Mass Spectrometry-based Plasma Proteomics: Considerations from Sample Collection to Achieving Translatable Data

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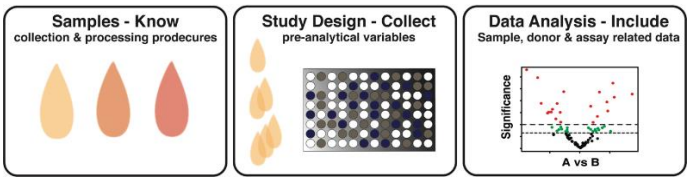
Abstract

The proteomic analysis of human blood and blood-derived products, particularly plasma, offers an attractive avenue to translate research from the laboratory into the clinic. However, due to its particular protein composition, performing proteomics assays with plasma is challenging. Plasma proteomics has though regained interest due to recent technological advances, but challenges imposed by both the complications inherent to studying human biology (e.g. sample variability) and the limitations in available technologies and methods remain. As part of the Human Plasma Proteome Project (HPPP), we bring together key aspects of the plasma proteomics pipeline. We provide considerations and recommendations concerning the study design, plasma collection and quality metrics, plasma processing workflows, data acquisition by mass spectrometry (MS), data processing and bioinformatic analysis. With exciting opportunities in studying human health and disease via the plasma proteomics pipeline, a more informed analysis of plasma will accelerate interest and possibilities for the incorporation of proteomics-scaled assays into clinical practice.



Keywords

Plasma, serum, blood, Human Plasma Proteome Project (HPPP), study design, sample collection, quality metrics, plasma processing workflows, data acquisition by mass spectrometry (MS), data processing, bioinformatic analysis.

Introduction

Whole blood is an easily accessible tissue that affords a significant opportunity to learn about human biology in a minimally invasive manner. Recent interests in using whole blood as a form of "liquid biopsy" for personalized medicine and more effectively monitoring response to therapeutic treatments are driving the discovery of new disease-specific biomarkers [1,2]. However, blood is a complex mixture, consisting of cells, exosomes, nucleic acids, proteins, and metabolites, amongst many other components, and plays a central role in facilitating diverse biological processes. The liquid component of whole blood, termed plasma, is obtained after centrifugation of whole blood in the presence of an anti-coagulation agent (e.g. EDTA, heparin, or sodium citrate). This isolation process eliminates the cellular material and leaves the cell-free components available for characterization. In this review, we focus on proteins found in plasma and discuss how to achieve robust data using mass spectrometry (MS)-based approaches.

Plasma proteomics has undergone a revival in the last five years. The need for more clinically translatable biological insights is driving an increase in the number of MS-based proteomic studies[3]. There are more than 150 FDA-approved and laboratory developed tests (LDTs) that utilize plasma for protein-based assays, such as C-reactive protein (CRP) levels for coronary disease and insulin levels for diabetes[4]. This existing clinical infrastructure and familiarity with plasma allows for the translation of new discoveries from the laboratory into the clinical setting[5].

Plasma is a challenging biological matrix, due to both a large dynamic range in protein expression and the capabilities of analytical methods. For example, the plasma peptidome[6,7] or those peptides carried by the human leukocyte antigen (HLA) molecules[8] represent low abundance, small molecular weight species. To mention other than MS-based methods, proteins in plasma can also be profiled by a variety of immunoassays[9] such as those tailored for high-sensitivity and throughput[10]. For the circulating antibodies, multiplexed protein or peptide arrays are common to study reactivity towards the autoimmune components of the proteome[11] and their post-translational modifications[12]. Today there are > 1000 autoantigens found in a large variety of human conditions [13]. Numerous studies have focused on plasma processing workflows towards achieving a more comprehensive characterization of the plasma proteome[3,14]; some are described in the Plasma Processing Workflows section below. While the basic-research community might focus on improving depth of coverage to detect low abundance plasma proteins (i.e. <1 ng/mL), the clinical community might emphasize reproducibility of measurements and low coefficients of variation to support actionable clinical decisions. In the former case, considerations for translating findings from the laboratory into the clinic can be limited as the added sample processing schemes may create hurdles to widely adopting the method. In the latter case, while the focus could be on high abundance proteins (i.e. >1 ug/mL)4, that have a precedence for some clinical indications (e.g. CRP levels), such protein markers may not be sufficiently sensitive for applications such as early disease detection. Perhaps there is a balanced approach that can satisfy the needs of the entire plasma proteomics community?

