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STATE OF THE ART ISTH 2021

Proteomics in thrombosis research

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Abstract

A State of the Art lecture titled "Proteomics in Thrombosis Research" was presented at the ISTH Congress in 2021. In clinical practice, there is a need for improved plasma biomarker-based tools for diagnosis and risk prediction of venous thromboembolism (VTE). Analysis of blood, to identify plasma proteins with potential utility for such tools, could enable an individualized approach to treatment and prevention. Technological advances to study the plasma proteome on a large scale allows broad screening for the identification of novel plasma biomarkers, both by targeted and nontargeted proteomics methods. However, assay limitations need to be considered when interpreting results, with orthogonal validation required before conclusions are drawn. Here, we review and provide perspectives on the application of affinity- and mass spectrometry-based methods for the identification and analysis of plasma protein biomarkers, with potential application in the field of VTE. We also provide a future perspective on discovery strategies and emerging technologies for targeted proteomics in thrombosis research. Finally, we summarize relevant new data on this topic, presented during the 2021 ISTH Congress.

KEYWORDS
biomarker, mass spectrometry, plasma protein, proteome, proteomics, thrombosis, venous thromboembolism, VTE

Essentials

- There is a need for novel plasma biomarkers for diagnosis and risk prediction of thrombosis.
- Recent technology developments have enabled large scale analysis of plasma protein profiles.
- We describe current and emerging technologies and strategies for identification of biomarkers.
- We review reports where these technologies are applied in the field of thrombosis research.

1 | INTRODUCTION

Venous thromboembolism (VTE), comprising both deep vein thrombosis (DVT) and pulmonary embolism (PE) is a common, multicausal disease with serious short-term and long-term complications. It is associated with high mortality in the first year, especially within the first 30 days (~30% for PE) and a high risk of recurrence, with a cumulative incidence rate of 25% within 10 years.[1-4] There is a need for better clinical tools for diagnosis and risk prediction of VTE; identification of VTE specific plasma biomarkers that could be routinely analyzed within existing clinical settings could transform clinical management. However, in contrast to acute coronary syndrome and heart failure, where plasma biomarkers such as high sensitivity troponin and proBNP represent the cornerstone in diagnosis and clinical decision making, biomarkers with similar characteristics in VTE remain to be identified. D-dimer is the only established biomarker in clinical management routines, but it is not specific for VTE. Because the predisposing common risk factors and clinical presentation of VTE are consistent with multiple other conditions, particularly in the case of PE, diagnosis of acute VTE can represent a clinical challenge. Current VTE diagnostic workup includes assessment of clinical probability (e.g., using the Well score) in combination with measurement of the plasma biomarker D-dimers.[5,6] Because of its low specificity for VTE, D-dimer is limited to ruling out VTE in low-probability cases, whereas diagnostic imaging is necessary to rule out or confirm diagnosis in medium- or high-probability cases. With less than 20% of computed tomography pulmonary angiograms performed on suspicion of PE confirming the diagnosis,[7-9] more specific biomarker-based tools implemented in the diagnostic workup have potential to reduce unnecessary imaging. Several studies have proposed biomarker candidates for acute VTE (e.g., p-selectin, microRNAs),[10,11] but none have yet translated to clinical implementation. Prediction of recurrence represents another clinical challenge where improved biomarker-based tools could facilitate treatment decisions for the individual patient (e.g., length of anticoagulant treatment). Risk scores based on D-dimer levels together with clinical risk factors have been developed for prediction for risk of recurrence,[12-16] but, again, none are yet routinely integrated into clinical practice. When incorporating genetic variants contributing to VTE into such risk scores, including recently discovered common genetic variants,[17,18] they still lack sufficient precision for individual risk prediction.[19,20] This likely reflects the interplay between persistent and transient risk factors in VTE development, including genetics, acquired risk factors, and environmental exposures.[21]

Blood plasma is an easily obtainable sample for analysis in a clinical setting that reflects active secretion, release, shedding, or leakage from cells and tissues in direct or indirect contact with blood. Because VTE is a disease of the intravascular compartment, the blood proteome could reflect combined environmental, genetic, and epigenetic contributors to risk variation between individuals. However, compared with other disease states, a limited number of plasma proteomics studies has been reported, where novel biomarker candidates for VTE are identified.[22-29] Today, several of the plasma proteins reportedly associated with VTE, and subsequently evaluated when incorporated into risk scores, have emerged from hypothesis driven analysis of a single, or limited set, of proteins based on known links to thrombosis (e.g., P-selectin[30]). Systematic profiling of a larger portion of circulating plasma proteins could lead to discovery of novel protein biomarkers with potential clinical utility for VTE diagnosis and/or prediction. Recent technology developments in the proteomics field have made such high-throughput screening more accessible through commercial systems available as fee for service at core facilities and/or through commercial providers. Here, we will provide an overview of existing and emerging technologies and review the current literature on their relevance in VTE research and provide future perspectives.

2 | TECHNOLOGIES FOR PLASMA PROTEOMICS

Blood plasma is complex to analyze because of the very large concentration range (10^{12}) between most abundant (e.g., albumin, immunoglobulins) and least abundant (e.g., interluekin-6, interferon gamma) proteins. Indeed, less than 1% of the individual proteins found in plasma constitute more than 90% of the total protein concentration.[31-34] Thus, analysis of the full protein profile in plasma, the circulating proteome, remains a challenge with currently available technologies. Today, analysis of the plasma proteome is typically performed using mass spectrometry (MS) or affinity-based methods. MS-based methods inherently have a high specificity for detected proteins through direct identification of generated peptides, but the detection of low-abundance proteins remains challenging, and practical aspects limit sample throughput capacity. Affinity-based methods have gained popularity because of greater throughput capacity, multiplexity, small sample size, and reduced processing requirement (e.g., high abundance protein depletion or fractionation is not required), and a higher capacity than MS-based methods for the detection of low-abundance proteins.[33,35,36] Here, we will discuss the

different approaches, their associated limitations, and the degree of consistency between the described platforms.

2.1 | Affinity proteomics: Technologies

Affinity proteomics is based on the use of molecules (antibodies or aptamers) with specific binding capability to proteins of interest. Over the years, affinity-based assays have moved from the detection a single protein at a time, as in Western blot or enzyme-linked immunosorbent assay (ELISA), to the measurement of large protein panels for the identification of signatures associated with specific disease conditions or health status.[31,33,35,37] Here, we discuss the three in-solution technology platforms for large-scale screening of biomarker candidates that, to date, have been most commonly used for plasma proteomic analysis: the antibody-based suspension bead array (SBA),[38] the proximity extension assay (PEA),[39] and the aptamer-based SomaScan assay.[40] All three platforms allow for highly multiplexed profiling of proteins in many samples using a plate-based format. The conceptual principles of these methods are shown in Figure 1.

