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Published in:
Translational Proteomics

DOI:
10.1016/j.trprot.2015.01.001

Publication date:
2015

Document version:
Final published version

Translational Proteomics 7 (2015) 40–48

Contents lists available at ScienceDirect

Translational Proteomics

journal homepage: http://www.elsevier.com/locate/trprot

Plasma proteomics to identify biomarkers – application to cardiovascular diseases

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ARTICLE INFO

Article history:
Received 1 October 2014
Received in revised form 12 December 2014
Accepted 2 January 2015
Available online 12 January 2015

Keywords:
Cardiovascular disease
Proteomics
Protein biomarkers
Plasma

ABSTRACT

There is an unmet need for new cardiovascular biomarkers. Despite this only few biomarkers for the diagnosis or screening of cardiovascular diseases have been implemented in the clinic. Thousands of proteins can be analysed in plasma by mass spectrometry-based proteomics technologies. Therefore, this technology may therefore identify new biomarkers that previously have not been associated with cardiovascular diseases. In this review, we summarize the key challenges and considerations, including strategies, recent discoveries and clinical applications in cardiovascular proteomics that may lead to the discovery of novel cardiovascular biomarkers.

1. Introduction

Cardiovascular disease (CVD) refers to any disease that affects the cardiovascular system such as cardiac disease, vascular diseases of the brain and kidney, and peripheral arterial disease. The underlying pathology is often atherosclerosis, but may also relate to other changes in the arterial systems (i.e. aneurysms, increased stiffness) and different pathologies in the heart as well (valvular alterations, cardiomyopathies etc.). CVD is the most common cause of mortality in the western countries and the prediction of cardiovascular events most often relies on the monitoring of prevalent risk factors such as smoking habits, diabetes, hyperlipidemia and hypertension [1]. Today, the cornerstone diagnosis and monitoring of CVD among individuals are clinical assessment of patients, imaging modalities together with electrocardiography (EGG). Moreover, the prevention of CVD is based on a strict management of the traditional risk factors of CVD; smoking, diabetes, hyperlipidemia, and hypertension. Although successful risk diminishing and effective treatment is available this strategy is not viable for all CVD. For example, the majority of individuals who actually develop manifest coronary heart disease have rarely more than one of the conventional risk factors and thus falls in to the low risk or intermediate risk group, which complicates the risk stratification before manifest disease occur [2]. Therefore new proteomic biomarkers are needed to support the information obtained from the conventional risk factors to improve the stratification of patients and to provide treatment at a personalized level. The recent advances in method development, bioinformatics and instrument speed, sensitivity, resolution and dynamic range of mass spectrometers used in proteomics hold the promise that the proteomics field will be a significant contributor of plasma markers enabling the prediction of early onset of CVD and treatment of the specific CVD at a personalized level.

In present work we summarize the proteomics platforms applied in cardiovascular plasma proteomics and also review the recent achievement in plasma proteomics related to cardiovascular diseases that may lead to new protein biomarkers with the potential for developing into a future clinical protein test.

2. Biomarkers for cardiovascular disease

A biomarker may serve a variety of functions when used in a clinical context. It has been defined as a "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic intervention" [3] and serve various functions corresponding to different stages in disease evolution. Thus, biomarkers may be considered as indicators of disease trait (risk factor or risk marker), disease state (preclinical of clinical), or

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http://dx.doi.org/10.1016/j.trprot.2015.01.001

disease rate (progression) [4]. Therefore, biomarkers can be grouped into (1) risk stratification biomarkers (identifying the risk of developing a disease), (2) screening biomarkers (screening for subclinical diseases), (3) diagnostic biomarkers (recognizing overt disease), (4) staging biomarkers (categorizing disease severity), or (5) prognostic biomarkers (predicting future disease course, recurrence and therapy response) [3]. A biomarker for CVD is not limited to a specific molecule (e.g. protein, RNA, or a metabolite) measured in a biological sample such as body fluids (plasma, urine, cerebrospinal fluids) or tissue, but may also be a recording of physical parameters such as a person's blood pressure, electrocardiogram, echocardiogram or computerized tomography (CT) scan.

Ideally, a biomarker for a CVD should enhance the ability of the clinician to manage the patient in an optimal way. Although proteomics technologies can identify and quantify thousands of proteins and several studies have proposed a variety of protein biomarkers for CVD only a limited number of plasma or serum biomarkers have been implemented successfully into cardiology practice – most of them being of diagnostic markers (Table 1). These include, for example, the myocardium-specific structural proteins troponin I and troponin T for the diagnosis of myocardial injury [5] and B-type natriuretic peptide (BNP) that is a hormone released from the ventricles during myocardial stress and used for the diagnosis and management of acute congestive heart failure. They form the cornerstone for the diagnosis of myocardial infarction and myocardial stress. Moreover, it has been demonstrated that – when measured with high sensitive assays – these markers have the potential to be risk prediction markers. In fact, several studies have demonstrated that within the reference level there is a risk gradient corresponding to an increased risk of an adverse cardiac event with increased troponin levels [6–8]. However, none of the routinely used protein biomarkers for CVD have initially been found in proteomics discovery experiments.