Can MS-based plasma proteomics overcome the current challenges?

A key challenge in human health, and an unmet need of medicine, is early disease detection, which is almost entirely dependent on more specific biomarkers, better patient stratification, and methods for predicting patient response to treatment. MS-based plasma proteomics can deliver solutions to many of these challenges when applied in an appropriate manner. In a recent update, the Human Plasma Proteome Project (HPPP) reported 3500 detectable proteins in 2017 from plasma samples emerging from almost 180 studies with a protein-level FDR of 1%[15]. This number nearly doubled from the 1929 proteins reported in 2011[16,17] and points to improvements made in analytical sensitivity when studying the plasma proteome. However, challenges still remain due to the low consistency in reproducible observations among these plasma proteomics studies. In fact, in studies performed between 2005 and 2017, the 500 most abundant plasma proteins were only detected in 50% of studies (Figure 1), hence missing data and coverage of protein abundance across all study subjects will be important aspects of plasma proteomic studies. While some of these differences can be explained by the different instruments, plasma processing techniques, and sample collection methods used in the studies, it also raises an additional question about which factors most strongly influence plasma protein

detectability? Towards answering this question, and improving the overall performance of plasma proteomics experiments, we recommend that researchers consider the key components of a plasma proteomics workflow, outlined in Figure 1.

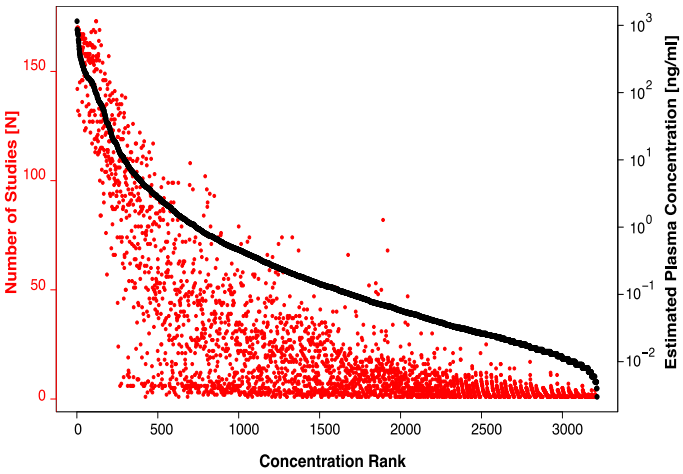


Figure 1. Frequency of protein identification in relation to plasma concentration. Using data collected for the 2017 draft of the human plasma proteome (hosted by PeptideAtlas[15]), proteins (in red) are plotted as a function of their concentration rank (x-axis) and the number of studies in which they were identified (y-axis, left). The identified proteins (solid black line) are also plotted as a function of their concentration rank (x-axis) and their estimated concentration (y-axis, right).

Components of a plasma proteomics study

There are many considerations when planning and executing a plasma proteomics study. In this review, we focus on the elements that span a full project: (1) study design, plasma collection and processing, (2) data acquisition by MS, (3) peptide identification and quantification (4) and bioinformatic and statistical analysis of the data. As summarized in Figure 2, we introduce these different layers of information that are contained within these four areas. Lastly, we discuss how each one can impact the outcome of a particular plasma proteomics study.

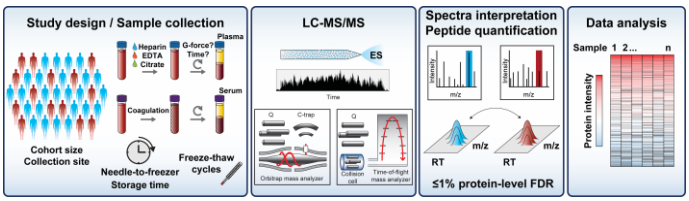


Figure 2. Components of a plasma proteomics workflow. Profiling proteins in plasma begins with collecting the samples in a standardized manner, reporting pre-analytical variables related to the sample and information about the blood donors. After protein digestion and peptide purification, the peptides are separated by liquid chromatography (LC) and ionized by electro spray (ES) for the analysis in the mass spectrometer (MS). Appropriate MS workflows and peptide identification and quantification tools are then applied. For protein identifications, the HPP guidelines recommend a protein-level FDR of ≤1%. Lastly, data analysis should consider how many peptides and proteins were identified and their consistency across samples.