The SBA technology is run on a commercial platform provided by Luminex Corp. (Austin, Texas, USA), which measures relative protein plasma levels using a flow cytometer-based analysis of multiplexed suspension bead arrays of protein-specific antibodies (Figure 1A). The Luminex platform (FlexMAP3D) can analyze up to 500 proteins in 384 samples, using as little as 3 µl of plasma per sample run. However, reliability depends on the specificity and functionality of antibodies used and any findings should be orthogonally validated. Advantages, compared with the current PEA and aptamer-based platforms, include full flexibility in designing custom antibody panels in a scalable fashion.[33,41] This technology platform has been used in proteomics studies for health assessment and various disease states,[33,42-44] including VTE.[22,23]

The PEA technology platform (Olink Proteomics AB, Uppsala, Sweden) uses selected paired antibodies, labeled with complementary DNA linkers, for the recognition of proteins (Figure 1B). A semiquantitative signal readout is generated either by quantitative polymerase chain reaction[39,45,46] or sequencing.[47] Conceptually, the application of dual antibodies targeting different neighboring epitopes increases specificity and reduces the risk that the signal reflects off-target binding. The unique sequences of the DNA linkers attached to the antibodies allow for a high level of multiplexing without the extensive cross-reactivity that would occur using conventional ELISAs. Still, differences in amplification efficacy and lack of quantitative standards mean that the measurements are semiquantitative and can only be used for relative comparisons of protein levels. PEA is commercially available as a high-throughput platform in a 96-plate format with multiplex immunoassays panels containing 48, 92, or 384 markers that are measured simultaneously. The potential for agnostic interrogation of the plasma proteome to discover entirely novel biomarkers is somewhat limited by the reliance on predefined panels designed based on prior knowledge. Currently, Olink offers 15 target panels (up to 92 protein markers/panel) designed as "disease oriented" (e.g., cardiovascular panels) or pathway oriented (e.g., immune response, "cardiometabolic"). There is not a panel designed with a specific thrombosis/hemostasis focus, and a comprehensive interrogation of pathways and systems with known role or relevance for thrombosis requires the analysis of several different panels in combination (e.g., cardiovascular disease, cardiometabolic, cytokines). Four larger "exploratory panels" targeting approximately 1500 unique proteins have become available, with a focus on cardiometabolic-, oncology-, neurology-, and inflammation-associated biomarkers, and more recently a combined Explore 3072 panel targeting approximately 2950 unique proteins has been launched. This technology has been applied to biomarker profiling studies of VTE.[24-26]

The aptamer-based SomaScan technology platform (Somalogic, Boulder, Colorado, USA) has emerged as an attractive alternative to antibody-based approaches[48](Figure 1C). Whereas, like Olink, it relies on predefined panels, the most recent Somalogic platform v4.1, with a library of SOMAmers targeting roughly 7000 unique proteins, conceptually allows for a semiagnostic interrogation of the plasma proteome using only 55 µl of plasma, potentially revealing novel pathways. With increasing numbers of studies using this platform, limitations inherent to the technology is attracting attention.[49] Similar to SBA, it is a single binder assay in which findings should be orthogonally validated. Furthermore, compared with antibody-based assays, aptamer-based assays are likely more vulnerable to nonspecific protein binding and to the effect of missense single nucleotide polymorphisms because of the resultant modification in electric charge (through amino acid substitutions), which can affect binding of the negatively charged aptamers.[50] This was recently demonstrated for a protein-altering variant in the soluble urokinase plasminogen activator receptor, where results were strongly influenced by an epitope-binding artefact when using an aptamer assay.[51] Pairwise comparisons of protein measurements with SomaScan and conventional immunoassays have shown highly variable correlations,[52] with some reporting poor agreement with standardized assays on validated laboratory instruments for established VTE-associated biomarkers, such as D-dimer.[53]

2.2 | Affinity proteomics: Considerations

In affinity proteomics, the signals generated cannot automatically be assumed to reflect target protein abundance because of potential for interference with the target/binder interaction (Figure 2).[54] Antigen occlusion through posttranslational modifications, complex formation, differential splicing, or single nucleotide polymorphisms in the binding epitope can affect signal strength. Missense mutations directly affecting epitopes, or protein structure and binding accessibility, can affect the protein recognition in a manner in which genetic differences drive the associations, rather than protein levels.[55,56] In addition, the measured signal can be generated by off-target low-affinity binding of a high abundant protein, outcompeting

