinical trials, biomarker validation should utilize randomized, blinded, and even placebo-controlled protocols, in case of validation of compound biomarkers. Systematic bias during validation phase can be reduced by using samples collected at multiple medical centers [97]. Likewise, centralized biobanks have been established to facilitate storage and distribution of high-quality clinical samples [99].

The majority of novel putative biomarkers discovered in academia rarely proceeds to the validation phase due to long duration of biomarker development, availability of clinical samples, high cost of biobanking, publication pressure and insufficient funding of translational work by research grants. Due to the immense resources and extensive collaborations required for biomarker validation, large collaborative networks and trials, such as the Early Detection Research Network [100], the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial [101], the European Randomized Study of Screening for Prostate Cancer [102] and others were established.

10. Development of clinical laboratory tests

Immunoassays remain the method of choice for protein quantification in clinical samples. Despite being a gold standard of clinical assays, immunoassays have certain limitations, including cross-reactivity, difficulty of identification of individual protein isoforms, batch-to-batch variability, lack of certified reference materials and challenges of multiplexing. Development of monoclonal antibodies and ELISA for a novel protein is laborious and may take many months.

Mass spectrometry assays are emerging as a vital alternative to ELISA [103–105]. SRM assays have certain advantages, such as high specificity, low sample volume, quick assay development and excellent multiplexing capabilities [106]. SRM assays have inter-laboratory variability of 20% or better for the analysis of medium- and high-abundance proteins [105,107] and 30% for low-abundance proteins, when combined with additional protein or peptide enrichment approaches [104]. Low sensitivity, fair sample throughput and lack of standardization across clinical laboratories remain major limitations of mass spectrometry-based assays.

High specificity of mass spectrometry allows for measurement of proteoforms with disease-specific SNPs and post-translational modifications [108]. Likewise, development of clinical SRM assays should consider possible SNPs and chemical and post-translational modifications which can affect measurements. For example, the rs2003783 variant of KLK3 gene results in L132I substitution in the tryptic peptide LSEPAEL^{132}TDAVK, the peptide commonly used for SRM quantification of KLK3 (PSA) protein. The frequency of the minor allele with this variant in the general population is around 10% [109]. This could result in the inaccurate measurement of protein concentration by an SRM assay which targeted a single peptide of KLK3. It is a lucky coincidence, however, that L132I variant of this peptide has an isobaric mass, so the results of KLK3 assays are not affected.

In the United States and the European Union, the development and marketing of commercial tests are regulated by the FDA and the European Medicines Agency, respectively. The application process required for a new commercial test to obtain FDA premarket approval (PMA) may take many years. Class III device PMA application, such as application for a novel diagnostic test or drug, involves preparation of many volumes of documents describing clinical trials. Many diagnostic tests never make it to the clinical trial stage and very few new proteins have been FDA-approved in the last twenty years. Between 1993 and 2008, only 22 novel protein-based tests were approved [30].

To develop a robust clinical laboratory test, a set of analytical features such as precision, linearity and limits of detection (LOD) and quantification (LOQ) should be assessed. The Clinical and Laboratory

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Standards Institute (CLSI) provides definitive guidelines evaluated and recognized by the FDA. Documents EP05-A2, EP06-A and EP17-A2 regulate evaluation of assay precision, linearity and determination of LOD and LOQ [110–112]. A novel guideline C62 "Liquid Chromatography–Mass Spectrometry Methods" emphasizes particular areas related to mass spectrometry-based assay development and presents a standardized approach for assay validation.

11. Integration of -omics approaches to facilitate protein biomarker discovery

Recent advances in genomics, epigenetics, transcriptomics and proteomics resulted in global profiling of genes, mRNA and proteins in health and disease. Proteomics incorporates qualitative and quantitative disease-specific changes translated from the upstream -omics levels through direct and indirect mechanisms, such as somatic mutations and gene fusions, copy number gains and losses, epigenetic regulation, non-coding RNA regulation, alternative splicing, altered signal transduction and post-translational modifications (Fig. 4). While qualitative genomic alterations resulting in rare SNP proteoforms or mutated proteins are not amenable to detection by ELISA, such proteoforms are readily measured by mass spectrometry [113]. Integration of disease-specific genomic, epigenetic, transcriptomic and proteomic alterations provides a comprehensive approach to discovery of protein biomarkers as well as elucidation of molecular mechanisms leading to such qualitative or quantitative changes.

