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REVIEW

Clinical translation of MS-based, quantitative plasma proteomics: status, challenges, requirements, and potential

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ABSTRACT

Introduction: Aided by the advent of advanced mass spectrometry (MS)-based technologies and methodologies, quantitative proteomics has emerged as a viable technique to capture meaningful data for candidate biomarker evaluation. To aid clinical translation, these methods generally utilize a bottom-up strategy with isotopically labeled standards and a targeted form of MS measurement.

Areas covered: This article reviews the status, challenges, requirements, and potential of translating current, MS-based methods to the clinical laboratory. The described methods are discussed and contrasted within a fit-for-purpose approach, while different resources for quality control, quantitative analysis, and data interpretation are additionally provided.

Expert commentary: Although great strides have been made over the past five years in developing reliable quantitative assays for plasma protein biomarkers, it is crucial for investigators to have an understanding of the clinical validation process, a major roadblock in translational research. Continued progress in method design and validation of protein assays is necessary to ultimately achieve widespread adoption and regulatory approval.

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1. Overview and significance of biomarker analysis

The prevalence of noncommunicable disease (e.g. cardiovascular, cancer, and diabetes) is increasing worldwide and is projected to escalate further in the coming years [1–4]. Modern medicine is therefore highly dependent on measures for detecting, stratifying, and monitoring disease. Such biological (or physiological) indicators of disease are referred to as biomarkers [5]. Examples of biomarker detection techniques include electrophysiological measurement (e.g. electrocardiogram for ischemic heart disease), isotopic medical imaging (e.g. positron emission tomography for neurological disease or malignancy), and mass spectrometry (MS) detection of analytes in biosamples (the focus of this review). Analytes can serve as effective markers of disease, provided that they demonstrate high clinical sensitivity and specificity.

Mass spectrometry is a fundamental and versatile quantitative technique for analyte detection in biological samples due to its speed, specificity, sensitivity, and throughput [6]. Principally, compounds of interest (including metabolites, proteins, peptides, lipids, and nucleic acids) are typically ionized and introduced into the gas phase where they are analyzed according to their mass-to-charge (*m/z*) ratio in various MS modes. The general modes of operation are the MS survey (for precursor ion acquisition) and the tandem MS (MS/MS, for product ion acquisition of the precursor ion fragments) scan. The additional dimension of molecular specificity afforded by MS/MS fragmentation is important in biomarker screening to help facilitate improved discrimination of target and non-target ions. The current modes most applicable for clinical biomarker screening are selected/multiple reaction monitoring (SRM or MRM; performed typically on a triple quadrupole mass spectrometer [7]) and parallel reaction monitoring (PRM; performed on a hybrid quadrupole-orbitrap [8] or a quadrupole time-of-flight [9] mass spectrometer). It should be noted that MALDI-TOF analyzers are in wide clinical use for microbial identification, but are only beginning to be used for quantitative biomarker measurements [10]. In classical MRM and PRM, MRM involves the *a priori* detection of precursor-product ion pairs (referred to as transitions) at defined chromatographic retention times (typically obtained from reversed-phase separations), whereas PRM selects specific precursor ions for complete product ion detection in a high-resolution, accurate-mass (HRAM) analyzer (e.g. orbitrap or time-of-flight). In select studies, MRM and PRM have been demonstrated to provide comparable linearity, dynamic range, precision, and sensitivity in protein quantification [11–14]. While the analyte specificity and multiplexing between these techniques differ, both are suitable for biomarker evaluation at any stage of the pipeline (from discovery to clinical validation; see Figure 1) [15]. Further to this, the mass

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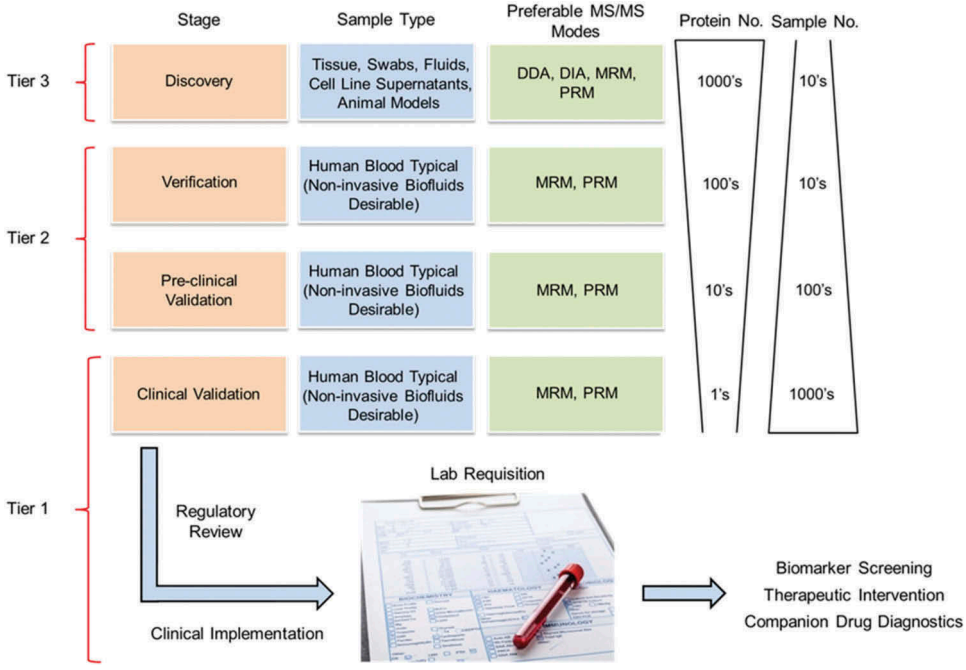

Figure 1. Workflow for translating candidate protein biomarkers from discovery to the clinic with MS-based technology. Shown are the various sample types and MS/MS acquisition modes available to interrogate the protein and sample numbers required at the different stages/tiers of biomarker development.

spectrometers used in MRM and PRM acquisitions have the ability to provide sufficient scan speeds to accommodate high-flow electrospray ionization (approaching 1 mL/min). These techniques therefore provide the necessary throughput to analyze the high sample numbers required at clinical validation and expected during routine screening.