3. The plasma proteome – the never-ending challenge

Circulating blood is a convenient source for sampling and can be collected without any interventional procedures. This is probably the main reason that most proteomic biomarker discovery studies have been done on circulating biomarkers and not on site-specific blood samples. Although advantageous, this has also some serious drawbacks; the discovered biomarkers – unless organ or disease-specific like troponin T for myocardial damage – may reflect the disease state of any organ in the body. Moreover, the circulating biomarker will also suffer from a poor signal-to-noise ratio. This may lead to poor predictive value and limited clinical use of the identified biomarker. Signal-to-noise ratio can be increased by sampling the blood distal to the diseased tissue, where the biomarker concentration most probably will be highest [9]. This strategy for blood sampling requires an interventional procedure and will not be feasible in most plasma biomarker discovery projects.

The most challenging feature of the plasma proteome with respect to proteomics biomarker discovery, however, is the presence of an exceptional wide concentration range of the proteins comprising this proteome. Today, more than 10,000 proteins have been identified in human plasma (http://www.plasmaproteomedatabase.org). The extreme concentration range of proteins (12 orders of magnitude) ranging from >600 μM to the low femtomolar level per litre of blood [10] and the set of extremely high abundant proteins (such as serum albumin, immunoglobulins and complement factors) that constitute more than 99% of the total protein amount makes the plasma proteome to the most heterogeneous and complex sub-proteome of the human body [11,12]. As a consequence, discovering and validating novel protein biomarkers for CVD such as atherosclerosis in plasma is very challenging. This dramatically impacts the results that can be obtained by proteomic discovery experiments on plasma samples that should not be compared with the results from proteomic discovery studies on tissue or cell models with the respect to number of identified and quantified proteins. These facts have driven the evolvement of several proteomic experimental approaches that have been applied for the discovery of new plasma biomarkers for CVD diseases. Many of these have applied highly advanced analytical strategies to overcome the two major obstacles in plasma discovery proteomics; the complexity of plasma (number of proteins and the presence of many post-translational modified proteins) and the extreme dynamic range of plasma proteins confining the number of identifiable and quantifiable proteins. Another important issue is the number of

Table 1
Commonly used diagnostic and prognostic plasma protein biomarkers for cardiovascular diseases.

Protein biomarker	Origin	Pathology	Use	References
Brian natriuretic peptide (BNP)	Secreted from membrane granules in the cardiac ventricles – cardiac specific	Congestive heart failure, acute coronary syndrome	Diagnostic, prognostic	[61,62–64]
Troponin T (TnT)	Cardiac-specific isoform, released upon myocardial injury	AMI, myocardial injury	Diagnostic	[65,66]
Troponin I (TnI)	Cardiac-specific isoform, released upon myocardial injury	AMI, myocardial injury	Diagnostic	[65,66]
Creatinine kinase/CK-MB	Primarily released from the cardiac muscle-not tissue specific	AMI, myocardial necrosis	Diagnostic	[67]
C-reactive protein	Liver-produced non-specific acute-phase reactant	Atheromatic plaque vulnerability, coronary artery disease, coronary vasospasm, left ventricular dysfunction, angina pectoris, myocardial infarction.	Screen for risk of CVD	[68–70]
Myoglobin	Cardiac muscle – tissue unspecific	Tissue necrosis, AMI, MI	Diagnostic	[71]
Apolipoprotein (A)	Liver, intestine	None	Prognostic, screen for risk of CVD	[72–74]
Apolipoprotein (a)	Liver, intestine	None	Prognostic, screen for risk of CVD	[72–74]
Apolipoprotein-B100	Liver	None	Prognostic, screen for risk of CVD	[75,76]

samples (individuals) to be included in a study and if data about biological variation should be addressed already in the initial discovery experiments. Most plasma proteome studies rely on mixing of individual samples, i.e. typically pooling of samples from healthy individuals and samples from diseased and comparing proteins in the two pooled samples by proteomics in an unbiased and quantitative manner. This approach does however not give information on biological variations of the individual protein concentrations. Since some highly relevant biomarkers display low inter-individual variation and only small differences in average values between cases and controls (for example albumin as a marker for disease activity in many diseases) [13], and since many proteins have extremely high inter-individual variation and may display apparently large (but insignificant) fold changes between different groups [14], both false positive and false negative results occur in typical pooling experiments. It is therefore important to take biological variation into account at a point in the biomarker discovery pipeline, optimally as early as possible. Nevertheless, only a minor part of studies rely on the quantitative proteomic analysis of a number of individual samples (often a limited number), which gives some insight into biological variation. In any case, follow-up studies, where a number of potential candidates are quantitated by immunoassay or multiplexed MRM, are needed to ensure that selected candidate biomarker proteins can be validated. Thus, with the present most used approach, which include pooling of samples in the initial steps of the proteomic discovery design, it is likely that many potentially clinical useful biomarkers with relative small absolute differences between groups are not considered due to false-negative neglections based only on fold changes.