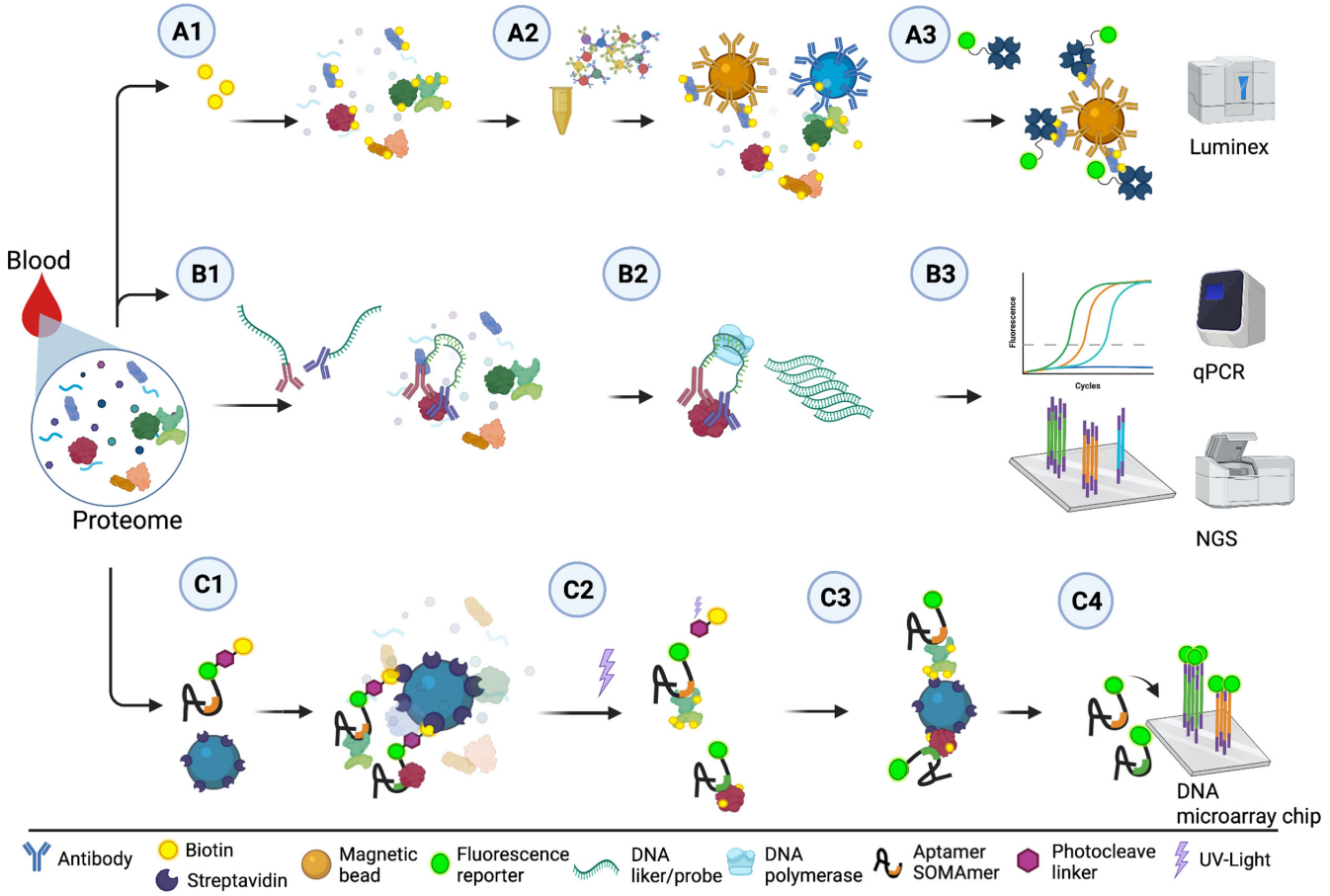

FIGURE 1 Affinity proteomics technologies. (A) Suspension bead array assay (Luminex): All proteins within the plasma sample are labelled with biotin for subsequent detection (A1) before incubation with a suspension of multiplexed protein-specific antibodies, each coupled to a unique color-coded micrometer-sized bead (A2). Unbound proteins are removed by washing and fluorescent-labeled-streptavidin is added to detect the protein biotin tag (A3). The suspension is analyzed by a cytometry-based instrument (Luminex), where the color of the antibody-bound bead provides the antibody identification, and the mean fluorescence intensity provides a relative measure of the corresponding target protein levels. (B) Proximity extension assay (Olink): for each biomarker, a matched pair of antibodies recognizing neighboring epitopes on the same target protein are linked to unique complementary oligonucleotides. Multiplex panels of matched antibody pairs are added to plasma (B1). When binding in close proximity on the protein target, the stretch of nucleotides hybridizes. DNA polymerase is added, and the annealing product is extended and amplified (B2) and detected by either quantitative polymerase chain reaction or next-generation sequencing (B3). The readout provides a relative measure of respective protein level in plasma. (C) SomaScan Assay (SomaLogic): for each biomarker, a single-stranded oligonucleotide, aptamer, folded into a tertiary structure, binds the target protein with affinity and specificity comparable to antibodies. The SomaScan platform is based on slow off-rate modified DNA aptamers (SOMAmers) labeled with a fluorescence reporter, photocleavage linker, and biotin. Multiplex panels of SOMAmers together with streptavidin coated beads bind respective target proteins in plasma (C1). Following washing to remove unbound proteins, fluorescent-labeled aptamer-protein complexes are released from the beads by ultraviolet-induced photocleavage, labeled with biotin (C2) and captured on fresh streptavidin-coated beads added to the sample, followed by washing to remove nonspecifically bound aptamers (C3). Protein-bound aptamers are released in denaturing buffer and detected by hybridization to complementary oligonucleotide probes on a microarray chip with the fluorescence intensity (C4) providing a relative measure of respective protein level in plasma. Figure created with BioRender.com

the true target.[33] Compared with PEA/Olink, which rely on dual antibodies targeting two different epitopes, both SBA and SomaScan are conceptually more vulnerable to reporting off-target binding effects. Such effects can be highly platform and binder dependent because binders may target different regions of the protein and/or be differently affected by changes in protein structure. Furthermore, neither the SBA, PEA, or SomaScan technologies provide absolute quantitative protein concentrations, only relative values, and each on different scales. Thus, individual signals cannot be directly compared between different studies, even when assayed with the same assay panels. Therefore, for results obtained with affinity proteomics, orthogonal quantitative measurements (e.g., conventional immunoassays, targeted quantitative MS) are needed to confirm an association between protein level and disease/phenotype. For example, immunoaffinity pulldown where the binder is coupled to magnetic beads and used to pull down the target from plasma before it is eluted, digested, and analyzed by liquid chromatography (LC)-MS, a method called immunocapture MS (IC-MS), can be

used to technically validate the target of the binder (Figure 3).[54,57] This can detect coenrichment of the target protein with other proteins, which can reflect complex binding with the intended target, cross-reactivity, or parallel on-target and off-target binding. Thus, identification of the intended target in IC-MS data does not exclude that a nontarget protein contributes to the signal associated to a trait or phenotype.

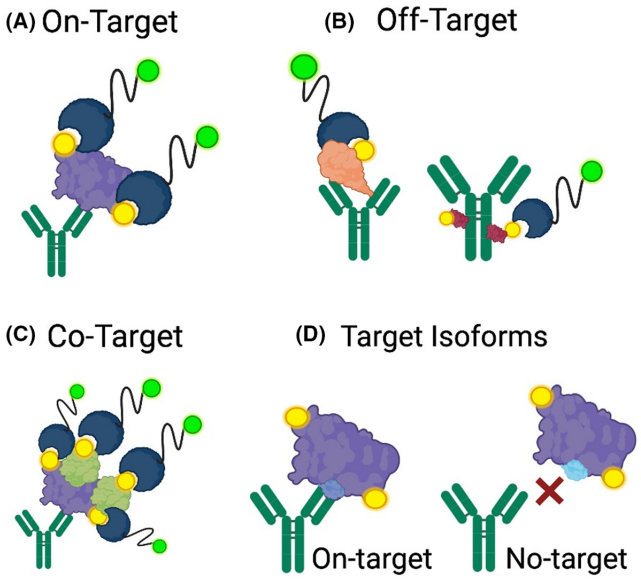

FIGURE 2 Representation of the possible binding scenarios for protein binding to the affinity reagent in a complex plasma matrix. (A) On-target: the antibody specifically binds the intended target protein. (B) Co-target: the antibody specifically binds the intended target protein in a protein complex. (C) Off-target: the antibody binds a nontargeted protein, either through binding to an epitope with similar linear amino acid sequence or conformational epitope structure, or a background signal is generated by unspecific binding. (D) Target isoforms: the antibody binds only one isoform of the intended target protein. This can occur when a coding genetic variation (missense single nucleotide polymorphism) cause an amino acid substitution in the epitope, or changes conformation. Figure created with BioRender.com