The most notable implementations of tandem MS in the clinical laboratory have been in peripheral blood screening (typically from dried blood spots of newborn infants) for inborn errors of metabolism (IEM; e.g. amino acid, organic acid, fatty acid) [16,17] and for the measurement of small molecules, such as vitamin D [18,19], steroid hormones [20,21], and immunosuppressant drugs [22]. IEM result in the abnormal accumulation of precursors caused by the absence of proteins/enzymes in the biochemical pathway of interest. Through MS/MS, panels of IEM-centered biomarkers can be screened [23]; although the exact number is jurisdiction-specific [24]. Nonetheless, these represent the best examples of 'omics in clinical medicine.

In protein biomarker analysis, plasma/serum has been favored since it contains the broadest concentration range of analytes (≥10 orders of magnitude, ranging from low mg/mL to low pg/mL [25]) that are secreted, released, or leaked from neighboring cells, tissues, or organs. It is important to briefly note the distinction between plasma and serum. Although both are derived from whole blood, plasma is collected in the presence of an anti-coagulant (e.g. K_2·EDTA, sodium citrate, or lithium heparin), while serum is the fluid that remains after coagulation and centrifugation. Since the coagulation process can invoke variability and cause cell lysis, as well as concomitant removal of nonspecific analytes [26], plasma has been selected over serum in many protein biomarker quantification studies and is thus the focus of this review.

Protein quantification can be performed in a relative or absolute manner. Absolute quantification, however, is preferred in clinical studies since it provides endogenous concentrations in physical units (e.g. in ng/mL or nM) as opposed to fold changes in abundance levels, as frequently reported in biomarker studies. Experimentally, 'absolute' protein quantification is normally achieved by a bottom-up experimental method involving protein denaturation (typically with urea, other surfactants and chaotropes are possible), disulfide bond reduction and alkylation (e.g. with dithiothreitol and iodoacetamide, respectively), proteolytic digestion (typically with trypsin at 10:1 to 100:1 substrate : enzyme ratios for 16 h at 37°C), peptide separation (typically by reversed-phase liquid chromatography, RPLC, using a C18-bonded silica stationary phase and a FA/ACN-based mobile phase), and MS/MS detection (normally by scheduled MRM). In this generalized approach (reviewed in detail by Dittrich et al. [27]), peptides serve as surrogates for the proteins of interest, with their reproducibility in preparation being an important factor of peptide selection. Figure 2 delineates a general schematic of the method options, and is discussed in detail in the following section. For consistent proteolysis and improved liquid chromatography (LC)-MS processing, the target peptides should be selected in accordance with sequence-specific (e.g. be unique within the plasma proteome, devoid of missed tryptic cleavages, and 7–20 residues in length) and residue-specific (e.g. absent from oxidizable and modifiable residues) selection rules. The reader is referred to [28] for further details on these selection rules, along with guidelines on peptide handling and measurement. For reduced variability, the multistep sample preparation workflow should be automated, while routine quality control (QC) measurements (using commercially available, standardized kits) should be performed to help pinpoint

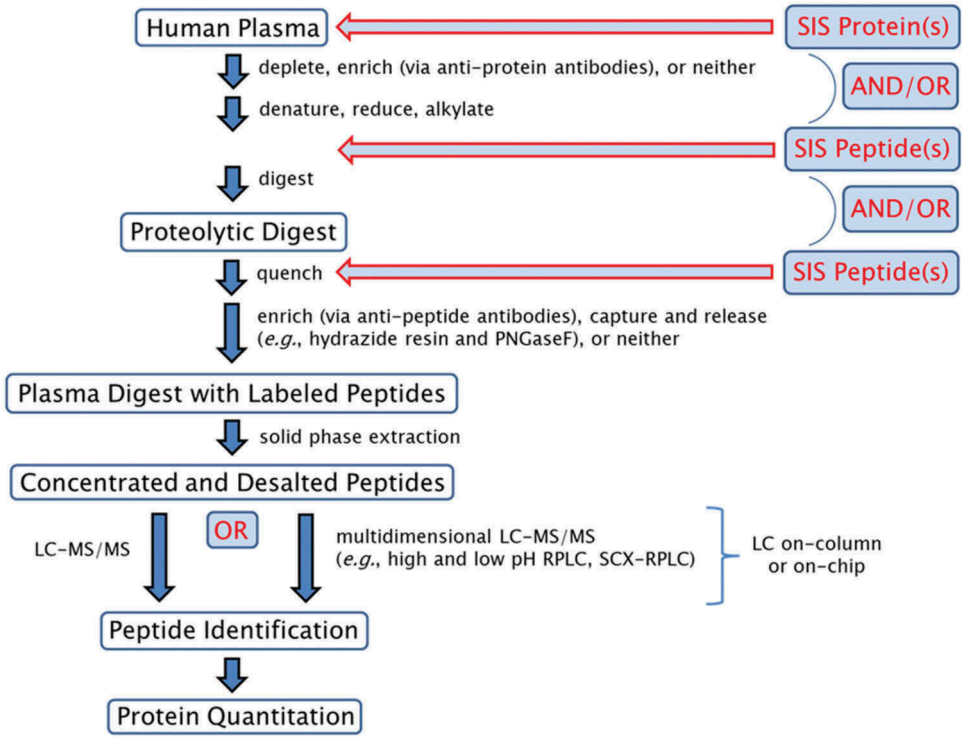

Figure 2. General workflow schematic for bottom-up LC-MS analysis of candidate protein biomarkers. Although the method options available are numerous, those discussed in this review are highlighted. The type of isotopically labeled standard(s) and purpose of experiment dictates its point(s) of insertion. Identification and quantitation of the resultant data can ultimately be accomplished with Skyline and its suite of tools.

source(s) of analytical variability (discussed further in the 'Requirements for Clinical Translation' section).