Following the completion of the Human Genome Project which provided the reference genome [114], fine-scale structural and functional elements of the human genome were investigated by large international projects. For instance, the International HapMap Project was initiated to identify genes and SNPs that affected both health and disease conditions [115]. The Encyclopedia of DNA Elements (ENCODE) project was aimed at discovering functional elements in the human genome sequence, such as regions of transcription, chromatin structure, transcription factor association, and histone modifications [116]. The 1000 Genomes Project was launched with a goal to discover genetic variations, such as SNPs, insertions, deletions, and copy number variations in 1000 individuals from 14 different populations [117]. Causal mutations for more than 4000 Mendelian disorders were cataloged in the Human Gene Mutation Database [118]. The International Cancer Genome Consortium will provide a comprehensive description of genomic, transcriptomic and epigenomic changes in 50 different tumor types and subtypes [119]. Similar large-scale projects included the Cancer Genome Project initiated in the United Kingdom [120] and the Cancer Genome Atlas project initiated in the United States [121–123]. Epigenomic changes in disease are currently investigated by the NIH Roadmap Epigenomics Mapping Consortium [124]. Differential transcriptomic changes and mRNA alternative splicing under different biological conditions are compiled in the microarray- and RNA sequencing-based NCBI Gene Expression Omnibus (GEO) [125] and EBI Array Express [126] databases. Finally, the Human Proteome Project launched in 2011 was presented as a global effort to catalog abundance, subcellular localization and function of all human proteins [7].

Since variation of protein levels may not always be predicted through profiling of the upstream -omics levels [69], integration of -omics databases would complement selection of biomarker candidates. Databases with data on the tissue-specific expression of genes and proteins are another complimentary source to search for biomarkers with potentially high diagnostic sensitivity. Abnormal changes in concentration of tissue-specific proteins are a clear indicator of a pathological process in the relevant tissue [56]. For example, the success of PSA protein, a prostate cancer biomarker, is mostly due to its high tissue specificity. Tissue-specific transcripts and proteins are cataloged in a number of databases including the Human Protein Atlas (www.proteinatlas.org) and BioGPS (www.biogps.org). BioGPS database is based on mRNA expression profiles of all human genes in 84 tissues and cells, while the Human Protein Atlas is the most comprehensive proteomic database; its current version 12.0 includes immunohistochemistry-based protein expression profiles of 16,621 genes based on 21,984 antibodies in 82 human tissues and cells.

The immense -omics data, however, has not yet been fully utilized for clinical diagnostics. Little is known yet as to which genomic, epigenetic and transcriptomic alterations in disease are translated to the protein level. Also, little is known as to which of these alterations can be measured in biological fluids and tissues and thus be useful for diagnostics. However, there is a little doubt that integration of multiple -omics technologies will facilitate protein biomarker discovery through stratification of disease subtypes, rational design of discovery and validation, and elucidation of molecular mechanisms contributing to altered abundance of protein biomarkers in tissues and biological fluids.

12. Common limitations of biomarker development projects

Shortcomings in biomarker project design as well as pre-analytical, analytical, and post-analytical issues [4] were stated as main reasons for biomarker failure during validation phase [127]. Pre-analytical issues emerging from sample collection bias are often claimed as primary reasons of the failure of putative biomarkers upon stringent validation. Standardized sample collection protocols are intended to reduce such bias. The history of biomarker discovery narrates that even slight variations in sample collection may lead to

