Proteomics in cardiovascular disease: recent progress and clinical implication and implementation

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Abstract

Introduction: Although multiple efforts have been initiated to shed light into the molecular mechanisms underlying cardiovascular disease, it still remains one of the major causes of death worldwide. Proteomics approaches are unequivocally powerful tools that may provide deeper understanding into the molecular mechanisms associated with cardiovascular disease and improve its management.

Areas covered: Cardiovascular proteomics is an emerging field and significant progress has been made during the past few years with the aim of defining novel candidate biomarkers and obtaining insight into molecular pathophysiology. To summarize the recent progress in the field, a literature search was conducted in PubMed and Web of Science. As a result, 704 studies from PubMed and 320 studies from Web of Science were retrieved. Findings from original research articles using proteomics technologies for the discovery of biomarkers for cardiovascular disease in human are summarized in this review.

Expert commentary: proteins associated with cardiovascular disease represent pathways in inflammation, wound healing and coagulation, proteolysis and extracellular matrix organization, handling of cholesterol and LDL. Future research in the field should target to increase proteome coverage as well as integrate proteomics with other omics data to facilitate both drug development as well as clinical implementation of findings.

Keywords: cardiovascular disease, vascular disease, proteome, biomarker, clinical proteomics

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide with a high mortality rate and an increasing incidence annually. Since it represents a major contributor to the global disease burden, gaining insights into the mechanisms of CVDs is emergent. CVD is a complex disorder describing pathophysiological conditions of the heart and blood vessels and includes a wide range of diseases such as coronary artery disease (CAD), heart failure (HF), myocardial infarction (MI), abdominal aortic stenosis, ischemic stroke, cardiomyopathy etc [1]. Several factors such as lifestyle, diet, (epi)genetics, dyslipidemia, hypertension, and inflammation are associated with the onset and development of CVD. Addressing the standard risk factors for CVD including hypertension, diabetes mellitus, smoking, and hypercholesterolemia is a major aim in the prevention of CVD. Nevertheless, the current treatment schemes, by addressing these individual risk factors, have not fully provided either a significant progress in curative treatment or a full understanding of the disease. Over the past years, efforts to elucidate the key molecular mechanisms underlying CVD were undertaken. Its complex etiology and the plethora of the different factors that are responsible for the observed pathology, require the development of more holistic treatment approaches targeting the underlying mechanisms [2]. The development and application of systems biology approaches for CVD investigation is an active and challenging research field [2-4].

Integrated omics approaches, including genomics/epigenomics, transcriptomics, proteomics, metabolomics have gained momentum the past few years. They provide information at multiple biological levels from the DNA and RNA to the protein and metabolite patterns, offering a different perspective on the molecular and cellular networks of whole organ system. Although, genomic and transcriptomic strategies have been recently applied in CVD

research, the weakness of these strategies is that they cannot correlate easily the observed changes to the expression of proteins and additionally, they cannot characterize the post-translational modifications (PTM) which play critical role in the regulation of many biological processes. These shortcomings can be addressed by the application of proteomic approaches. In fact, proteomic changes are likely the cause of most non-communicable diseases, including CVD, and interfering drugs generally act via targeting specific proteins. As graphically depicted in **Figure 1**, based on these facts it appears evident that the proteome will inform with superior accuracy on (any) disease, and on the best suited therapeutic intervention, leading towards personalized medicine.

Two main strategies appear of relevance in the exploitation of proteomic changes in the context of CVD: 1) obtain information about molecular pathophysiology and enable identifying relevant targets for intervention, and 2) identification of biomarkers that support patient management and guide therapy. As depicted in **Figure 2**, these two strategies do have different requirements. While implementation of a biomarker requires very high confidence in a biomarker and the test to be used (needs to be verified in large cohorts, likely exceeding 1000 subjects, and needs to demonstrate a significant improvement over the current state of the art), knowledge on the specific role of the biomarker in the molecular pathology is not a prerequisite for its employment (e.g even if we do not know why a patient has fever or elevated blood pressure, we nevertheless typically act upon detection of a significant change from the reference range in these biomarkers). In contrast, identifying drug targets requires in depth knowledge on molecular pathology. A major prerequisite to unravel the biological processes and pathways (and to consequently enable identifying the best suited drug targets) is a comprehensive coverage of the proteomic changes in the respective disease. To enable such in depth coverage, reduced stringency in

statistical evaluation needs to be accepted; it is impossible to verify ideally thousands of (sometimes) subtle changes observed in disease.