For improved detection confidence and measurement precision, stable isotope-labeled standards (SIS) having the same physicochemical properties as their endogenous counterparts should be added as internal standards. While variations are possible (e.g. recombinant QconCATs added predigestion [29]), these standards are typically $^{13}C/^{15}N$-labeled and are incorporated at either the beginning of the analytical workflow, in the case of proteins, or following proteolysis, in the case of peptides. The choice of normalizer ultimately depends on the intended purpose of the measurement. Using a fit-for-purpose approach [30], clinical (or Tier 1) assays require internal standard addition at the earliest stage of the sample preparation process in order to account for the variabilities that can arise at all steps of the experimental procedure (including proteolysis). This helps address the issue of quantitative accuracy, which is a requisite for the absolute determinations required in Tier 1 assays. Subsequently, quantification is evaluated with standard response curves generated in a normal (i.e. constant SIS peptide levels with variable synthetic endogenous levels) or reverse (i.e. constant endogenous peptide levels with variable SIS levels) manner, with performance metrics (e.g. limits of detection and quantification, respectively abbreviated LOD and LOQ) obtained for each target analyte. In addition to the analytical performance considerations, the clinical performance of the candidate biomarkers must also be validated in Tier 1 assays. This is normally accomplished through receiver operating characteristic (ROC) curves (plots of sensitivity vs. 1-specificity [31]). Through such curves, the diagnostic accuracy and optimal decision threshold of a biomarker can be effectively evaluated.

To date, twenty-three protein tumor markers have been approved by the US FDA for use in clinical practice [32]. Diagnostic examples include prostate specific antigen (PSA) for prostate cancer, carcinoembryonic antigen (CEA) for colorectal cancer, carbohydrate antigen 19–9 (CA 19–9) for pancreatic cancer, and cancer antigen 125 (CA-125, also referred to as mucin 16) for ovarian cancer. The majority of these protein biomarkers is currently detected and quantified using an immunoassay, which is typically accomplished by automated sandwich immunoassay with chemiluminescent detection. There is a notable discrepancy, however, in the number of clinically approved markers in comparison with the amount of novel biomarkers reported in the literature. The causes are diverse and encompass issues with experimental design and validation, as extensively reviewed by Drucker et al. [33]. Additional explanations for the decline in the introduction of new protein biomarkers include poor performance in subsequent clinical testing for disease screening or failure to report clinically relevant diagnostic performance metrics, such as sensitivity and specificity. Investigators should thus be cognizant of both the analytical and clinical requirements for novel biomarkers.

This article provides both a retrospective review and prospective view of the MS detectability/application of quantifying human plasma proteins for novel biomarker development in a clinical setting. Our focus was on literature published between 2011 and 2016, unless a seminal article is relevant that predates this period. The methods to be reviewed are primarily from Tiers 1 and 2, which refer to clinical assays for biomarker screening in the former and research assays for biomarker evaluation (i.e. preclinical verification and validation) in the latter (see Figure 1). Pertinent exploratory assays from Tier 3 that possess translational

potential are also discussed. As expected, stricter requirements in experimental design (i.e. use of labeled internal standards) and characterization (e.g. quantitative precision and accuracy) are required for assays designated for diagnostic laboratory tests than for biomarker discovery efforts [30]. In addition to discussing the status and potential of translating candidate protein biomarkers from research to the clinic, the challenges and requirements therein will be noted and expert commentary/recommendations will be provided.

2. Status and challenges of current methods

A recognized limitation of quantifying proteins in human plasma is its inherent complexity. Apart from the broad concentration range, the top 22 proteins in abundance (e.g. albumin, fibrinogen, serotransferrin) represent 99% of the total plasma protein mass [25]. This creates a substantial 'masking' effect in the measurement of lower abundance plasma proteins. Further adding to the complexity is the digestion event that is required in a bottom-up (or peptide-centric) methodology. Proteolysis results in thousands of plasma proteins being split into millions of peptides, which increases the potential for chemical interference during detection. Despite these challenges, plasma proteins covering the top six orders of magnitude (low mg/mL to mid ng/mL) have successfully been quantified using a direct LC-MRM/MS approach [34–38]. In a recent development, 142 proteins were measured in a single analysis, with myeloblastin at 44 ng/mL (LOQ at 10 ng/mL) being the lowest quantified protein in a multiplexed plasma panel [34]. To quantify proteins at low-to-sub ng/mL levels, which are often considered more suitable for biomarker screening, sample pretreatment (via depletion, enrichment, or fractionation) is required (see Table 1 for a comparison of the general, method advantages and disadvantages).

One strategy for improving the sensitivity of detection involves removing a subset of the top 22 proteins through immunoaffinity depletion [39–42]. In one example, differentially expressed proteins from discovery experiments were quantified in a plasma pancreatic cancer cohort (n = 60) using a bottom-up LC-MRM/MS method that involved up-front depletion of albumin and immunoglobulin G (IgG; via a ProteoPrep® kit, Sigma-Aldrich, St. Louis, MA, USA) [43]. This delivered a >20-fold improvement in sensitivity compared to an undepleted plasma analysis, with three moderate abundance proteins (namely gelsolin, lumican, and tissue inhibitor of metalloproteinase 1) found to be differentially expressed (i.e. >0.75 area under the ROC curve). However, as is the case for many such studies, further verification is required with larger sample sizes to validate this panel as clinically useful markers of pancreatic cancer. Up-front depletion of albumin and IgG was also performed prior to bottom-up LC-PRM/MS in a separate biomarker development [44]. This enabled the isotype- and protein-specific quantification of serum amyloid A in human plasma samples of non-small cell lung cancer patients (n = 27). In a second development example, the 14 most abundant plasma proteins, constituting ca. 95% of the total protein mass [42], were depleted (via a MARS Hu-14 column, Agilent Technologies, Santa Clara, CA, USA) prior to bottom-up RPLC-MRM/MS [45]. Here, the use of a long LC gradient (3 h) performed on a long ultra-high performance liquid chromatography column (30 cm in length, 75 µm internal diameter, 1.9 µm particles) maintained at non-ambient temperature (50°C) enabled the highly multiplexed quantitation of >400 endogenous peptides (with three transitions/peptide

Table 1. Key merits and detractions of the described, bottom-up LC-MS/MS methods for biomarker verification and validation of human plasma proteins. Enrichment is accomplished with either anti-protein or anti-peptide antibodies, while multidimensional fractionation is performed on-column or on-chip.