Study design

A well-designed plasma proteomics study requires a clear research question along with pre-specified hypotheses. By defining and deciding on these factors early, the subsequent study protocols and experimental design decisions can be appropriately made. Furthermore, this deliberate approach will ensure that data processing and bioinformatic analysis are executed in a purposeful manner; this also ensures that if subsequent analysis is performed (e.g. after reviewing the preliminary results), it can be correctly categorized as *post-hoc* analysis. Here, we discuss study design considerations that can be grouped into the following categories: study settings, cohort selection, and reference samples.

Study settings (e.g. specific disease, healthy, or drug investigation)

Plasma proteomics studies to date, and especially in the last five years, have converged in three areas: (1) techniques to improve proteome coverage (i.e. credibly detect the largest number of plasma proteins), (2) solutions that are applicable for clinical applications (e.g. sample throughput, reproducibility, and costs), and (3) studies investigating diverse diseases (e.g. cardiovascular diseases, cancers) or the effect of therapeutics on the plasma proteome (e.g. chemotherapy).

Cohort selection - sample size

Historically, plasma proteomic studies have had small sample sizes, typically less than 100. This can be attributed to difficulties in sourcing plasma samples with sufficient quality, high sample processing costs (e.g. depletion and fractionation), or even limitations in data acquisition throughput. More recently, efforts to generate large sample biobanks for proteomic analysis [18,19], the introduction of automated and high-throughput sample preparation workflows [20–22], and improvements in liquid chromatography are facilitating larger cohort studies[23]. Some developments combine rapid sample preparation protocols, multiplexing strategies, automated platforms, and optimized HPLC setups [20,23–25]. Beyond these technical aspects, there is a growing recognition that separating biological signal from sample variability often requires large sample cohorts. Accordingly, in an ideal situation, sample size itself would not be a limiting factor during the study design process. Impressively, this has allowed researchers to measure the proteome in cohorts of hundreds to one thousand samples [26–34].

While large sample sizes can facilitate better powered proteomic studies, they introduce additional experimental considerations to avoid the introduction of bias into data analysis. In particular, large sample numbers result in an increased data acquisition time, either on one or across multiple instruments. Appropriate design of technical and experimental considerations is required to group samples into processing batches in a balanced and randomized manner, minimizing the introduction of bias that could result from acquisition time, run order, operator, or instrument. Typically, a combination of instrument performance, sample-related variables (e.g. age of sample, inclusion order, time point of collection), and donor-related metadata (e.g. age, sex, ethnicity, disease state) are used to set the maximum number of samples within a processing batch, and the randomization of samples across those batches. When executed well, large-scale studies will shift research from small-scale discovery phase studies to the so-called "rectangular study designs," where large sample numbers can be analyzed in both the discovery and validation stages of biomarker research or related projects [3]. In this way, large cohort studies could enable a significant paradigm shift in the utility of plasma proteomics for clinical applications.

Cohort selection - age (adult vs. pediatric)

According to the 2019 Revision of World Population Prospects, 25% of the world's population will be under 15 years of age in 2020, 16% between 15 and 25, 50% between 25 and 64 years of age, and 10% above 65 years of age [35]. Despite this distribution, an often-underappreciated aspect of previous plasma proteomics studies is that a majority have focused on adults, with only a small proportion of published studies targeting children (approximately 0.6%). Researchers should keep this age balance in mind when selecting samples to include in their own study. This is especially important when considering the fact that early disease detection is critical for children, especially when trying to limit both the short- and long-term effects of a disease. Additionally, a recent proteogenomic study revealed that newborns have three times the number of unique proteins as their mothers, further suggesting that differences in the plasma proteomes between adults and children could lead to novel biological results [36]. Studies focusing on sick children are critical in understanding underlying population-specific pathophysiology and may help reduce the guesswork in medical interventions that are currently associated with drug dosages and predicted patient responses. A recent review by McCafferty et al. summarizes the 35 plasma proteomic studies focused on biomarker discovery in the pediatric population [34].