2.3 | Mass spectrometry-based proteomics: Technologies

The most commonly used method for peptide identification when using MS is the shotgun proteomics strategy, which often is used for discovery studies. This unbiased method can measure more than 1000 proteins in plasma from single samples, using a peptide prefractionation strategy.[58] Discovery workflows allow researchers to profile proteins in numerous samples in an unbiased manner and to compare differentially abundant proteins between samples. However, the accuracy and precision of label-free shotgun proteomics methods are often affected by analytical biases introduced during the sample preparation process. Small deviations and uncertainties in chromatography and ionization, in combination with the highly complex nature of clinical samples, make the precursor selection process complex and a source of error affecting the quantitative performance. It is often described as a stochastic process when selecting which ion to select and identify through tandem MS (MS/MS), making peptide identification and quantification inherently difficult to reproduce.[59-61] Therefore, data-independent or targeted proteomics approaches have become an attractive alternative to the shotgun method for validation studies where the same set of proteins are quantified across hundreds of samples. The data-independent acquisition,[62,63] or sequential window acquisition of all theoretical fragment ion spectra MS,[64] was introduced to improve the quantitative accuracy of peptide quantification across many samples. This is a step toward the

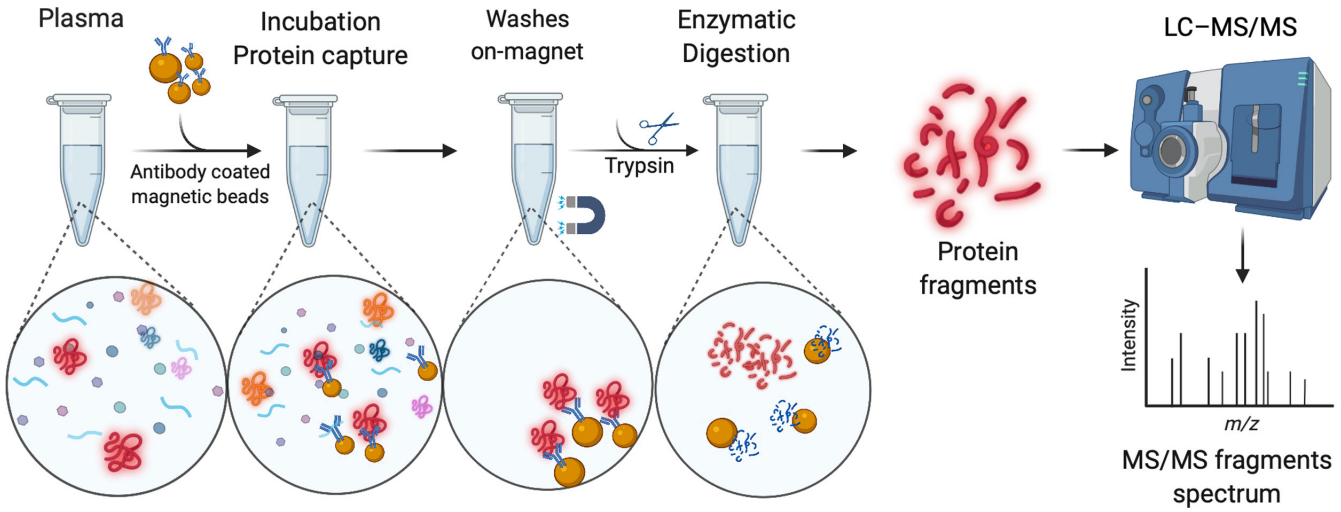

FIGURE 3 Workflow for ImmunoCapture-mass spectrometry (IC-MS). Plasma samples are incubated with magnetic beads coupled with the affinity reagent (e.g., antibody). Beads with captured proteins are separated by applying a magnetic field, and nonbound proteins removed through sequential washes. Proteins bound to the antibody-coupled beads are digested with trypsin, and after bead removal, the resulting sample with peptide fragments are injected into liquid chromatography tandem MS for analysis. Figure created with BioRender.com

rationale behind targeted proteomes, where specific and well-characterized ions are used to quantify a predefined set of peptides. One of these more targeted methods is the parallel reaction monitoring (PRM) ion sampling strategy, in which targeted acquisition is performed to simultaneously analyze all fragment ions of a preselected list of peptides.[65,66] The more stringent type of targeted proteomics method is the selective reaction monitoring (SRM) strategy.[67] The SRM method demands a lot of preanalytical work spent at the assay generation stage, but when optimized, the assay can deliver excellent quantitative performance. The analytical method shows the highest degree of assay linearity, low limit of quantification, and high repeatability, which is in accordance with many existing clinical assays, making SRM particularly useful when studying a predefined set of proteins.[67,68] To achieve absolute quantification, an accurately determined amount of a standard peptide (either as a peptide or as part of a protein), which is an isotopologue of the endogenous analyte of interest, is added to the sample as detailed previously.

Targeted proteomics is a powerful MS-based technique used for high-quality protein quantification in complex matrices, such as human blood plasma.[69] The method is an attractive alternative to many affinity-based methods because it can quantify proteins of interest without the need for any affinity reagent. The analytical strategy provides both high sensitivity, reproducibility, and quantitative performance over a broad dynamic range.[70] Recent efforts of standardizing bottom-up proteomics workflows, where proteins are digested into peptides by trypsin, has enabled characterization and identification of hundreds of proteins in undeleted human plasma, including many Food and Drug Administration-approved biomarkers.[71-76] This, in combination with the unsurpassed specificity of mass spectrometers,[77] over many other molecular technologies, has made it an attractive choice for precision medicine and future diagnostic applications.

Stable Isotope Standards (SIS), in the form of either peptides or proteins, can be added either before or after the proteolytic digestion step that facilitates quantification of the target peptide analyte. The peptides selected as reference standards must be unique to the protein of interest and suitable for quantification. The SIS peptide and the peptides originating from the endogenous protein behave identically throughout the sample preparation and so the relative ratio provides quantitative information because the peptides can be distinguished by the mass spectrometer. The absolute concentration of the target protein can be calculated using the ratio of heavy (standard) and light (target) peptides, and can potentially replace traditional serology, using only 1 ml of input plasma. The use of SRM to quantify proteins in blood plasma generally range from 31 mg/ml for albumin down to 18 ng/ml for peroxidredoxin-2.[78] As many as 267 proteins have been quantified in multiplex by SRM, including 61 Food and Drug Administration-approved targets.[79] Mohammed et al.[80] developed a multiplexed PRM based quantification of a panel of 31 coagulation and hemostasis markers and found good agreement with results from conventional standardized laboratory assays.