Sample pretreatment	Advantages	Disadvantages	References
None	• Minimal sample and reagent requirements (typically <10 µL plasma) • Wide linear dynamic ranges in quantification • High multiplexing • Robust • High throughput	• Limited quantification potential below 40 ng/mL	[34–38]
Depletion	• Low ng/mL quantification attainable	• Expensive depletion kits • <100% protein recovery • Decreased throughput • High variability	[39–46]
Solvent precipitation	• Effective removal of high and medium MW proteins • Inexpensive	• <100% protein recovery • Low throughput	[47]
Enrichment	• Low pg/mL quantification possible • Wide linear dynamic range in quantification • Robust • High throughput	• Long lead time for developing well-functioning antibodies • Low multiplexing • Expensive	[48–71]
Multidimensional Fractionation	• Mid pg/mL quantification possible	• Low throughput • Reduced peptide recovery between dimensions • Low multiplexing • Potentially high RT variability • Difficult to transfer between laboratories	[81–90]

RT: retention time.

corresponding to 2400 transitions) in a single run. Relative to standard LC-MRM/MS methods implementing 12 cm HPLC columns with 3 µm particles, the long LC column enabled higher loading capacity (four- to six-fold increase), as well as improved peak capacity (two-fold increase) and sensitivity (four-fold improvement in median LOQ). Overall, despite the merits in signal-to-noise ratios and LOQs, depletion can cause select target proteins, such as cardiac troponin T and PSA, to be partially or completely removed through nonspecific binding [46], which affects the quality of the results. Due to additional drawbacks in terms of cost, throughput, and variability, depletion is generally not recommended for protein biomarker analysis, where reliable and accurate determinations are required for disease screening or diagnosis.

A separate approach to depletion involves the removal of high and medium molecular weight (MW) proteins by precipitation. This comparatively inexpensive alternative to depletion allows the low MW proteome to be effectively interrogated. In one example, a two-step precipitation procedure, involving organic solvents and liquid-liquid solvent extraction, was used prior to LC-MRM/MS in the quantitation of five low MW target proteins (namely IGFs 1 and 2, IGFBPs 2 and 3, and A2GL) implicated in breast cancer diagnosis and growth hormone management [47]. These moderate abundance proteins were quantified at average concentrations ranging from 12.3 µg/mL (for A2GL) to 183.9 ng/mL (for IGF1) in control plasma samples (n = 20). Given their processing throughput of 17 min run times, ca. 80 samples could be evaluated per day, which is considered low throughput compared to clinical immunoassay platforms that run 500–1000 samples/day. An additional limitation of the solvent-based precipitation approach is the <100% recovery obtained from the salting-in/salting-out process. While this may not be important in Tier 3 assays, deficient recovery can impact the absolute quantification required in Tier 1 assays. Method design must therefore weigh this parameter in order for the assay to ultimately fit its intended purpose.

Through the use of antibodies, immunoaffinity enrichment has also served as an effective tool to enhance the detection of lower abundance plasma proteins [48]. The stage at which such reagents are used is governed by the nature of capture. In general, anti-protein antibodies are incorporated at the beginning of an analytical workflow for protein enrichment [39,49,50], while anti-peptide antibodies are employed postdigestion for tryptic peptide enrichment [51–53] (see Figure 2). In an example of the former, anti-protein antibodies were covalently bound to a monolithic column (as opposed to magnetic beads) [50] for protein enrichment and detection of the target proteins or peptides in a top-down [54] or bottom-up [55] manner, respectively. This immunoenrichment approach, referred to as a mass spectrometric immunoassay (MSIA™), enabled the multiplexed targeted quantification of 16 plasma proteins (related to seven different disease states, e.g. cardiovascular, Alzheimer's, and cancer) at concentrations ranging from mg/mL to pg/mL [50]. In a recent study, the MSIA-SRM approach was used to robustly and sensitively quantify several domains of PCSK9 (proprotein convertase subtilisin kexin 9) gain-of-function mutations in which are responsible for some cases of familial hypercholesterolemia [56]. This MSIA methodology has also been used to measure insulin [57] and the apolipoprotein C family (includes C-I, C-II, C-III, and it's proteoforms) [58] in patient sera/plasma at 85 pg/mL and low µg/mL levels, respectively. Overall, the quantitative limits of these assays were found to lie largely within clinical reference ranges and yield strong correlations to commercial immunoassays in the sample analyses; thus providing clinical translational potential.