4. Important considerations for the experimental design of a plasma proteome study

A plethora of pre-analytical variables influence the outcome of the final discovery analysis of a plasma sample, and the lack of standardized procedures in all pre-analytical steps account for the vast majority of errors during this process. Hence, for a successful production of validated plasma biomarkers utmost attention should be paid on the process of blood collection, the nature of the sample container, lag-time prior to sample processing, temperature (storage conditions and no. of freeze–thaw cycles), and processing [15]. For example, the needs for pre-analytical standardization was demonstrated by Marshall et al. [16] in a study where MALDI-TOF was used for the analysis of blood samples from patients with myocardial infarction. They found that the detected changes in the protein profiles were caused by protease activities rather than a result of the disease processes. It should also be noted that the type of sample and sample processing may be quite different in the different phases of biomarker development and biomarker validation. For example, a typical plasma proteome discovery project includes multiple sample processing steps such as depletion, enrichment, chemical labelling, and pre-fractionation prior to MS analysis (Fig. 1). By contrast, the sample processing in the validation phase applying multiple reaction monitoring mass

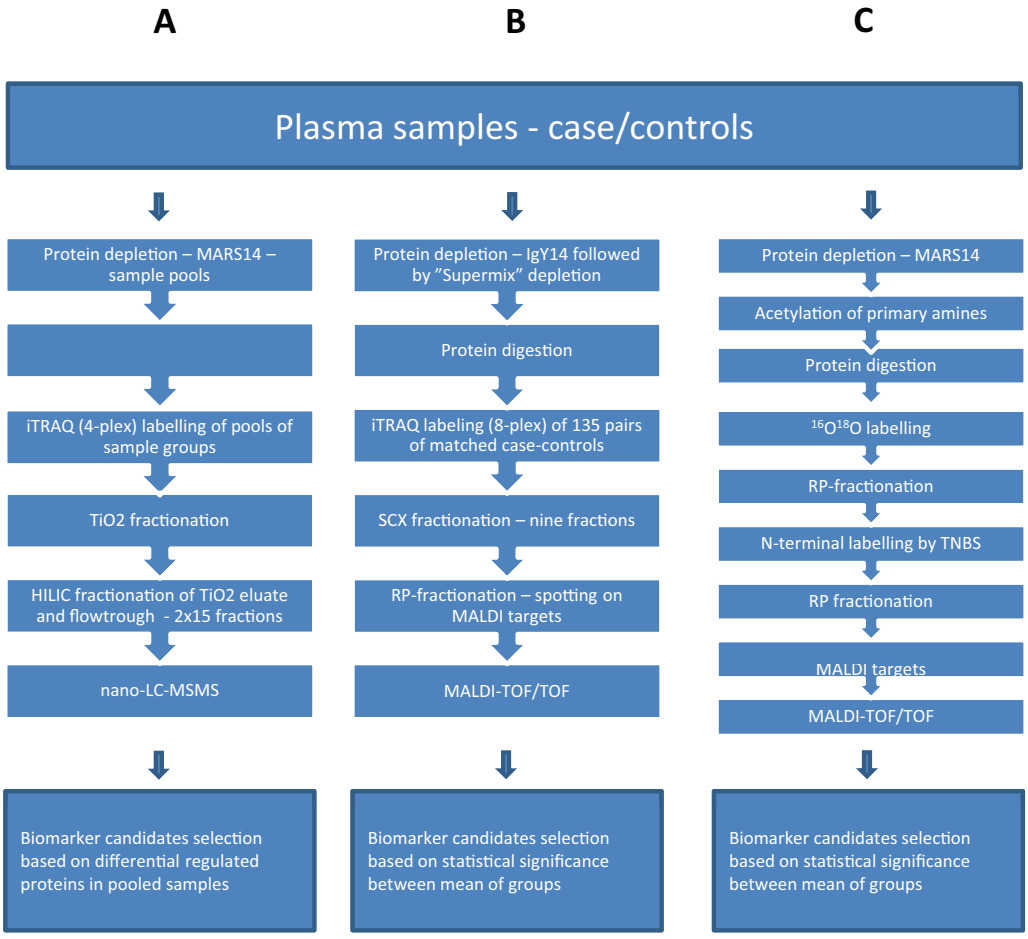

Fig. 1. Schematic representation of recent proteomic experimental strategies oriented to the identification of plasma biomarker of CVD.

spectrometry (MRM) most often only include the final sample processing step of the discovery phase: reduction and alkylation followed by proteolytic cleavage [14].