2.4 | Mass spectrometry-based proteomics: Considerations

Despite the identification of many possible biomarkers from studies using quantitative MS-based proteomics experiments, very few have made it to the clinic. Many quantitative, bottom-up proteomics workflows are affected by biases introduced throughout the sample preparation process, which includes protein denaturation, reduction, alkylation, digestion, and peptide separation, taking place upfront the mass analyzer.[61] This can be minimized through strict, standardized approaches[81] to limit variation and ensure high statistical significance when evaluating biomarkers of interest.[82,83] However, systematic biases are introduced and are often accounted for by the introduction of stable isotope standards that are added directly to the sample of interest, which will act as a reference point of calibration. Addition at the peptide level cannot account for differences introduced at the digestion step,[84] but alternative strategies have been implemented. SIS guidelines can combat this, including flanked peptides (cleavable site), either by extension or by combining multiple protein targets combined into a single recombinant protein fragment (Quantification concatemer, QconCAT).[85] The most reproducible, but most expensive, alternative is a full-length SIS recombinant protein[86] (Figure 4A). This performance can be mimicked by the QPrESTs technology[87] where a shorter, 50 to 150 amino acid sequence that contains prototypic peptides can be spiked to the plasma instead (Figure 4B). In contrast to spiked peptides, spiked SIS proteins or protein fragments generate multiple proteotypic peptides to be added before the trypsin cleavage, ensuring that uncleaved endogenous peptides will not affect the quantification, as long as the digestion efficiency of the protein standard is the same as that of the endogenous protein target. Addition of PrESTs show very robust with interday coefficient of variations around 5%.[88] The PrEST technology has been used to quantify more than 100 proteins in human plasma[89] with addition-only protocols. This has introduced a new and more precise quantification rationale of proteins present in body fluids with good quantitative accuracy (<15%)[90] if compared with fully labeled protein standards.

2.5 | Comparisons of plasma proteomics assays in clinic study sample sets

Multiple studies have observed variable degrees of agreement between protein quantification in identical samples using different high-throughput proteomics platforms or immunoassays (conventional single-protein ELISA and optimized multiplexed assays).[50,52,91,92]

Liu et al.[52] compared concordance between SomaScan and conventional immunoassays in two sets of samples obtained from the same 294 subjects undergoing cardiac surgery (pre- and postoperatively). For 26 proteins measured both by immunoassay and SomaScan assay, 20% and 35%, respectively, showed high Spearman correlation ($rs \geq 0.75$), whereas 53% and 41% had low correlation

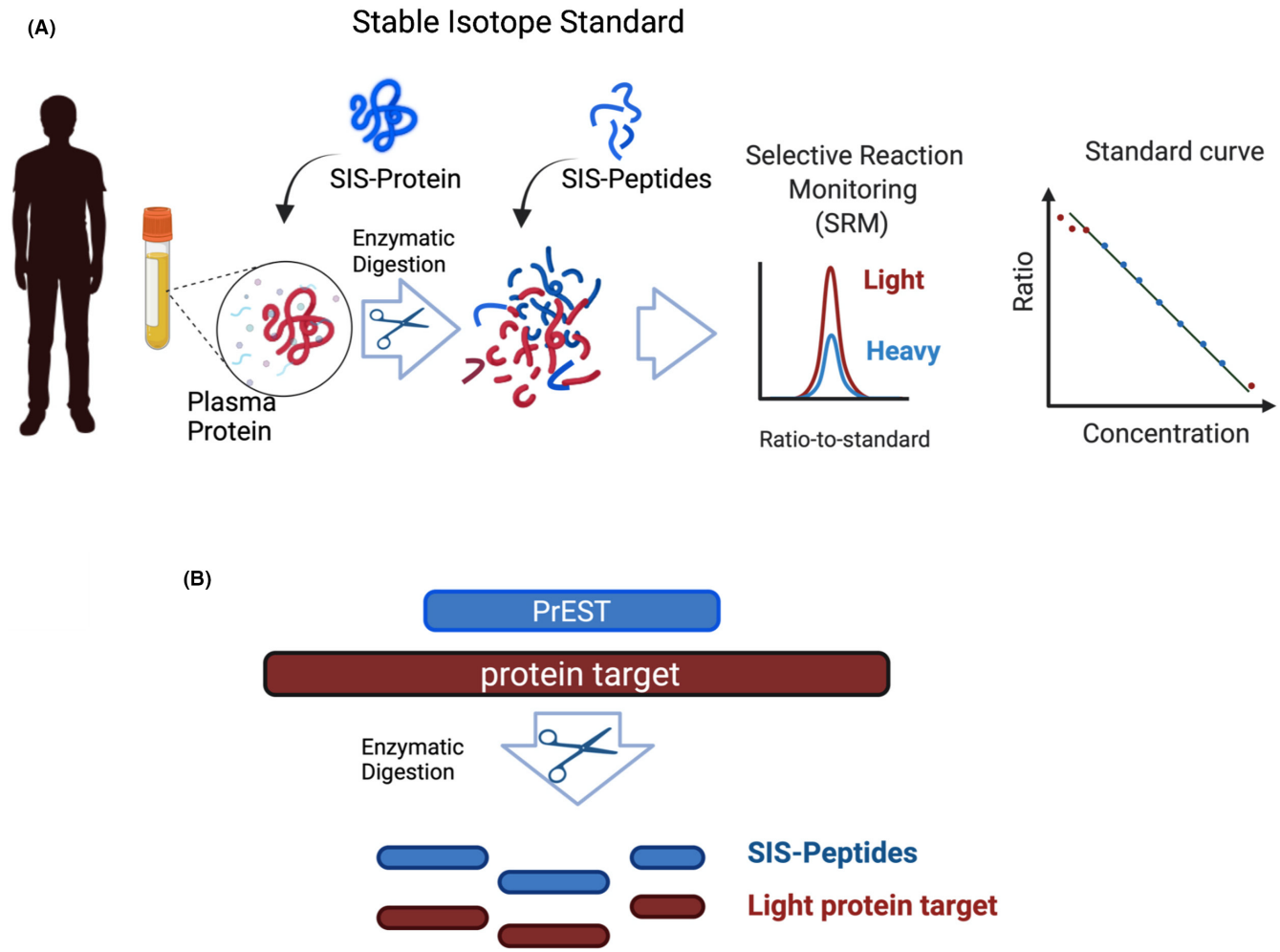

FIGURE 4 Targeted proteomics experiment using either spiked protein or peptide standards. (A) Stable isotope standards (SIS), incorporating an amino acid labeled with the stable isotopes, are added directly to the plasma sample prior to digestion. Alternatively, SIS peptides can be added upfront to liquid chromatography tandem mass spectrometry analysis. Target peptides are eluted and measured using selective reaction monitoring analysis. The endogenous (light) peptide concentration is calculated as a ratio between the heavy standard peptides of known concentration. A separate standard curve is established to define the dynamic range, limit of detection, and limit of quantification. (B) Illustration of the principle of SIS recombinant proteins (PrEST) as internal standards. SIS PrEST have amino acid sequences that uniquely align to the endogenous target protein. On tryptic digestion, proteotypic peptide fragments are released from the SIS PrEST and its target and the standard can be added upfront enzymatic digestion

(*rs* < 0.5), respectively, in the two sample sets. They also tested for association with acute kidney injury (AKI) using both methods; only about one-half of the AKI associations found using immunoassay data were replicated in the SomaScan data, with statistically significant differences in odds ratio for paired proteins generally found in the group of markers with low or medium correlations *rs* < 0.75. Strong interplatform correlations and more consistent biomarker AKI odds ratio tended to be observed when biomarkers had a higher plasma concentration,[52] suggesting that these platforms are more likely to replicate with concordance for abundant proteins, rather than those with than low abundance.