Affinity-based peptide enrichment with anti-peptide antibodies has been termed SISCAPA (i.e. stable isotope standards and capture by anti-peptide antibodies) [53], iMRM [59], and immuno-MRM [60]. Over the past few years, this approach has been applied to precisely quantify panels of disease-related proteins in human plasma [51,52,61–65]. In a recent study, 40 novel immuno-MRM assays (for 27 proteins) were developed and validated in accordance with the characterization guidelines for Tier 2 assays [66]. Characterization involved standard curve generation (for assay performance metric determination) and repeatability/reproducibility measurement (for intra- and inter-day precision evaluation), as outlined previously [30,67]. The response curves revealed LOQs from 0.18 to 35.9 ng/mL and wide linear dynamic ranges of three orders of magnitude, while the total variability of the analysis was found to be 20.6, 8.2, and 6.3% CV for samples spiked with low, medium, and high SIS peptide concentrations, respectively. Since the polyclonal anti-peptide antibodies commonly used in these multiplex iMRM assays have short lifetimes, a novel method was developed to extend it [61]. Through acid washing, 44 antibodies in the test panel (which in turn target 60 peptides) were found to deliver uncompromised assay performance (reproducibility: <15% CV, on average) over 10 analyses. The increased longevity of the affinity reagents helps reduce assay cost, a factor of exceeding importance in Tier 1 and 2 measurements. In a separate proof-of-principle study, the performance of recombinant antibody fragments (Fabs) were evaluated against traditional monoclonal antibodies as well as full-length IgG antibodies [68]. As revealed from standard curves, the Fabs provided comparable linearity (3–4 orders of magnitude), precision (ca. 10% average CV at LOQ for all antibodies), and sensitivity (0.3–2.9 ng/mL range for LODs from all antibodies) for each proteotypic peptide from osteopontin, E-selectin, and ADAM17. Due additionally to equivalent recovery (e.g. 87% vs. 84% with monoclonal for osteopontin) and reduced development time (ca. 12 weeks vs. 6–9 months for traditional antibodies), Fabs provide significant potential for future immuno-MRM assay developments and applications. This antibody development includes *in vitro* generation (using RapMAT technology [69]) and expression (using tags, such as Strep, for magnetic bead binding), as well as assay characterization (e.g. for recovery, precision, and accuracy), in order to ensure that the recombinant anti-peptide antibodies specifically capture the peptide(s) of interest. Despite the development lead time, once generated and demonstrated to be well functioning, antibodies can be an effective means of increasing the sensitivity of analysis. To date the peptide-capture approach has been successfully applied to the clinical measurement of thyroglobulin [70,71], a marker for thyroid cancer recurrence in which endogenous autoantibodies occurring in a subset of patients interfere with conventional immunoassays.

In the absence of antibodies, enrichment of posttranslational modification (PTM)-containing proteins/peptides can be achieved with lectins [72,73] or by chemical immobilization (via hydrazide chemistry [71,74,75]). Since PTMs are thought to highly correlate with disease incidence and progression, such investigations could prove quite promising in revealing novel biomarkers for early detection and monitoring. In one study, N-linked glycopeptides were targeted in plasma toward the discovery/verification of new markers for Parkinson's disease [76]. Experimentally, following tryptic proteolysis, the resulting glycopeptides were captured on an Affi-Prep hydrazide resin (Bio-Rad, Hercules, CA, USA), purified, released by N-glycosidase F (PNGase F), then separated and detected by LC-SRM/MS (see Figure 2). Using SIS peptides for normalization, the results revealed low ng/mL detection of 11 disease-related glycoproteins (from 12 N-linked glycopeptides), with a four peptide panel (from MEGF8, HSPG2, PRNP, and NCAM1) providing the diagnostic sensitivity (90.4%) and specificity (50.0%) necessary to screen for disease (n = 282 total samples). Although further verification is required, the results provide a promising means of detecting neurodegenerative disorders from plasma, as opposed to more invasive alternatives, such as cerebrospinal fluid (CSF, requires a lumbar puncture). Another promising finding involving N-glycan analysis came from a recent study by Aebersold et al. [77]. Their development revealed a 5-protein biomarker panel (includes CP, TIMP1, LRG1, PON1, and SERPINA3) that can sensitively detect and classify colorectal cancer in patient plasma samples. Further evaluation of the panel is, however, required to develop a fit-for-purpose clinical grade assay. In a separate example, endogenous plasma glycopeptides were quantified in the presence of standards by a MS-based, data-independent acquisition (DIA) technique referred to as SWATH (i.e. sequential window acquisition of all theoretical mass spectra) [78]. In the SWATH approach, MS/MS scans are collected systematically and independently of precursor ion information over sequential and consistent *m/z* ranges (typically 25 Da precursor ion isolation windows) [79]. This mode creates complex spectra that can be mined postacquisition. However, the complexity increases the potential for chemical interference and therefore requires sophisticated software tools, such as SWATH2stats [80], for de-convolution and statistical analysis. In this study, while a high degree of reproducibility was obtained for the 37 target glycopeptides (14.9% CV, on average), the sensitivity was approximately three-fold inferior to standard SRM assays. This reduced sensitivity may limit the detection and quantification of low abundance biomarkers.

Fractionation has also been used to enhance the sensitivity of detecting lower abundance plasma proteins. In an example of this antibody-free approach, dimensions of chromatographic separation and a carefully selected fraction pooling strategy was used to obtain altered peptide selectivity and improved peptide detectability [81]. The approach that provides the greatest orthogonality involves two dimensions of RPLC separation, with the first operated at pH 10 (e.g. with ammonium formate or hydroxide) and the second at pH 3 (with formic acid) [82]. In a development example, an off-line 2D LC-MRM/MS method was used with SIS peptides to quantify 253 plasma proteins (from 625 interference-free peptides) in 13 pooled fractions [83]. Experimentally, two-fold higher volumes of sample and SIS mix were used compared to a conventional, unidimensional LC-MRM/MS method [34] for quantification via single point measurement. Limitations of this multidimensional LC-MRM/MS methodology include decreased throughput and high volume requirements for quantitation via 7-point standard curves [84]. Nonetheless, at the low end of the quantification scale, 31 proteins were found to have concentrations between 452 pg/mL and 10 ng/mL in the control plasma measured. In a separate example, the 'PRISM' (high-pressure, high-resolution separations coupled with intelligent selection and multiplexing) technique was implemented to quantify select proteins in depleted [85] and undepleted [86] human serum/plasma. The LOQs were found to be at low pg/mL levels with prior depletion (via IgY14) and at mid pg/mL levels without this form of pretreatment. In an interesting application of PRISM, a collection of amyloid-β peptides were quantified in human plasma with impressive reproducibility (<5% CV, on average) and sensitivity (LOQ of 570 pg/mL for amyloid- $\beta_{1\text{-}40}$) starting from 10 µL of human plasma [87]. Since amyloid-β peptides represent one set of current targets for diagnosing Alzheimer's disease, this method provides an attractive alternative to CSF measurement worthy of further exploration. It is important to note that these two examples of 2D RPLC methodologies differ in the choice of mobile phase compositions, concatenation strategy, eluent flow rates, and target panel. Despite these differences, improvements in peptide recovery and sample throughput are still required before these techniques can be applied to large-scale biomarker screening.