Furthermore, features that are unique to cardiovascular conditions will also influence the overall experimental setup for a given study. A chronic condition that has evolved over decades may often lead to an altered gene expression and therefore also a change in the plasma protein levels of specific plasma proteins produced by the liver or proteins related to the endothelial cells, arterial smooth muscle cells or the extracellular matrix of the arterial matrix. For example, atherosclerosis evolves over decade with an equivalent slow change in the composition of the arterial protein matrix and plasma protein composition. Here, the timing of sampling is less important. By contrast, in acute cardiac conditions or when acute insults induce rapid post-translational modifications (PTM) of pre-existing proteins, the promptness of the plasma proteome modulation should be taken into account and the timing for collecting becomes more critical. Ideally, blood samples should be collected before under and after the onset of the disease allowing each subject to serve as its own control. For example, in a study aiming the identification of early biomarkers of cardiac injury blood samples were collected directly from the hearts of patients undergoing a planned myocardial infarction for treatment of hypertrophic cardiomyopathy hearts, before, during and after a controlled myocardial injury [17]. This design secured the enrichment for candidate protein biomarkers of myocardial infarction and concomitant proteomic analysis revealed more than 100 novel candidates for this disease by a label-free, relative quantification approach. A targeted proteomic approach based on accurate inclusion mass screening actually qualified a fraction of these candidates in peripheral plasma. A verification study of peripheral plasma from controls and patients with planned myocardial infarction or spontaneous myocardial infarction by MRM or immunoassays suggested a specificity of the analysed markers towards myocardial infarction. Another important cause of variation to be considered in proteomic plasma biomarker discovery is the biological variation (between subject variation) for a specific plasma protein within the individuals (case and control group) of the study on top of the experimental variation of the proteomic platform applied in the study. In ideal cases both the baseline variation over time within individual and also the variation between multiple individuals for each of the analysed plasma proteins are determined. Practically, this is a rare case in plasma proteome discovery studies as these most often are performed on pooled samples (pool of samples from healthy individuals' vs. group of diseased). Pools are then analysed with technical replicates or a rather low number of individual samples (healthy and diseased analysed individually). Candidate biomarkers are given by a pre-defined ratio (most often a factor of 1.5–2) relative to the pooled sample from healthy individuals or by statistical evaluation of relative peptide ratios [18]. This approach results in the identification of several biomarker candidates in typical discovery experiments. There is a risk, however, that the selected biomarker candidates will turn out to be not statistical significant due to the high inter-individual variation in protein abundance among the samples analysed or that the sample pooling will push individual proteins below the detection limit of the applied technology [19]. Moreover, it is also possible that this approach will exclude further investigations of candidates, which may be important since many relevant biochemical changes in plasma components does not necessarily show high fold change differences between healthy and disease. In cell models or tissue samples a typical proteomic experiment often quantifies >2000 proteins. Of them many hundreds meet the pre-defined criteria for a potential biomarker candidate that should be considered for further evaluation using targeted quantitative approached such as MRM or immuno-chemical methods (ELISA) in a rather large patient cohort as a first step of validation. This strategy is in many cases not viable due to very high cost and enormous workload required for validating this number of candidate with the current standard methods (MRM and/or ELISA). Therefore, the list with candidates is reduced by means of bioinformatics analysis to identify likely disease-relevant candidates, by selecting on the basis of an intellectual assessment by the researcher, or simply just by selecting the most regulated ones. Each of the three mentioned approaches will result in an erroneous exclusion of "real" disease-relevant candidate markers thus leading to the selection of a relative high number of "false positive" candidates for further validation. This is mainly because the selection biased due to "expected" findings or the fact that the regulation of disease-relevant protein markers not necessarily correlate with pathological impact. Therefore it may be advantageous that the proteomic discovery experimental setup is based on samples from individuals instead of pooling samples. The major advantages of this approach is a significant reduction in "false positive" candidates as the biomarker candidate is selected on the basis of statistical significance that also take technical and inter individual variation into account. This can in principle be done by both label-free methods and labelling-based methods. Label-free methods include spectral methods (counting the number of peptide assigned to a protein in an MS/MS experiment) or area under curve (AUC)/signal intensity measurements based on precursor ion spectra [20,21] whereas common labelling methods include dimethyl labelling [22] and isobaric tags for relative and absolute quantitation (iTRAQ, TMT) [23,24].