Raffield et al.[91] compared overlapping sets of analytes analyzed with Olink platform and the SOMAscan 1.1k array, in the same set of 48 samples from a cohort of 10 myocardial infarction patients. For the 425 proteins measurements obtained in both platforms, Spearman correlation ranged from −0.58 to 0.93; only 56 (13%) proteins were highly correlated (*rs* ≥ 0.7), whereas 179 (42%) were poorly correlated (*rs* < 0.3). They also compared data for a set of 63 proteins that were assayed both with the SomaScan 1.3 k platform and multiplexed conventional immunoassays in two separate chronic obstructive pulmonary disease (COPD) cohorts ($n = 371$ and $n = 176$).[91] Here, Spearman correlation ranged from −0.13 to 0.97, with a median of ~0.5. In contrast to Liu et al.,[52] Raffield et al.[91] found that the abundance of the individual proteins did not affect the degree of correlation in their measurement across platforms. The 63 proteins analyzed in the COPD cohorts were assessed for presence of cis pQTL to provide a measure of aptamer specificity for the respective target proteins. Of the 63 proteins, 31 (49%) had evidence of a cis pQTL in at least one of the two COPD cohorts, using data obtained with either of the two assays (SomaScan or immunoassays).

Furthermore, using public data, 42 (67%) were found to have a reported pQTL in either of two large studies.[56,93] However, the median correlation in the subgroup of proteins with a pQTL ($r = 0.5$) was comparable to the overall correlation. Furthermore, they did not observe any systematic pattern for presence/absence of common missense variants among low- or high-correlating proteins. In general, presence of concordant cis pQTLs for a protein (suggestive of binding the annotated target) did not ensure high correlation across assays; however, discordant cis pQTLs were generally found only for low-correlating proteins. This indicates that although pQTL analysis is a useful tool to assess if a signal measures an on-target binding, further validation (e.g., orthogonal quantification of protein level) is necessary.

In a more recent study, Pietzner et al.,[50] used the SomaScan v4 platform covering 4775 unique human protein targets, together with the Olink platform covering 1069 unique proteins to measure samples from the same 485 individuals. Of 871 overlapping proteins that were successfully was assayed by both platforms, they found a median correlation of 0.38 (interquartile range 0.08–0.64), spanning a wide range, from highly concordant ($r = 0.95$) to inversely correlated ($r = -0.48$). When combining with genome-wide association studies data, they identified in total 504 pQTL, of which 306 (61%) were shared with both platforms, whereas 198 (39%) were platform specific. Platform-specific cis-pQTLs were more likely to have low Spearman correlation, lower binding affinity of the SOMAmer reagent to the protein target, or to be in linkage with a missense variant, pointing to epitope or structure altering variants affecting affinity reagent binding.

Studies comparing performance of MS and affinity proteomics platforms include that by Petrera et al.,[92] where 173 human plasma samples were analyzed using Olink assays (eight panels with 736 nonredundant proteins) or MS-based platforms, either with data-independent acquisition (734 proteins) or data-dependent acquisition (368 proteins). Of 35 overlapping proteins, 23/35 (65%) had a correlation >0.5 between the two MS platforms, whereas only 6/35 (20%) of proteins had a correlation >0.5 in all three platforms. The difference in sensitivity to lower abundance proteins is likely to explain part of the low overlap between the MS and affinity proteomics platforms.

3 | PLASMA PROTEOMICS IN VENOUS THROMBOSIS RESEARCH

A rather limited number of studies have been reported using plasma proteomics for identification of novel plasma biomarkers associated with VTE. Both affinity-based proteomics and MS-based proteomics methods have been used, with very few overlaps in terms of identified proteins between studies, which could reflect that several reports are based on small studies. The more relevant publications are summarized in Table 1.

We used multiplex antibody SBAs targeting 408 selected candidate proteins[22] to perform a proteomics discovery screen in 88 cases and 85 matched controls in the Venous Embolism BIOmarker study, where patients were sampled after discontinuation of anticoagulant treatment for a first-time VTE. With access to the large resource of antibody reagents generated as result of the Human Protein Atlas covering >85% of the proteins encoded in the human genome[94] (www.proteinatlas.org), we custom designed suspension bead arrays using 755 antibodies, targeting 408 candidate proteins that were selected for (1) their known roles in the coagulation/fibrinolysis cascade and/or intermediate traits of relevance to thrombosis, (2) their specific expression in endothelial cells (a key cell type involved in thrombosis physiopathology), or (3) encoded by genes identified in pangenomic studies as associated with several cardiovascular disease-linked biological pathways (e.g., platelet function, renal function, inflammation). Following a replication in 580 cases and 589 controls from the French FARIVE study,[22] platelet-derived growth factor β (PDGFB) was identified as a novel VTE-associated biomarker, together with von Willebrand factor. To verify the target specificity of the PDGFB capture antibody, we used IC-MS and an ELISA assay.[22] In another study, Razzaq et al. identified Plexin-A4 (PLXNA4) as a new susceptibility gene for PE using an original integrated proteomics and genetics strategy, based on a proteomics analysis of samples from 1388 VTE patients from the MARTHA study, generated with a custom designed SBA of 376 protein-specific antibodies targeting 234 plasma proteins,[23] selected using similar criteria to that described previously.[22]

Using the PEA technology, Ten Cate et al. profiled 444 proteins with five 96-plex Olink immunoassay panels (Cardiovascular II and III, Cardiometabolic, Inflammation, Immune Response) in 532 individuals with VTE, sampled at diagnosis, in the Genotyping and Molecular Phenotyping of Venous ThomboEmbolism (GMP-VTE) study.[24] They identified five proteins as more specifically associated with an isolated PE phenotype compared with DVT or DVT-associated PE, of which three (interferon-γ), glial cell-line derived neurotrophic factor, and interleukin-15Rα were replicated in Olink data from 5778 individuals in the Gutenberg Health Study. Target specificity for the corresponding Olink assays was validated using cis pQTL analysis. In a subsequent study, using the same Olink panels to analyze VTE patients sampled at diagnosis and at 12 months after the index event in GMP-VTE, they identified a body mass-associated proteomic signature of 11 proteins that were consistently related to body mass index in plasma of VTE patients sampled at diagnosis and at 12 months after the index event.[25] However, this signature did not explain the obesity paradox in VTE patients, but leptin was inversely associated with the combined endpoint of recurrent VTE and death.