Additional fractionation approaches have been developed for plasma protein quantification using alternative combinations of LC separations. In one example, a multidimensional LC-SRM method was developed with an internal peptide standard for the low ng/mL quantification of endogenous PSA in undepleted male plasma [88]. This specifically involved anion exchange chromatography (on a solid phase extraction cartridge) sandwiched between dimensions of RPLC and HILIC configured in an off-line arrangement. Although satisfactory precision (CV range from repeatability tests: 5.6–7.9%) and sensitivity (LOQ: 1 ng/mL) was obtained for the MS workflow, the total analysis time was lengthy (40 min) and the multiplexing capacity was not evaluated. An example of an on-line 2D LC-SRM method designed to overcome the sample throughput and recovery issues present in off-line 2D methods was developed by Krisp et al. [89]. Using strong cation exchange and RP liquid chromatography, a 90% improvement in the peptide responses of abundant plasma proteins was obtained compared to their unfractionated peptides. This enabled the detection of low abundance proteins and demonstrated one of the merits of configuring the multidimensional separation dimensions for on-line MS/MS operation.

LC fractionation has also been performed on microchips to selectively enrich peptides from plasma proteolytic digests. In one example, a Chip/Chip/SRM platform was developed for the low ng/mL quantification of PSA in plasma samples of male prostate cancer patients [90]. The method specifically involved initial immunodepletion (of albumin and

IgG) followed by SIS peptide addition, tryptic digestion, isoelectric focusing (on a digital ProteomeChip), RPLC (on a microfluidic chip), and SRM/MS. While the derived concentrations were found to correlate with a commercial ELISA (R^2 = 0.946), the number of compared samples was small and not in the analytical range of greatest clinical value (<10 ng/mL). Additionally, this technique is considerably more complex and has a low sample throughput, limiting its potential extension to the larger sample numbers required for biomarker evaluations.

3. Requirements for clinical translation

In considering the biomarker pipeline, there exists an inverse relationship between the number of proteins and the number of samples that can be interrogated at each stage (see Figure 1). At the clinical validation end point of biomarker evaluation, a small number of qualified biomarker candidates must potentially be validated against thousands of patient samples. To be considered fit-for-purpose, the assay's must be well validated (i.e. through standard curves, repeatability, reproducibility, and parallelism experiments) to deliver highly precise and accurate quantitative data. Compliance with such regulatory guidelines as the Clinical Laboratory Improvement Amendments (CLIA), the US FDA, or the European Medicines Agency are recommended at this stage. For example, the FDA guidelines for bioanalytical method validation of standard curves defines specific thresholds that should be obeyed in assessing the precision (average CVs of <20% at LOQ and <15% for higher levels) and accuracy (deviations of <20% at LOQ and <15% for higher levels) of the technical replicates at each qualified level (minimum four in total) [91]. However, precision performance of routine protein biomarkers should be much better than 15% and lie in the analytical range of use in clinical practice [92,93]; therefore, higher expectations must be sought in development. In addition, the FDA requires curve QC checks to be run at low, medium, and high concentrations that are distinct from the curve concentration levels utilized. In application, the standard curve and its QCs should be constructed from a matrix digest that has been spiked with a known concentration(s) of internal standard(s). While this approach is feasible with reverse curves (utilize SIS peptides only), it is not practical with normal curves (employ SIS and synthetic endogenous peptides) since endogenous target peptides from the matrix digest are additionally present. In this latter case, the standard curve and QCs should be prepared from an alternate matrix, such as buffer or chicken plasma. The choice of matrix ultimately depends on performance (in terms of quantitative accuracy, linearity, and dynamic range, for example); however, the peptide targets must also be verified to be sequence unique within the matrix selected. Further to the standards, while several options exist, full-length SIS proteins may be preferred, if properly folded and characterized, since they may best account for the variabilities that can arise throughout sample preparation (i.e. from denaturation to detection) [94]. Since variability can impact the accuracy of quantification, controlling and correcting for such variabilities is essential, as indicated in the outcome of a mock 510(k) premarket submission of targeted proteomic assays to the FDA [95]. Also of declared importance from this submission are standardized methods that can be executed by nonexperts, the use of FDA-approved instrumentation/software, and the analysis of interference-free peptides with well characterized standards used as surrogates. Further to the standards, for clinical implementation, their short- and long-term stability at the intended storage condition/temperature must also be established, as well as their stability over freeze-thaw cycles.