A viable and affordable experimental labelling approach is to apply an amine reactive mass tag-based strategy that in addition to, samples from individuals (healthy and diseased) included in the study also includes an internal standard (a laboratory control sample, e.g. a pool of all samples to be analysed in the study or a pool of the healthy or diseased individual of the study). Currently, this chemistry allows the assessment up to eight (iTRAQ 8-plex reagent [25]) or ten individuals (10-plex TMT reagent [26]) in a single experiment thereby reducing the number of samples to be analysed dramatically. For example, Yin et al. [27] in their search for new-onset plasma biomarkers for atherosclerosis used this approach in an 8-plex iTRAQ experimental setup where plasma samples from 136 case–control pairs were randomly tagged by six of the eight iTRAQ channels throughout all iTRAQ mixes (46 in total) to eliminate age, gender, and examination bias while ensuring that case-control pairs were assayed in the same iTRAQ mix to maximize the precision of pair-wise comparisons (Fig. 1B). The remaining two channels producing the m/z 113 and m/z 117 reporter ions were assigned to the reference samples (pools of case and control samples). This experimental design allowed the researcher to select candidate biomarkers for further validation on the basis of statistical analysis; paired t-test to compare means between cases and controls, and conditional logistic regression models.

5. Reducing complexity and protein dynamic range in plasma is essential in proteomics

Reducing sample complexity is crucial to achieve sufficient analytical depth of the plasma proteome. It has been estimated that blood contains maybe more than 1,000,000 proteins when taking the number of variants due to proteolytic processing, PTM, splice variants, and SNIPS (single-nucleotide polymorphism) in account [28]. On top of that, many biological interesting proteins relevant to CVD are present at very low levels (nanomolar range). For example, the cardiac markers troponin I and troponin T are found in the low nanomolar range and the tumour necrosis factor

in the femtomolar range in pathological states and even lower when measured in healthy states. Despite the tremendous developments in the MS technology in this century the sample complexity of the plasma proteome still exceeds the capability of the MS instruments for several technical reasons. First, as the number of peptides that elute from the chromatographic column into the MS instrument in a given time increases with sample complexity a higher proportion of the peptides will remain unsequenced by the MS instrument. Secondly, the huge dynamic range of the plasma proteome (10^{-12}) far exceeds the dynamic range of any MS instrument which at best is around four to five orders of magnitude [29]. Consequently, on top of the instrumental limitations such as dynamic range and speed of the MS instruments different approaches to reduce sample dynamic range and sample complexity and have evolved during time; depletion of high abundant proteins using chemical or immune chemical methods and/or chromatographic pre-fractionation of the sample. Depletion of high-abundant plasma proteins is most often based on immune depletion methods that are essentially based on multi-affinity removal systems (MARS) that target up to top 20 proteins and in the plasma sample or even serial immune depletion by MARS depletion followed by "SuperMix" depletion where the second depletion step targets "flow through" proteins from the first depletion step (Fig. 1B) [30]. The use of depletion systems cause loss of approximately 99% of the sample and enables – when combined with multidimensional separations approaches such as strong cation exchange chromatography (SCX) or hydrophilic interaction chromatography (HILIC) with nano-LC–MS/MS analysis – the identification of more than 1000 unique proteins [31]. Loss of sample (plasma proteins) rarely causes experimental problems as plasma on average has a protein concentration of approximately 70 μg per μL and the available amount of plasma available in clinical settings is often several millilitres. Even in cases where only very limited amount of plasma is available (mouse experiments, plasma from new born, etc.), e.g. 10 μL plasma samples will leave one with approximately 35 μg protein per individual for a proteomic experiment which is sufficient for a multidimensional fractionation proteomic experiment – for example, a typical first dimension fractionation employing HILIC has a capacity of 30–40 μg providing 15–20 2 μg fractions that subsequently are analysed by nano-LC–MS/MS analysis resulting in the quantification of at least 400–500 unique proteins (2 high confident peptides per protein) in iTRAQ experiments (unpublished results, our laboratory [14]). By contrast, when applying similar multidimensional approached to tissue or cells as many as 6000–7000 confidently identified proteins are obtained (unpublished results, our laboratory).

Another promising technology for the proteomic detection of medium- and low-abundant proteins in plasma include the combinatorial peptide ligand library (CPLL) approach [32]. This technology uses a stoichiometric peptide library (say millions of peptides) immobilized on a solid support (chromatographic beads) that – in theory – binds all proteins in a proteome with similar affinity resulting in an enrichment of very dilute protein species whereas sites (peptides) with affinity for high abundant protein species rapidly saturates resulting in a dilution of the high abundant proteins. This enrichment approach was for example used to detect circulating protein biomarkers for carotid atherosclerosis in coronary patients [33], and to search for plasma biomarkers for aortic stenosis [34].

Other depletion approaches have been reported such as dithiotrethiol precipitation of serum albumin [35], enrichment of low molecular weight serum proteins by acetonitrile precipitation [36], affinity enrichment of specific plasma proteins by chemical proteomics [37], and more recently, hydrogel particle trapping of high-abundant plasma proteins [38]. Although promising, none of these approaches have proven their robustness and reproducibility in a clinical setting. A well-recognized concern when using any protein depletion technology is the unintended removal of low abundant proteins that specifically or nonspecifically bind to the proteins targeted for depletion. Several studies demonstrate that such losses occur when using immune-depletion columns but at the same time also showed that such losses are merely neglectable [39,40]. The reproducibility and robustness and applicability in an automatic setup using LC or FPLC equipment suggest the commercially available depletion columns to be the technology of choice in a clinical biomarker discovery and validation setup.