Cohort selection - reference samples

The selection of reference samples is an important, study-specific consideration for all plasma proteomic projects, and typically forms the basis for any comparative data analysis. For example, consider a study trying to identify plasma biomarkers of mild traumatic brain injury (mTBI) to identify those subjects who will have delayed outcomes. In this case, the ideal cohort consists of pre-trauma samples from all subjects to assess individual baseline values, the mTBI subjects who have delayed outcomes, and reference samples consisting of mTBI subjects who do not have delayed outcomes, matched for age, sex, and ethnicity. While this approach seems appropriate, it is important to recognize that epidemiological studies over the last several decades have revealed that there are sometimes additional variables to consider when matching study groups, such as medication, disease history, or state of hydration. Matching for these variables will reduce their impact on the bias in the data, and hence stratified randomization becomes a viable solution. However, some of these variables could remain unknown during the planning phase of the study (denoted "hidden variables"). Accounting for hidden variables is challenging, and sometimes the only course of action is with a sample size large enough that the study will be robust to these potential effects. Depending on the contribution of the experimental batches on the generated data, total randomization can be preferable over a stratified randomization.

In addition to reference samples for matching conditions/groups, the use of "healthy/normal" subjects as a reference group also needs to be made cautiously. First, a subject included in a "healthy/normal" group may not be representative of the general population or intended use population (in the case of clinical tests), as their selection and inclusion may be based on practical rather than clinically useful criteria. Second, if the "healthy/normal" group is only tested to be negative for the particular disease or condition being studied (e.g. mTBI without delayed outcomes) they could still be positive for other conditions. Consequently, the categorization as "diagnosis-free" may be more appropriate, as it implies that other diseases or conditions may still exist. One approach to mitigate against this risk in the comparative analysis between cases and controls is to compare within groups of cases (e.g. mild versus severe disease states), rather than those that would otherwise have no condition. For example, a study investigating biomarkers of aging may not need a specific negative control group; instead, study samples could be representative of the age spectrum in question and ensure that patient variables (e.g. sex and ethnicity) are balanced across those age groups.

Reference ranges

In addition to selecting the correct set of reference samples, understanding the range of expected values for the reference samples is an equally critical component of planning any plasma proteomics study. For example, the field of developmental proteomics has demonstrated that biological systems (e.g., hemostatic and inflammatory) in the healthy population undergo age-specific changes in protein expression, from neonates to adults [37,38]. This is particularly important in diagnostic testing and biomarker discovery applications, where adequate information about the "healthy/normal" group should be collected as a continuum from birth until adulthood. Moreover, protein expression-levels can be individual-specific, where they can be stable over time within an individual but very different between individuals [30,31]. To help control for this variability, it might be necessary to define individual-specific (often called "personalized") references values rather than population-level values [3,4]. This approach can be seen in longitudinal and multi-omics studies [39], where subjects are followed temporally and self-controlled, helping distinguish whether differences between study groups are due to a phenotype of interest or to other traits (e.g. medication, genetics, or lifestyle).

Plasma generation and quality metrics

Pre-analytical decisions can have a large impact on the quality and consistency of plasma proteomic data. It is therefore recommended that researchers obtain a clear understanding of how plasma samples were, or should be, collected, processed, and stored. During blood collection, factors that need to be considered or monitored include the phlebotomy procedure (e.g. needle gauge, number of times subject is exposed to blood sampling), the blood collection tube type (e.g. vacuum container, coagulation activators or inhibitors). Likewise, during the plasma processing step, factors that can affect quality include the centrifugation speed, duration, braking rate, and temperature; the delay from blood collection to plasma processing and storage; and where samples were collected.

Apart from the generation of plasma, storage conditions and the number of freeze-thaw cycles add further pre-analytical variables [40]. In particular, extensive delays of several hours prior to separating the blood cells from the fluid can alter the plasma proteome composition due to erythrocyte and platelet cell degradation [41]. Protein degradation may further occur due to instability or the action of proteases or loss of inhibitors (e.g. SERPINs). At the same time, other studies suggest that a large variety of plasma proteins are relatively stable to post-centrifugation delays and freeze-thaw cycles [42,43] which may indicate that sedimented cells or the use of gel plugs to keep the layer of cells apart from the fluid may be beneficial for protein detectability. Nevertheless, it is important to establish standard operating procedures (SOPs) and strictly follow them in order to minimize systematic pre-analytical variations that otherwise can result in significant changes in the plasma proteome.