Apart from the analytical requirements of methodological assays, QCs must be available for benchmarking method and system performance. To date, a number of QCs have been commercialized for human plasma proteomics on an array of instrument platforms [96,97]. The ProteusQC™ standard (Cambridge Isotope Laboratories, Andover, MA, USA), which contains an isotopically labeled mixture of human apolipoprotein A-I and a subset of its peptides, is a recent example. Interestingly, the analytes in this mix have the potential to do more than just QC monitoring. They can be used to index retention times (RTs), evaluate digestion efficiency (of apolipoprotein A-I), and perform absolute quantification. Further to the latter, the peptides derived from the ^{15}N-labeled ApoA-I SIS protein can serve as global internal standards for the endogenous proteins in the user's target panel, as was demonstrated previously for an array of moderate abundance proteins in SRM and PRM applications [12,94]. If clinical translation is to be achieved, data analysis tools are required for peak selection, integration, quantification, and interpretation by nonexperts. To that end, an open source platform – Skyline [98] – is available for inspection and analysis of all types of MS/MS acquired data. Skyline is vendor-independent and is capable of standardizing many of the processes expected in quantitative proteomic analyses. Examples of external tools available through Skyline include AudIT (for chemical interference screening [99]), QuaSAR (for quantitative results determination [100]), and MSstats (for statistical visualization and interpretation [101]). Additional, platform-independent, open source tools for quantitative analysis and interpretation are MRMPlus [102] and Qualis-SIS [103]. While both tools are designed for analysis of Tier 2 assays, Qualis-SIS was developed with the FDA requirements for standard curve generation and analysis in mind, as required for assays seeking FDA clearance/approval [95], and therefore also serves as a good resource for Tier 1 assay measurements. To help evaluate QC performance data, a statistical visualization tool – termed Statistical Process Control in Proteomics (SProCoP) [104] – is available through Skyline. By tracking the chromatographic (e.g. RT, peak symmetry) and mass spectrometric (e.g. peptide ion intensity) performance metrics over time (e.g. in a control chart matrix; see Figure 3 for a process summary), sources of variation can be diagnosed and corrected before real sample analyses are performed.

Regarding the diagnostic accuracy required at clinical implementation, while high sensitivity and specificity are ideally targeted, this is challenging to achieve as an increase in one is typically obtained at the expense of the other [32]. As indicated earlier, this paradigm is best visualized with ROC curves. In practice, the area under the ROC curve indicates the analytes diagnostic performance, while threshold values can be defined through Youden index estimations [105]. In theory, while no set threshold values have been defined by

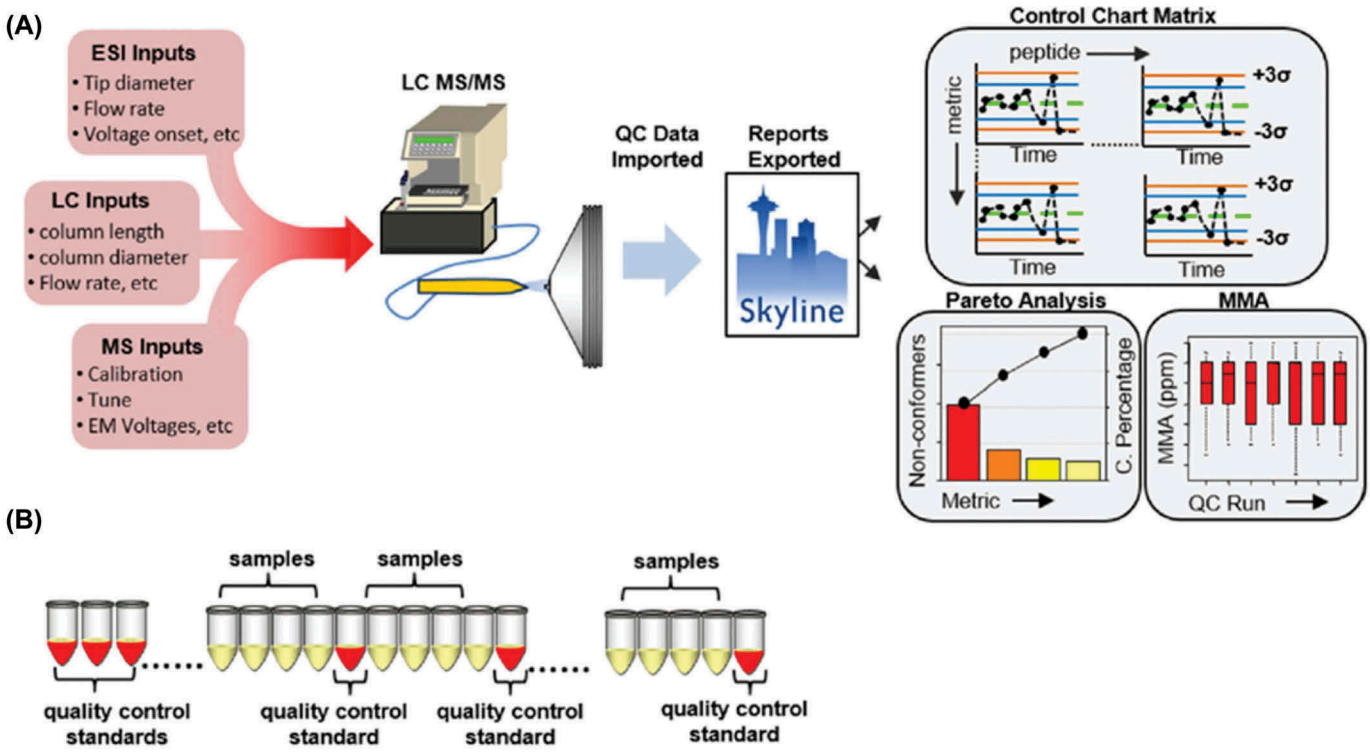

Figure 3. Utility of the Skyline-enabled, SProCoP tool to display MS proteomic data from performance experiments with QC standards. The LC-MS metrics under evaluation in (A) are tracked over time, as shown in (B), through various plots and matrices against empirically derived thresholds. Reproduced from [104], with permission.

the FDA, the greater the area under the curve (i.e. close to 1.0, indicating sensitivity and specificity approaching 100%), the better the discriminating power of the marker(s).