In an earlier study, Memon et al. used a single Olink panel, Cardiovascular III, to measure 92 proteins in a small study of 45 patients with acute DVT and 45 controls. They identified seven proteins with significant association with acute VTE.[26] These included three known VTE-associated markers: p-Selectin, tissue factor pathway inhibitor, and von Willebrand factor, together with four novel markers: transferrin receptor protein 1, osteopontin, bleomycin hydrolase, and ST2 protein. Tala et al. used the SomaScan assay targeting 1317 proteins, together with conventional immunoassays

TABLE 1 Publications of relevance for proteomics-based thrombosis research

Bruzelius et al., *Blood* 2016[22]	This report describes the first large-scale affinity proteomics study in the venous thrombosis field. A total of 408 proteins are targeted in a discovery case/control study (VEBIOS, $n = 88$ cases and 85 controls) with validation in an independent case/control study (FARIVE, $n = 580$ cases, 589 controls). It describes the application of immuno-capture mass spectrometry to validate assay target specificity. Plasma level of platelet-derived growth factor β is identified as associated with venous thromboembolism risk.
Ten Cate et al., *Blood* 2021[24]	This report describes the application of machine learning techniques to analyze affinity proteomics data. A total of 444 proteins are targeted in a discovery cohort (Genotyping and Molecular Phenotyping of Venous ThomboEmbolism, $n = 532$ cases) with validation in an independent population cohort (Gutenberg Health Study, $n = 5778$). It describes the application of cis pQTL analysis to validate assay target specificity. Plasma levels of interferon-γ, glial cell-line derived neurotrophic factor, and interluekin-15Rα proteins are identified as associated with isolated pulmonary embolism.
Razzaq et al., *Sci Rep*. 2021[23]	This report describes an original integrated affinity proteomics and genetics strategy using a neural network approach, based on proteomics and genome-wide association studies data in the MARTHA study ($n = 1388$ cases) with replication in the EOVT study ($n = 339$ cases). PLXNA4 is identified as a new susceptibility gene for pulmonary embolism.
Iglesias et al., *Arterioscler Thromb Vasc Biol* 2021[37]	This report describes a novel endothelial cell centric affinity proteomics strategy targeting 216 proteins with endothelial enriched expression in a population-based cohort (SCAPIS $n = 1008$). Plasma levels of 38 endothelial-derived proteins are identified as associated with cardiovascular disease risk.
Deutsch et al, *J Proteome Res* 2021[36]	This publication provides a comprehensive overview of technological developments and applications of mass spectrometry- and affinity-based plasma proteomics methods, summarizing recent advances and challenges for translating plasma proteomics into clinical utility for precision medicine. It presents the Human Plasma PeptideAtlas build 2021-07 and the Human Extracellular Vesicle PeptideAtlas 2021-06.

(e.g., ELISA) for 16 hemostasis biomarkers (e.g., coagulation factors, von Willebrand factor), to analyze plasma from 59 critically ill adolescents, of whom nine developed incident DVT.[27] CD36 molecule, macrophage inhibitory cytokine-1, and erythropoietin receptor were marginally associated with DVT using the SomaScan data. Jensen et al. used untargeted tandem mass tag-synchronous precursor selection-mass spectrometry-based proteomic profiling to analyze plasma from 100 cases of incident VTE and 100 controls, reporting transthyretin, vitamin K-dependent protein Z, and protein/nucleic acid deglycase DJ-1 as plasma proteins most strongly associated with incident VTE.[28] Orthogonal validation of the results from these studies, or replication in other cohorts, have not yet been reported.

Zhang et al. performed a proteomic analysis of serum in a small study of 24 patients with acute PE and 24 controls.[95] A discovery in a subset of nine cases and nine controls was analyzed using two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight MS analysis, which identified eight proteins associated with disease, of which one, haptoglobin, was orthogonally validated with ELISA in the full sample set. Han et al. applied data-independent acquisition MS and antibody array proteomic technology in two small case control studies ($n = 13$ and $n = 32$) of PE patients and healthy controls, with orthogonal validation by ELISA quantification in a separate study of 50 patients and 26 controls.[29] Five proteins including serum amyloid A-1, calprotectin, tenascin-C, gelsolin, and histidine-rich glycoprotein were identified with significant differences between PE and controls.

Several of the studies reported analyze a small number of cases,[27,29,95] which limits their statistical power to identify novel biomarkers for VTE, and when not combined with independent and adequately powered replication cohorts, results should be interpreted with caution.

4 | PLASMA PROTEOMICS IN ATHEROTHROMBOSIS

Mass spectrometry-based methods have been used to identify novel plasma biomarkers of acute atherothrombosis using a two-step approach of unbiased proteomic discovery analysis in a smaller sample set, followed by targeted validation of selected markers in a larger sample set. Shin et al. used LC-MS/MS in a discovery proteomics analysis of plasma from 50 patients with acute coronary syndrome (ACS) and 50 controls, followed by validation in 120 ACS and 120 controls by targeted MS proteomics for absolute quantification of seven

identified top candidates,[96] replicating four (AGP1, C5, LRG1, vitronectin) as significantly increased and one (gelsolin) as decreased in ACS. All four upregulated proteins are expressed predominantly in liver, not myocardium, and thus possibly linked to the pathogenic mechanisms in the acute arterial thrombotic event, rather than reflecting cardiomyocyte injury. Pan et al. used LC-MS/MS in a discovery proteomics analysis of plasma from 12 patients with acute ST elevation infarction (STEMI), 12 non-ST elevation myocardial infarction and 8 healthy controls, followed by validation in 75 STEMI, 75 non-ST elevation myocardial infarction, and 75 controls by ELISA for nine selected candidates.[97] Of these, the noncardiomyocyte proteins serum amyloid A-1, S100A8, Ficolin-2, and lipopolysaccharide-binding protein were identified with significant differences between STEMI and non-STEMI, thus partly overlapping the proteins identified by Han et al. as associated with PE.[29] Endothelial dysfunction, injury, and vascular inflammation are shared features of both cardiovascular and thrombotic disease, and comprehensive interrogation of vasculature-derived proteins in plasma by targeted proteomics could reveal novel plasma biomarkers linked to underlying pathogenic mechanisms in the vasculature. Proteomics platforms that allow for flexibility in developing large-scale multiplexed custom designed assay panels (e.g., affinity-based Luminex SBA and MS-based multiplexed PRM assays) facilitate strategies focusing on vascular cell-type specific proteins. Using a novel bioinformatic approach for deconvolution of RNA bulk sequencing, Butler et al.[98] identified a core endothelial cell-enriched transcriptome. These candidates were explored in the VEBIOS study[22] and more recently in the population-based Swedish CArdioPulmonary bioImage *Study*, *SCAPIS*, identifying endothelial cell proteins associated with cardiovascular disease risk factors and the Framingham risk score.[37] With a similar strategy, Ishizaki et al. developed a multiplex SRM MS assay for quantitation of a panel of 135 biomarker candidates for vascular inflammatory disease that included 87 endothelium-related proteins predicted to be present in blood through *in silico* screening of public databases.[99] The SRM panel was used to analyze paired plasma samples from 23 and 29 patients with vasculitis before and after treatment, identifying nine markers that were validated by conventional ELISA in 169 patients.[99]

5 | ISTH CONGRESS REPORT

A limited number of studies aiming to identify biomarkers for VTE by applying state-of-the-art proteomics screening technology platforms (e.g., PEA and targeted MS) were presented at the ISTH 2021 virtual congress.