The protein dynamic range of plasma can be reduced by multidimensional fractionation prior to the proteomic experiment. The recent development in proteomic technologies and methods for sample preparation and enrichment techniques mostly rely on multidimensional liquid chromatography electrospray tandem mass spectrometry (2DLC–ESI–MS/MS). In most cases the peptide sample is off-line fractioned using separation methods that are orthogonal to the final reversed phase nano-LC–MS/MS analysis. At the peptide level, SCX [41], HILIC [42], high pH reversed phase chromatography [43] are the methods of choice, whereas and off-gel electrophoresis [44] and SDS–PAGE gel slides and liquid-phase IEF are the most commonly methods used at the protein level. Actually, most proteomic studies in plasma rely on a combination of sample depletion followed by pre-fractionation in order to gain sufficient analytical "depth" and to reduce sample complexity.

6. Recent discovery studies in cardiovascular plasma proteomics

The establishment of viable proteomics platforms evolved during the past decade – effective depletion and enrichment methods combined with pre-fraction methods and the evolvement from capillary LC systems with split-flow to highly robust direct nano-LC systems and the tremendous development of fast scanning, high resolution and high sensitive mass spectrometers – have severely outdated earlier plasma proteome studies in terms of speed, number of biomarker candidates identified in each discovery experiment. This clearly illustrated the fact that a total of only 289 proteins were identified in plasma in 2002 [11] to a number that exceeds 10,000. In other words, less much effort is required today to similar results as at the onset of this century as demonstrated by some recent proteomics discovery studies.

For example, a recent study by Yin et al. [27] searched for novel plasma protein biomarkers of new-onset atheroschlerotic CVD using a discovery mass spectrometry-based proteomic approach based on a dual-stage protein depletion strategy followed by 8-plex iTRAQ labelling of tryptic peptides and SCX fractionation into 9 fractions (Fig. 1B). Each of these fractions were then further fractioned into 304 fractions by reversed-phase HPLC and directly spotted onto a MALDI plate for MS/MS analysis by MALDI-TOF/TOF analysis. By using this approach 831 proteins in 135 myocardial infarction cases and 135 matched controls were quantified. Of them were 587 present in >40 case-control pairs. Single marker and multiple marker analysis adjusted for established clinical risk factors demonstrated that seven proteins in aggregate (cyclophilin A, CD5, MUC18, collagen-α1, [XVIII] chain, salivary α-amylase 1, C-reactive protein, and multimerin-2) were highly associated with myocardial infarct and significantly improved its prediction as compared with a statistical model with risk factors alone. Multiple marker analysis of data from targeted analyses of 59 proteins by MRM in 336 case-control pairs of atherosclerotic CVD revealed a combination of four proteins (α-1-acid glycoprotein, paraoxonase 1, tetranectin, and CD5 antigen-like protein) that predicted incident atherosclerotic CVD and also improved its prediction as

compared to a statistical model with risk factors alone. It is noteworthy that, despite the great efforts that have been made to reduce the dynamic range of the sample and reduce sample complexity by the application of dual-stage immune-depletion and peptide fractionation prior to MALDI-TOF/TOF analysis, only 587 proteins were quantified. Despite this approximately 10% of them (59 proteins) qualified for further testing by MRM in 336 case–control pairs whereof seven proteins in aggregation demonstrated a significant association with myocardial infarct ($P < 0.0001$). This study clearly demonstrates that it is possible to develop plasma protein biomarkers from a fairly low number of proteins quantified in the discovery study if only the number of individuals in the study is large enough.

In another study, Kristensen et al. [14] searched for early onset biomarker for atherosclerosis and used a quantitative proteomic approach based on immuno-depletion of the most abundant plasma proteins from pools of samples from individuals without any cardiovascular symptoms and no sign of coronary calcification, individuals with a high coronary calcium score, individuals that had surgery because of an atherosclerotic diseases, and individuals suffering from acute coronary syndrome ($n = 30$ in all four groups) and four-plex iTRAQ labelling followed by HILIC and titanium dioxide pre-fractionation, and nano-LC–MS/MS analysis. This experiment allowed the simultaneous investigation of proteomic changes occurring along with three manifestations of atherosclerosis. This experimental approach quantified 731 unique plasma proteins whereof 319 were only identified in the eluate from the titanium dioxide fractionation step. This demonstrates the power of adding an extra fractionation step that is selective towards a specific chemical structure present on a subgroup of peptides – in present case a phosphorylation. Using this approach 25 proteins demonstrated a significant altered expression as compared to the control group in at least the group with individuals diagnosed with acute coronary syndrome, several among them well-known markers for CVD including apolipoprotein (a) (Apa(a)), C-reactive protein (CRP), serum amyloid protein A (SAA), that previously has been associated with CVD such as coronary heart disease, myocardial infarction and stroke [45–48]. On top of the identification of well-described markers for CVD this study also identified one novel marker, vinculin – a multi-linker protein that acts as connector for cell–matrix adhesions and cell–cell-adhesions to the actin-based cytoskeleton. This protein was the top-most elevated protein identified in the study and its expression was validated at single patient level means of MRM whereas the known markers for CVD were validated using immunoassays. Apo (a), CRP, and SAA were validated by both immunoassays and MRM and this validation demonstrated a high agreement between the two methods applied for validation. Major drawback of this study is, however, that the discovery phase is performed on pooled sample of a relative low number individuals included in the study. Moreover, the validation of the top-most regulated biomarker candidates by MRM analysis performed on individual samples was performed on the same sample group as the discovery phase.