4. Expert commentary

The quantitative proteomic landscape has evolved over the past 5 years such that translational research investigating the clinical implementation of new biomarkers could soon be realized. The evolution has been shaped by advances in technology and methodology that were designed to improve sensitivity (e.g. enrichment provides a 3–4 order enhancement over neat analysis), specificity, and throughput (e.g. through shorter LC-MS/MS processing times). In one example, the throughput of SISCAPA assays was improved for the protein quantification of plasma samples by replacing the LC dimension with solid phase extraction (SPE) [106]. This SPE-MRM/MS platform provided an approximate 300-fold improvement in analysis time over conventional LC-MRM/MS methods, while maintaining the multiplexing capacity required for biomarker verification/validation. Overall, the criteria of importance are ultimately governed by the purpose of the intended assay, with more strict criteria generally required for Tier 1 assays than for Tier 3. Additional workflow improvements (e.g. automated sample handling and processing with robotics, such as the AssayMap Bravo [71] or the Tecan Freedom Evo [107]) have helped streamline development and application, while public repositories of validated MS/MS assays have been created to aid standardization [108–110].

As a whole, methodologies for biomarker analysis and biochemical pathway interrogation are beginning to be developed under a fit-for-purpose approach with the clinical end point in mind [111]. For a collection of methods, standard curves with QCs have been instituted to measure system and curve performance. Experiments have been conducted for the determination of repeatability and reproducibility, yielding average CVs of 15–20%. While this variability may satisfy the guidelines for Tier 1 and 2 assays, it is unsuitable for clinical practice. For some methods, large-scale analyses of quantitative proteomic assays have been performed across independent laboratories using a diverse set of LC-MS platforms [64,112–115]. These studies have collectively demonstrated the feasibility in measuring a broad range of plasma proteins (encompassing high-to-low abundance) using standardized practices for system suitability, quality control, and quantitative analysis. This collectively helps pave the way for their continued use in larger-scale assessment studies of candidate protein biomarkers using undepleted human plasma as the biological matrix. Overall, while significant strides have been made in deciphering the treasure trove of biomarkers present in human plasma, continued progress in method design and validation of protein assays is necessary to ultimately achieve widespread adoption and regulatory approval.

5. Five-year view

This review has sought to summarize and evaluate the advances that have been made in the translation of MS-

based quantitative proteomic methods for plasma protein biomarker screening to the eventual inclusion of these methods in a clinical diagnostics laboratory. It is clear that significant advances have been made such that the major technical issues that surround assay performance have been, or are capable of being, addressed. These issues include sample preparation, reproducibility, sensitivity, and scale (i.e. throughput), while the solutions presented are operable within a tightly controlled quality framework in order to ensure that the multiplexed protein measurements are fit-for-purpose in a clinical decision-making situation. As one might anticipate, there is significant hope and expectation that these advances will be translated into clinical practice. This will be the much and long awaited outcome of the detailed and carefully executed technical advances reported here. The compelling need for biomarkers with superior clinical performance that produce improved healthcare outcomes for patients, while enhancing the cost-effectiveness of healthcare systems, drives the continued determination and optimism to deliver proteomics methods for clinical use. In the next 5 years, it is not unrealistic to anticipate the emergence and adoption of:

i) automated, streamlined workflows for sample preparation that are customized to specific analytes and tests;
ii) FDA-approved instrumentation and software such that hospitals and other 'general' laboratories can access the power and potential of MS-based protein measurements. This will ideally support the use of MS by hospital laboratory staff who are accustomed to making routine measurements of clinical importance;
iii) standardized reagents and methods for performance assessment that satisfy the metrics defined by regulatory agencies, such as the FDA.

In summary, the outlook appears very promising from a technical and proteomic perspective. However, what has been lacking to date is the alignment of the important developments outlined in this review with clinical needs. Investigators should continue to strive to improve collaboration with clinicians, clinical pathologists, and clinical chemists to identify targets that have sufficient and high potential healthcare impact to provide the clinical and monetary incentives to help propel development forward. Alternatively, the application of the methods may occur in niche medical areas and through innovative approaches that have, as yet, been unanticipated by mainstream efforts. Nonetheless, we remain optimistic that over the next 5 years, an alignment of technical, clinical, and regulatory advances will emerge to the benefit of patients through the delivery of multiplexed protein tests performed on mass spectrometers.

Key issues

- Few protein biomarkers discovered by proteomics have been clinically validated and FDA approved for disease diagnosis, prognosis, and risk stratification.
- Current plasma markers, such as PSA and CA 19–9, lack the specificity/sensitivity required for accurate detection leading to false positives/negatives and invasive follow-up procedures for confirmation.
- The most promising strategy for verification/validation centers on targeted MS/MS with SIS peptides or proteins employed within a bottom-up workflow; although, top-down workflows are likely to also play a role.
- For clinical adoption, quantitative methods must be well characterized (through standard curves and repeatability/reproducibility experiments, for instance) and evaluated to ensure that stable system performance is maintained throughout application.
- Over the past 5 years, improved quantitative proteomic methods, such as immunoaffinity enrichment coupled with MS, have been developed for biomarker assessment and systems biology investigations in human plasma samples.
- Tools for quality control, quantitative analysis, and data interpretation have additionally been developed.
- To date, currently available methods enable the robust, multiplexed quantification of plasma proteins spanning the mg/mL to mid pg/mL range.
- Challenges in quantifying low abundance plasma proteins (including cytokines and interleukins) remain, while the inter-laboratory reproducibility of currently developed methods is required to demonstrate their transferability and to help expedite biomarker assessment.
- To satisfy clinical assay requirements, methods must be executable by non-experts and demonstrate reproducible/accurate quantification from interference-free peptides as protein surrogates using 1000's of patient samples.
- While further progress is required (*e.g.* instrumentation and software for FDA approval), the next 5 years are expected to have as many, if not more, advances than the past 5.

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Declaration of interest

Authors A Percy, T Agreste, and M Duffy are employed with Cambridge Isotope Laboratories, which has commercialized one of the standardization products - ProteusQC™ - discussed. S Pennington is founder of the UCD spinout biomarker company Atturos. D Holmes has given remunerated scientific talks for Immunodiagnostic Services and has sat on two single-day advisory boards for EMD Serono and Pfizer. N Anderson is the chairman, CEO, and founder of SISCAPA Assay Technologies, Inc. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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•• of considerable interest

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