In cases where retrospective sample selection is required, and the opportunity for strictly controlling the pre-analytical process for plasma generation is not possible, alternative strategies can be used to help minimize systematic bias within the study. In these "freezer studies", where samples are selected from biobanks around the world, it is necessary to evaluate the quality of available plasma samples. First, the centrifugation protocols for plasma generation should be reviewed because they directly influence the abundance and the types of cellular material that could be present in the plasma. Specifically, single-spun plasma typically contains many platelets, and these samples could be affected by the post-collection release of platelet-specific proteins. Alternatively, double-spun centrifugation protocols have the capacity to remove a significant number of contaminating cells, including platelets. In order to minimize the impact of post-collection platelet activation, double-spun plasma should be used whenever possible. Second, recently generated reference proteomes for erythrocytes and platelets and the comparison of plasma with serum resulted in three contamination panels against which one should benchmark samples[44]; high prevalence of cellular proteins might suggest contamination issues, and the sample can be flagged for further investigation.

Despite all efforts to control for pre-analytical variability, some degree of systematic bias is inevitable when working with human samples. However, such bias can be controlled through appropriate study design decisions to create randomized and appropriately balanced processing batches. Further, information about pre-analytical variables concerning the sample collection (e.g. needle-to-freezer time, timepoint of sample collection, center or geographical location of sampling) should be collected as a common procedure and considered as part of the data analysis and quality assurance process. Related issues with systematic bias are already known and controlled for in other proteomics applications, including large-scale affinity proteomics experiments[45,46].

Plasma is not equivalent to serum

The distinction between plasma and serum as different biological sample types is important when it comes to executing a proteomics study using blood-derived products[47]. For example, designing the right study plan, correctly interpreting the results, and appropriately comparing and contrasting results all require knowledge of the sample type used in the experiments. To clarify the distinction: plasma is obtained by centrifugation of whole blood, while serum is obtained after blood clotting and centrifugation. By removing the blood clot during the preparation of serum, some high abundance proteins such as fibrinogen will be drastically decreased in their concentration in serum but will be present in plasma, increasing the ability to detect some low abundance proteins in serum. At the same time, there are many proteins that are either actively involved in clot formation, nonspecifically adsorbed to clotting proteins, or randomly captured during clot formation [44,48]. For example, the process of whole blood coagulation induces protein secretion from platelets, amongst other cell types; inconsistencies in this process between samples can lead to false positives for differentially expressed proteins in serum. At the same time, clot formation and/or removal processes could be impacted by phenotype (e.g., disease or age), and therefore such differences could represent real biological signal.

While the differences between plasma and serum are largely appreciated in clinical practice, this is underestimated by the proteomics community, where the terms serum and plasma are sometimes used interchangeably and considered to

be comparable. However, understanding the factual differences between the two sample types is critical for the advancement of proteomics towards the clinic. Systematic comparison of plasma and serum by MS-based proteomics and affinity-based assays point out the clear differences between the actual composition of these blood preparation types [49,50].

Plasma processing workflows

Comprehensive characterization of the plasma proteome can be difficult. The large dynamic range of circulating proteins, combined with the diversity of known and unknown protein isoforms, can complicate any analytical method, including liquid chromatography coupled to mass spectrometry (LC-MS) [51]. However, motivated by the high value of identifying and characterizing plasma proteins, standard proteomics workflows have expanded to include prefractionation and labeling approaches to address these challenges [52–54].Here, we provide a brief overview of these pre-analytical methods and highlight some recent examples.

Depletion workflows

Although the abundances of plasma proteins span a large dynamic range, which can be greater than 10 orders of magnitude, albumin accounts for 50% of plasma proteins by weight; the top 22 abundant proteins account for 99% [51]. This characteristic can be utilized to improve detectability of low abundance proteins through the systematic depletion of high abundance proteins [55]. For example, immune-depletion spin columns, liquid chromatography columns, and magnetic beads have been developed to remove up to the 20 most abundant proteins [56–61]. This strategy has also been extended to deplete the moderately abundant proteins (e.g., Complement proteins, Fibronectin, and Plasminogen) using Affibody molecules, bead-bound peptide hexamers, or antibodies[62 57,63,64]. In addition to depletion strategies using affinity reagents, methods utilizing nanoparticles also exist [58,65,66]. These nanoparticles are designed to distinguish proteins based on their physical properties and can be tuned to both exclude (i.e., deplete albumin) and enrich (i.e., capture small proteins[67] or specific analytes) simultaneously. While depletion methods can help access lower abundance plasma proteins, using these reagents can lead to limitations, including increased sample handling, lower reproducibility and throughput, and carry-over concerns (i.e., when depleting multiple samples consecutively by reusing reagents)[58]. Moreover, it is important to recognize that many proteins are bound to albumin (albuminome) and other abundant proteins that have carrier functions, and removing these bound passenger proteins may result in off-target effects[63,64].