Panova et al. (OC 10.4) analyzed plasma proteomics profiles using PEA in 652 individuals from GMP-VTE study, of which 82 had cancer. Using the same Olink panels as in two previous reports based on the same study,[24,25] they identified 60 unique proteins (13% of interrogated proteins) that in a model together (with nine other variables, not described) differentiate between cancer-associated thrombosis (CAT) and noncancer VTE with an area under the curve (AUC) of 0.89. The 60 proteins were primarily related to complement, coagulation, angiogenesis, immune response, and cell growth regulation, which could reflect the preselection biases of the PEA panels (e.g., Cardiovascular, Immune Response). In another study from the same group, Ten Cate et al. (PB 0496) presented an abstract for their recently published study that identified five proteins as more specifically associated with an isolated PE phenotype compared with DVT or DVT-associated PE.[24]

Zwicker et al. (OC 23.1) used PEA assays to profile 1161 unique proteins in baseline samples for 183 gastric and lung cancer patients in the HYPERCAN study,[100] of which 32% developed CAT. A machine learning model identified 10 plasma proteins that, together with six clinical parameters, were predictive of future CAT (AUC 0.75 + 0.04). In comparison, the Khorana score[101] was not predictive of VTE in this study (AUC 0.52).

Buijs et al. (PB 0492) used quantitative MS-based targeted proteomics, with internal standards, to measure 269 proteins in plasma samples of 142 colorectal cancer patients sampled at initiation of chemotherapy, of which 12 (8.4%) subsequently developed VTE. They identified four proteins associated with risk of future CAT: angiotensinogen, apolipoprotein B100, CD5 antigen-like, and immunoglobulin heavy constant mu.

Because these studies had not been peer reviewed and results not validated in independent studies and/or were based on small number of cases and thereby limited in statistical power, the reported results should be considered preliminary.

6 | FUTURE DIRECTIONS

6.1 | Applications of plasma proteomics technologies in thrombosis research

The COVID-19 pandemic has resulted in thousands of publications reporting the effects of SARS-CoV-2 infection, including those featuring the technologies discussed here, to analyze changes in the serum or plasma proteome. A recent literature review identified 53 reports (peer reviewed, or available on preprint servers, up to June 2021),[36] that used either conventional immunoassays, MS-based proteomics, or affinity-based proteomics (SomaLogic and Olink platforms), or two of these technologies,[102] to analyze plasma from COVID-19 patients. With an urgent need for clinically applicable biomarkers predictive of short- and long-term prognosis for COVID-19 disease severity and complications,[36] and the central role of thrombotic complications in COVID-19 pathology, we are likely to see an increasing number of studies applying state-of-the art plasma proteomics strategies to investigate the increased risk of VTE in COVID-19.[103]

6.2 | Novel strategies for plasma biomarker discovery with targeted proteomics

Thus far, candidate biomarker selection for studies of VTE have been predominantly centered around preexisting knowledge,

with commercially available screening panels still primarily configured to detect proteins with known functions in pathophysiological processes and pathways involved in vascular disease. A significant number of candidates in such panels have wide tissue and/or cell type expression, which can complicate interpretation of pathophysiological relevance of identified markers. Indeed, a common feature of most clinically useful biomarkers is expression specificity to a particular tissue or cell type (e.g., plasma levels of a cardiac-specific isoform of intracellular troponin, such as TnT) is used to detect protein leakage from injured cardiomyocytes in myocardial infarction. Indeed, the discovery of clinical biomarkers in use today typically followed the identification of the tissue- and/or cell-specific expression patterns of the proteins. Developments in mRNA sequencing, of both tissue and single cells, has relatively recently transformed our understanding of the specificity of protein expression across the human body.[104] In the context of VTE research, the selection of potential candidate targets based on the discovery of their high specificity of expression in disease-relevant cell types, such endothelial cells[98] or others,[104] could uncover novel and more specific VTE biomarkers that could otherwise be missed by existing screening panels.

6.3 | Emerging technologies for targeted plasma proteomics and biomarker validation

Mass spectrometry is the leading technology to accurately measure proteins in complex samples on a large scale. Targeted proteomics, in combination with the spike in stable isotope standards, is considered the gold standard and can improve both the analytical precision and specificity of the assay. The targeted proteomics workflow is very flexible and highly suitable for biomarker validation studies because it can report absolute protein concentrations, which can be compared across sites and studies. The combination of targeted proteomics and stable isotope standards are now robust enough for precision medicine efforts, where protein quantification must be reproducible over time. Technical leaps in chromatography allow for up to 100 proteomes to be quantified within 1 day, with hundreds of proteins quantified with median coefficient of variations less than 10% at an absolute scale.[89] These panels can be either be increased to cover a more comprehensive list of medium to high abundant plasma proteins, or tailored toward few biomarkers with high clinical significance.

RELATIONSHIP DISCLOSURE

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

All four authors participated in writing the manuscript and contributed with respective expertise. Dr. F. Edfors is a junior group leader (Targeted Proteomics Group, KTH, SciLifeLab Stockholm), with expertise in targeted mass spectrometry-based proteomics. Dr. M.J. Iglesias is a senior researcher (Clinically Applied Proteomics group, KTH, SciLifeLab Stockholm) with expertise in application of affinity proteomics and biomarker validation. Assistant Prof. L.M. Butler is a group leader (Translational Vascular Research, UiT Tromsø, Norway, and Karolinska Institute, SciLifeLab Stockholm) with expertise in endothelial cell biology, in vascular disease, and in the application of novel strategies for biomarker discovery, based on cell or tissue type enriched proteomes. Prof. J. Odeberg is a group leader (Clinically Applied Proteomics group, KTH, SciLifeLab Stockholm) and senior consultant in acute medicine and hematology, with expertise in application of proteomics for identification of biomarkers for venous thrombosis. All authors participated in critically revising the manuscript and has approved of the final version.

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