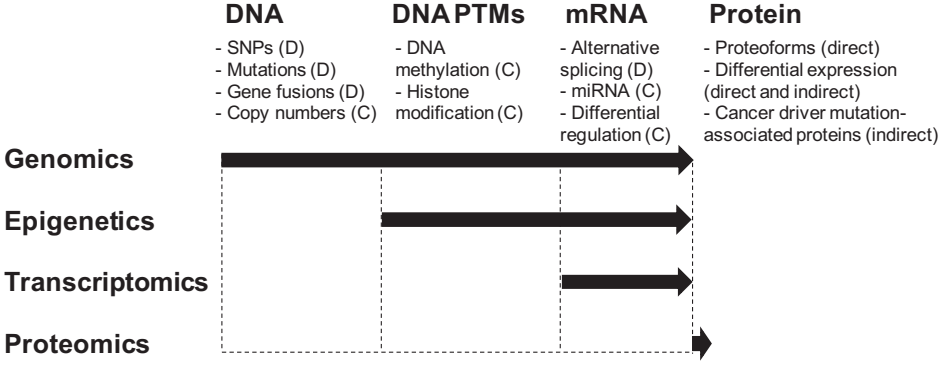

Fig. 4. Integration of genomic, epigenetic, transcriptomic, and proteomic approaches to select protein biomarker candidates. Disease-specific alterations at the proteomic level can be *qualitative* (discrete, D) and *quantitative* (continuous, C) as well as *direct* (affect the corresponding gene/protein) or *indirect* (affect other proteins in the disease-associated pathways). Global proteomic analyses typically identify *quantitative* alterations due to *direct* and *indirect* mechanisms, but ignore novel *qualitative* alterations. Integration of multiple -omics approaches provides complementary biomarker candidates and also facilitates elucidation of molecular mechanisms resulting in disease-specific proteomic alterations.

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false discoveries [128]. Standardization of instruments and protocols in proteomics is set to exclude analytical issues, such as false positive identifications, cross-reactivity, incomplete trypsin digestion, and fractional protein precipitation or sample loss. Post-analytical issues deal with proper statistical analysis of data, multiple hypothesis testing, and data over-fitting. Artificial neural networks, decision-tree analyses and random forest algorithms readily provide multiparametric patterns with near-absolute sensitivities and specificities, but often suffer from data over-fitting and thus should be always critically assessed with the independent sets of samples.

Added clinical value of a biomarker should be the major driving force of biomarker development projects. Even if a biomarker can detect disease early, it may not be useful in the clinic due to the absence of effective treatment or serious side effects of over-treatment and burden of over-diagnosis. Likewise, biomarkers with excellent sensitivity but average specificity will not be useful for population screening and early detection of rare cancers [18]. Indeed, with all efforts made to discover cancer biomarkers in the last few decades, none of the FDA-approved cancer biomarkers are recommended for population screening.

Financial aspects of biomarker development should also be carefully assessed. Practice shows that reimbursement for developing a new diagnostic test is relatively low. Unlike novel therapeutics which will directly improve the quality of life, the effect of new biomarkers would probably be indirect and thus less valuable. A novel cancer therapeutic drug that provides just a marginal increase in patient survival will still make it to the clinic and will compete with alternative therapies. A novel biomarker, however, should provide significantly better diagnostic performance to compete with existing diagnostic tools.

In order to alleviate the most common limitations and facilitate standardization of biomarker discovery and development, several initiatives such as CONSORT [129] and REMARK [130] were established to provide guidelines for biomarker study design, patient selection, sample collection, analytical assay development and statistical data analysis. Registration of unsuccessful clinical validations of biomarkers through centralized registries [99,131] should also diminish the bias originating from meta-analysis of databases and literature which contain only positive findings and avoid publishing statistically non-significant results.

13. Conclusions

Better understanding of the concept of biomarker development, standardization of pipelines, improvements in the robustness of mass spectrometry instrumentation and reproducibility of proteomic sample preparation protocols should eventually result in clinically useful biomarkers discovered by proteomic approaches. For example, few emerging biomarkers recently discovered by proteomics include YKL-40 (CHI3L1) protein as a biomarker of several diseases including cancer [132] and TEX101 protein as a biomarker of male infertility [56]. Many more promising biomarkers may emerge from integration of multiple -omics technologies, such as whole genome sequencing, next generation RNA sequencing, miRNA profiling and quantitative proteomics. It is also expected that some successful protein biomarkers will be first discovered for diseases with well-defined pathobiology and cancers with monoclonal origin. In the case of highly heterogeneous diseases, genomic approaches will first facilitate stratification of patients into distinct molecular subtypes, followed by the search of protein biomarkers using subtype-specific clinical samples.

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