A third recent proteomic study aimed for the discovery of novel diagnostic biomarkers for acute decompensated heart failure (ADHF) driven by the lack for suitable marker that could discriminate ADHF patients with or without a history of chronic heart failure (CHF) with sufficient specificity and selectivity (the former represent up to 80% of ADHF patients) [49]. Cardiac courses of ADHF are diverse and include left ventricular diastolic and/or systolic dysfunction, right ventricular dysfunction and/or acute ischaemia [50] and the existing markers for ADHF, B-type natriuretic peptides (BNP) released from stressed cardiomyocytes, can diagnose ADHF with high sensitivity but cannot discriminate between patients that have ADHF or patients with CHF but not ADHF. Here, the COFRADIC (combined fractional diagonal chromatography) technology was used to compare plasma samples of 10 ADHF patients with 10 controls in order to identify novel protein biomarkers for ADHF (Fig. 1C). With COFRADIC, the large dynamic range of plasma proteins is circumvented by an N-terminal enrichment strategy. This strategy included a two-step depletion approach based on immune depletion of the top-14 proteins followed by reduction and alkylation of free cysteine residues and acetylation of free amines (α- and ε-amines). The following trypsin digestion produces two types of peptides: internal peptides with a free α-amino group and acetyl-blocked N-terminal peptides. The free N-terminal peptides are then blocked with 2,4,6-dinitrobenzenesulfonic acid (TNB) to form highly hydrophobic peptides that enables the isolation of the acetyl-blocked N-terminal peptide fraction by reversed-phase HPLC. This fraction, that in principle contain only N-terminal peptides, is separated by nano-LC and directly spotted onto MALDI targets. A relative quantification of samples from ADHF patients with controls was done by means of O16/O18-labelling following the trypsin digestion step. Statistical analysis of microarray (SAM) of the proteomic data revealed 103 significantly differentially regulated proteins whereof 49 proteins were retained as potential candidates for further evaluation in a larger patient cohort. A proof-of-concept of the discovery platform was achieved by the detection of the "gold standard marker" of HF; the natriuretic peptide pro-BNP and other established markers such as C-reactive protein and cystatin-C. MRM-based quantitative assays were developed for 27 of the 49 selected candidates. The clinical performance of the 27 chosen candidates was tested in 267 prospectively collected individuals (76 patients with acute HF, 71 patients with non-cardiac dyspnoea, 80 stable CHF patients, and 40 healthy controls). Downstream analysis of the 27 marker candidates identified sulfhydryl oxidase 1 (QSOX1) to be highly sensitive and specific for ADHF diagnosis in patients with acute dyspnoea and also significantly reduced false positives and demonstrated the highest specificity for ADHF diagnosis in patients with dyspnoea. A major weakness of this study is, however, that the discovery part of the work only include 10 healthy individuals that is compared with 10 diseased individuals which is a rather low number of individuals to be included for a discovery study. It is also not clear how many proteins that a rather low number of individuals to be included for a discovery study. It is also not clear how many proteins that were quantified across all individuals. Furthermore, the protein identification and protein quantification is likely to be based on single peptide as – in theory – only the N-terminal tryptic peptide of each protein is present after sample preparation. On top of that, several steps in the sample preparation procedure include chemistries that may affect the accuracy of this method.

7. From discovery to clinical value: status and perspectives

Once a biomarker candidate has been discovered it should be confirmed and validated in hundreds to thousands of specimens before it can be brought into clinical (use) trials. Moreover, it should be specific, sensitive and reproducible. The improved robustness of proteomic discovery technologies has primed an enormous number of discovery projects, but disappointing few clinical useful biomarkers have been successfully validated for use in clinical routine or research practice [51]. In fact, despite a nearly exponential growth in the number of biomarker publications during the past 10–15 years, the rate of the introduction of new protein biomarker has remained surprisingly constant around 1–2 per year [52,53], and even more concerning, none of these have been discovered by proteomics technologies [53,54]. These facts strongly indicate serious deficiencies in the proteomic biomarker discovery process that were addressed to be due lack

of suitable analytical platforms to verify candidate protein biomarkers in large cohorts/sample sets [55], limited access to clinical samples that are well-matched according to factors such as CVD risk factors, medication, gender and age [56], the absence of a proper organized biomarker development pipeline [57,58], and a too empirical attitude to biomarker research [59].