Fractionation workflows

An alternative strategy to affinity-based depletion for improved plasma protein detection is sample fractionation, which can also be used in combination with depletion strategies. Although depletion methods could be considered a form of fractionation (i.e., separating the high abundance from the low abundance components), in such workflows the "high abundance" component is treated as waste. Here, fractionation is defined as a method that divides all plasma proteins into usable groups for subsequent characterization. In one approach, two-dimensional liquid chromatography (2D-LC) can be used to divide one plasma sample into many, less complex fractions, whereby the standard low pH reversed-phase (RP) LC conditions applied in most LC-MS workflows is coupled with an orthogonal LC method. While early implementations of 2D-LC used strong cation exchange chromatography prior to standard RPLC methods (e.g., multidimensional protein identification technology, or MudPIT) [68], there has been a shift towards high pH reversed-phase chromatography [69,70]. In both cases, the specific number of fractions can vary, as well as how those fractions are combined, if at all, prior to LC-MS analysis [71]. Additionally, these methods can be executed on-line with a mass spectrometer, or off-line where samples are first fractionated and then fractions are separately subjected to LC-MS. Fractionation can also be achieved by gel electrophoresis, including in-gel digestion or as a desalting mechanism, where unwanted salts or materials are removed [72]. Recently, a high-resolution isoelectric focusing approach was developed and utilized for MS-based plasma analysis[36]. Regardless of the implementation, one general concern with fractionation-based methods is the increase in the number of samples to measure, as this scales with the chosen number of fractions. Consequently, fractionation methods create challenges with sample throughput and normalizing data across multiple LC-MS runs.

Enrichment workflows

In cases where comprehensive characterization of the entire plasma proteome is not necessary, a strategy around enrichment and concentration might be better suited. In this approach, workflows are used to increase the sensitivity for specific proteins of interest [59]. For example, methods have been developed to focus on specific sub-proteomes (plasma glycoproteome and phosphoproteome)[73–75], proteins that have a specific activity (cytokines for signaling), or those that originate from specific compartments (membrane proteins) [76]. In addition to functional-based enrichment, physical properties of proteins have also been utilized to increase target-protein concentrations [66,77]. For example, proteins can be selectively precipitated by salts or organic solvents [78]; separated by size using chromatography, dialysis, membrane filtration concentrators, or gel electrophoresis [77,79,80]; and further separated by charge (e.g., abundant glycosylation). Finally, single or multiplexed enrichment is possible using immunoaffinity reagents (e.g., antibodies, aptamers, or derivatives thereof), peptides, or chemical baits [81–83], and they can be used to identify individual proteins or groups of interacting proteins in plasma [84].

Quantification workflows

Quantification is a critical aspect of nearly all plasma proteomics studies, and it can be reported in either a relative or an absolute manner. The relative abundance of proteins is typically measured by label-free or isobaric labeling techniques. The most common classes of reagents for isobaric labeling are the tandem mass tags (TMTs)[85] and the isobaric tag for relative and absolute quantitation (iTRAQ) [86,87]. These tags permit multiplexing of several samples per LC-MS run, commonly 4, 6, 8, or 10 samples, but more are possible [88]. To learn more about the use of isobaric labeling for plasma proteomics, please see a recent review by Moulder et al.[89] as well as its recent applications [36,90,91]. Multiplexing methods help compensate for throughput concerns associated with large sample numbers and some plasma processing workflows (e.g., fractionation), enabling the combination of several samples into one [92,93]. At the same time, isobaric-based labeling reagents have some drawbacks, including the lowering of target sensitivity (i.e., the signal from individual samples can be diluted below the detection limit if some samples have low signal) and a reduction in quantitation accuracy (i.e., co-isolated peptides can interfere with correct quantitation due to the ratio distortion phenomenon, also known as "ratio compression"). In the latter case, there are options available to help minimize the impact of ratio compression through software correction, narrow isolation windows, further fragmentation of the peptide fragment ions, and novel isobaric tags [94–96].

Relative protein abundance can also be measured using label-free techniques. While this approach does not require any additional reagents or sample processing steps, it does not allow multiplexing of samples. In contrast to both of these methods, the absolute abundance of specific target proteins can be determined by spiking heavy-labeled reference peptides or proteins at a known concentration. Such spike-ins are an attractive concept to serve as a reference between studies, instruments and laboratories. The reference can derive from either peptides or proteins and be added prior to or after digestion of the proteins. The utility of these concepts have recently been demonstrated by selecting suitable standards from a large library of protein fragments [97], or by spiking in peptides in an approach using stable isotope standards and capture by anti-peptide antibodies (SISCAPA) [98].