The biggest reason, however, for the proteomic technology over the past decades have not developed new CVD biomarkers and biomarkers in other fields as well, is most probably due to technological limitations. Despite the great excitement of the technological development of the 2D PAGE technology up through the 1980s and 1990s until 2002 the total number of proteins that were identified in human plasma was 289 [11]. In retrospect – with today's knowledge that the human plasma proteome comprises more than 10,000 proteins – the likelihood to discover novel plasma protein biomarkers for CVD was very low. At that time the technology shifted from being 2D gel-based to platforms consisting of multidimensional chromatographic separations of crude peptide mixtures combined with mass spectrometric analysis. With this shift in technology, a variety of new sample preparation methods, such as affinity purification of specific proteins or removing high abundant proteins by immune chemical methods, aiming the detection of low abundant proteins in human plasma samples, have evolved. This, in combination with the recent tremendous development in speed and sensitivity of mass spectrometers and development of robust direct nano-flow LC systems enabled the development of robust proteomic workflows and experimental designs (see, e.g. Fig. 1) that enabled the in-depth proteomic analysis of plasma.

Another reason for the lack of discovery of clinically useful protein biomarker by proteomics technologies may relate to the fact that plasma from cases and controls are often not sampled in the optimal situation, when considered from a clinical perspective. There is no doubt that factors like fasting, time of day, timepoint in the disease progression, comorbidities, and many others influence protein concentrations. It is therefore essential that samples for discovery studies, focused on finding clinical useful markers, are sampled in thoughtful way. Optimally, sampling for studies that aims at finding clinical relevant markers should therefore use samples taken in the exact population where the clinical question is relevant and at exactly the timepoint where the clinical question is relevant. For example, if the idea is to develop a marker that may help the clinician to discriminate between intracranial haemorrhage vs. arterial occlusion in patients with symptoms of stroke, it is important to search for biomarkers in samples taken at exactly the timepoint when this clinical question is relevant, for example in a population with relevant neurological symptoms at arrival at the hospital, not taken hours or days later. One challenge is that these optimal plasma samples are seldom available. Moreover, in such studies it is important to have good information concerning the current gold standard for the clinical situation for which a novel biomarker is intended.

Not every barrier to the discovery of new protein markers are, however, related to the technology and challenges in the proteomic analysis, it should also be mentioned that it is very important for the clinical community to define which markers could be of actual use either in research studies or in clinical situations. For example, it is important to realize that it is not necessarily important to search for and find better estimates for risk in a certain situation, if it does not help the clinician to suggest a better management of the patient. Aspects in relation to this issue should probably be part of the design of discovery studies, i.e. the samples that are used for the search should reflect the situation in which markers should be used. This is relevant for all kind of markers, i.e. screening, diagnosis, prognosis, prediction and also companion diagnostics. Another important barrier to the development of new CVD biomarkers could be that researchers historically were looking for the "one and only" biomarker and have not considered the potential of using combinatorial panels of markers until recent years where proteomics technology has enabled the quantification of 1000s of plasma proteins instead of a handful only one decade ago.

In fact, combining known biomarkers in a multiple biomarker approach might in fact improve the prediction of the risk of cardiovascular event. This was demonstrated by Wang et al. [60] who measured the levels of 10 biomarkers and found that levels of CRP, the urinary albumin-to-creatinine ratio, B-type natriuretic peptide, homocysteine, and renin strongly predicted risk of death whereas levels of B-type natriuretic peptide and the urinary albumin-to-creatinine ration. When these biomarkers were combined in multi-marker score, individuals with higher multi-marker scores had a significant increased risk of death and major cardiovascular events than individuals with low multi-marker scores. The impact of adding the multi-marker scores to the standard risk factors did only result in a slight increase in the ability to classify risk. This result was, however, somewhat predictable as the measured biomarkers in this study and the risk factors were beforehand correlated – thus interrogating similar pathways and thereby acts as a proof-of-principle experiment for the multi-marker approach and excellently demonstrates the potential of applying novel biomarkers in a multi-marker approach to improve the prediction of risk of cardiovascular events.

In conclusion, the recent developments in the proteomic discovery platforms allowing the detection of even the less abundant plasma proteins, the establishment of an increasing number of bio banks and collection of plasma samples, and the development of even more endowed experimental designs will undoubtedly lead to the discovery of CVD plasma biomarker candidates that – alone or when used in combination – have a larger potential to pass the validation phase and become the next generation biomarker for CVD in the clinic.

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