General workflow considerations

With any sample processing workflow, characterizing its technical performance is as important as the potential value that the method may afford. All too often, one method may work well in the hands of one researcher but cannot be reproduced by another researcher. This has had the unfortunate consequence of leading to opposing or orthogonal workflows for similar goals, making it harder for untrained experts to make informed decisions when implementing plasma proteomics workflows in their own labs. Additionally, the lack of technical performance information has led to assays with low reproducibility, resulting in hard-to-replicate results. Researchers should consider incorporating broader technical performance characterization into their method development process, and should understand features such as multi-laboratory repeatability and identify common sources of confounding in their method of choice [99,100]. This information can help the plasma proteomics community move towards the ultimate goal of improved plasma characterization for applications in human health and biology.

Data acquisition by mass spectrometry

There are broadly two approaches to measure peptides in plasma via MS: targeted and untargeted workflows.

Targeted plasma proteomics

Selected reaction monitoring (SRM) is the typical targeted approach, wherein target peptides must be selected in advance and the instrument programmed with the expected signatures of those peptides, thus enabling the measurement of relative ion abundances or upper limits for each of the desired targets in every sample[101]. Such an approach does require a potentially time-intensive, up-front process of initial target selection and signature transition optimization, although resources such as SRMAtlas [102] enable rapid selection of target peptides and their signatures. Targeting of peptides in plasma can be quite challenging since plasma is a very high dynamic range and complex background in which the target peptides must undergo careful validation procedures to be confirmed and quantified, as demonstrated by the SpecTRA study group[103]. A popular strategy relies on the use of spiked-in stable isotope standards (SIS) as reference peptides to help ensure the correct molecules are being identified[104]. Also, Carr et al. have proposed an important set of guidelines, organized by three tiers of rigor, for the application of SRM to biological samples[105]. In general, targeted proteomics is the method of choice when the number of analytes is relatively small and known in advance, and quantitative measurements are a crucial requirement of the experiment.

Untargeted plasma proteomics

For untargeted proteomics, there are two broad approaches: data-dependent acquisition (DDA) and data-independent acquisition (DIA). In DDA workflows, the mass spectrometer acquires survey scans to assess which precursor ions are currently entering the instrument, and then sequentially selects several of them to isolate and fragment in turn [106]. The fragmentation spectrum ideally contains the fragments of just a single precursor. In the DIA workflow (such as with SWATH-MS[107]), the instrument usually also acquires survey scans every few seconds, but then in between scans it steps through a series of selection windows, often 25 m/z units wide, producing fragmentation spectra of all precursors in the wide window multiplexed together [108]. The advantage of the DIA workflow is that the fragmentation patterns of all precursor ions within the selected mass ranges are recorded, unlike for DDA, wherein fragmentation data is only collected for selected precursors in a semi-stochastic manner. The disadvantage of the DIA workflow is that more complex and less mature software is required to demultiplex the very dense fragmentation spectra. However, the substantial advantage is that with a successful analysis there are typically far fewer missing values in the final data matrix. An additional substantial difference is that while DDA workflows are amenable to isobaric labelling, DIA workflows typically rely on label-free quantitation. The potential of both approaches has recently been demonstrated for plasma analysis using isobaric labeled DDA[93] and in studies using DIA[27,29–31,109].

Data processing and bioinformatic analysis

The data generated by mass spectrometers is generally quite complex and requires substantial downstream analysis with sophisticated software tools[110]. However, as far as these software tools are concerned, analysis of data sets derived from plasma samples does not differ substantially from that of other sample types, such as tissue or urine. For SRM data analysis, in addition to vendor-provided tools, Skyline[111] dominates the free and open-source software field. For DDA data analysis, there are many analysis tools available, including MASCOT [112], SEQUEST [113], MaxQuant [114], and X!Tandem [115], just to name a few; please see a recent review by Nesvizhskii et al. [116] for a more comprehensive list. For DIA data analysis, the options are far fewer than for DDA, with OpenSWATH [117], Spectronaut [118], PeakView, and DIA-Umpire [119] as the most frequently used tools. For most SRM and DIA analysis workflows, the tools for identification and quantitation are integrated and work together by default. For many DDA data analysis workflows, the identification and quantitation components are separate tools and the compatibility of those tools is important.

The proteomics community has already done a great job of lowering barriers and working towards freely accessible data in public databases. This approach to openness should continue. It is now common to deposit proteomics datasets in data repositories, most of which are members of the ProteomeXchange Consortium[120,121]. ProteomeXchange sets basic standards and minimum requirements for its members and fosters similar submission and dissemination policies. The main repositories of ProteomeXchange are PRIDE[122], PeptideAtlas[123,124] (with its SRM component PASSEL[125]), MassIVE[126], jPOST[127], iProX[128], and Panorama Public[129]. Researchers are encouraged not only to deposit their final datasets in a ProteomeXchange repository, but also to consider downloading and examining previously downloaded and generated datasets to inform the generation of their own data.

There are various formal guidelines that should be followed when submitting manuscripts describing a plasma analysis depending on the type of data and publication. Some journals have their own specific sets of guidelines, such as for the Journal of Proteome Research and Molecular and Cellular Proteomics [130]. Contributions as part of the Human Proteome Project (HPP) must follow the HPP MS Data Interpretation Guidelines [131]. Other guidelines are applicable to certain workflows, such as for DIA data [132] and targeted SRM data [105]. It is well worth preparing for the relevant guidelines in advance of data analysis, since complying with some guidelines after an analysis is complete may require redoing some of the work.

Conclusion

Here we summarize several key aspects about performing plasma proteomics experiments using MS. We provide insights into current capabilities but also raise awareness about the challenges that remain to be addressed. This complements other reviews on MS-based plasma proteomics and its route towards greater translational utility[3,14]. From the perspective of "How should you perform your plasma proteomics experiment?", we discuss several design elements that are often omitted when focusing on improving the technology rather than its application. While there is not a single "correct" way of performing plasma proteomics, comparative analysis of different methods, such as those proposed for antibody validation[133], would be a valuable path forward. In addition, we suggest reviewing the list of 1000 "popular" plasma proteins that can be detected by MS and affinity-based methods [15].

Ultimately, the usefulness of proteomics as a methodological approach is dependent on its clinical applicability as a tool to improve patient outcomes. Unfortunately, plasma proteomic studies have, for the most part, focused more on identifying the largest number of proteins rather than focusing on the proteins that can be detected consistently and that have a clinical utility (e.g., predicting a clinical outcome). This raises a couple of questions: Are we, as proteomics community, cooperating enough towards the common goal on translating plasma proteomics across research labs and into the clinics? Are we aware of the issues faced in the clinical setting and do we understand how proteomics can assist [134]? Is this the reason why proteomics is not as advanced as genomics or transcriptomics when it comes to translational research and clinical utility? Considering the technological advances in proteomics that are actionable from a clinical perspective is certainly a key component in getting proteomics into the clinic, and doing so more quickly and effectively. There is no question that plasma proteomics can have a clear and significant impact on improving clinical diagnostics.

The future of plasma proteomics in the context of diagnostic laboratories is highly reliant on knowledge of the normal, age-specific expression ranges for plasma proteins and their use for accurate diagnosis for our population as a whole. With advanced research in this field, plasma proteomics can provide a reliable, efficient, and highly capable approach to take proteomics to the clinic, to drive a truly personalized medicine experience, and, most importantly, to contribute to human health. While capitalizing from the rapid advances in mass spectrometry, a greater diversity of data from well-designed biomarker discovery and validation studies will become available. Hence, plasma proteomics is well on the way to developing a robust set tools for quantifying proteins across major diseases that will be translated into robust assays made available to diagnostic laboratories.

Notes

Krishnan K. Palaniappan is an employee of Freenome. All other authors declare no competing financial interest.

Acknowledgements

We thank the community for the providing open access data. We thank Conor McCafferty for assistance with this manuscript. This work was funded in part by the National Institutes of Health grants R01GM087221 (EWD), R24GM127667 (EWD), U54ES017885 (GSO), and U24CA210967-01 (GSO). JMS acknowledges support from The KTH Center for Applied Precision Medicine, funded by the Erling-Persson Family Foundation grant for Science for Life Laboratory, and the Knut and Alice Wallenberg Foundation. PEG acknowledges support from the Max Planck Society for the Advancement of Science and by the Novo Nordisk Foundation (grant NNF15CC0001).

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