To review the current status of proteomic biomarkers associated with CVD, we conducted a literature search in PubMed using the keywords [proteomic; biomarker; cardiovascular disease] or the search query [cardiovasc*and proteom* and marker or biomarker] in the Web of Science. Following this combined search, 704 studies from PubMed and 320 from Web of Science published between January 2010 and October 2016) were initially retrieved. Studies focusing on animal models and reviews were excluded. Only studies in human using proteomics technologies for the discovery of biomarkers in cardiovascular disease were included (all listed as supplementary table). Based on these articles, we discuss in this review the recent advances in the development of specific biomarkers for CVD through the application of state of the art proteomic technologies and the progress that was made towards their clinical implementation. The general workflow that is followed in these studies and the molecules that are currently being used as CVD biomarkers in the clinical routine is shown in **Figure 3**. A brief overview of main employed proteomics platforms and types of specimens analyzed in CVD research, followed by a more detailed presentation of biomarker findings, as retrieved from the aforementioned literature search, follow.

2. Overview of the state of the art proteomics technologies in CVD

Quantitative methods can be divided into global and targeted proteomic approaches. The global approaches compare two or more proteomes for the identification of the differentially expressed proteins under physiological and pathological conditions and can be applied to the unbiased discovery of biomarkers. Global approaches are further categorized into gel based and gel free [Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

based]. Gel based approaches, such as two-dimensional electrophoresis (2DE) and two-dimensional Differential-In-Gel-Electrophoresis (2D-DIGE), have contributed in CVD [5-8]. However, 2DE is of lower resolving power in comparison to LC-MS/MS, allowing, by now, to assess with good confidence > 1000 proteins in complex mixtures per experiment.

As a result, LC-MS/MS has become a commonly employed method due to its sensitivity and high resolving power. There are two main strategies to perform quantitative proteome analysis, by either using label-free strategies where the peptides from 2 or more samples are injected into the mass spectrometer and analyzed in individual LC-MS experiments or by using the isotope labeling approaches. Labeling proteomic approaches include stable isotope labeling by amino acids in cell culture (SILAC), in which isotopically labeled amino acids are incorporated into cell culture media, as well as isobaric tags for relative and absolute quantification (iTRAQ) [9] or tandem mass tags (TMT), applicable to the study of any type of biological samples (beyond cell cultures) [10]. In a recent study, employing multiplexed iTRAQ, intensive depletion of abundant plasma proteins, optimized fractionation methods along with the use of the latest mass spectrometry (MS) instrumentation on plasma samples from patients undergoing a therapeutic, planned myocardial infarction (PMI) for treatment of hypertrophic cardiomyopathy [11], about 5000 proteins were identified in one analysis.

In studies that focus on analyzing a large number of heterogeneous samples containing interfering compounds such as lipids, the use of capillary electrophoresis coupled to mass spectrometry (CE-MS) appears advantageous [12]. This technique has been applied for the proteomic analysis of urine and has enabled the identification of novel candidate biomarkers in CAD [13], stroke [14], HF [15] and deep vein thrombosis [16]. These studies are summarized in the following sections.

Targeted proteomic approaches characterize and quantify a limited number of known target proteins from a complex sample. Their application includes a follow-up on proteins identified from global/unbiased proteomics screening, conducting functional studies such as post-translational modification (PTM) analysis and validation of biomarker candidates. Selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) is frequently employed in targeted proteomics and has been applied for biomarker [17-19] and PTM quantification [20,21]. In some instances SRM has been combined with the Protein standard absolute quantification (PSAQ) strategy, which uses full-length isotope-labeled protein standards to quantify target proteins. The PSAQ-SRM method was used for the quantification of four biomarkers (LDH-B, CKMB, myoglobin and troponin I) in MI patients' sera with good accuracy and reproducibility [22].

In recent years, array-based techniques have also been applied in order to identify biomarkers in atherosclerosis [23,24], abdominal aortic aneurism (AAA) [25] and CAD [26]. Additionally, aptamer-based proteomics have been applied for the identification of low-abundance biomarkers in CVD [27]. However, due to the small sample size and the lack of validation studies, the value of these approaches cannot be assessed yet.

3. Samples for proteome analysis in CVD

3.1 Tissue

Tissue proteomics in CVD have generated insight into the proteome of atherosclerotic plaques [6,7] and thrombi [28]. Although a number of studies have focused on the proteomic analysis of the whole atheromatous tissue of carotid and coronary arteries, this approach suffers from the existence of different layers; the adventitial, the medial and the intima. The latter represents the layer where a wide variety of changes occur upon

atherosclerosis development and progression. To overcome this barrier, arterial tissue subproteomes isolated by laser microdissection (LMD) from human atherosclerotic and pre-atherosclerotic coronaries have been studied [7]. This selective approach is also considered essential for studying the different cell populations or the cellular/extracellular components to better understand their contribution in the pathogenesis of the disease [29]. Finally, tissue secretome represents an alternative sample source. Tissue explants directly obtained from healthy and diseased arteries are cultured and their supernatant which is enriched in secreted proteins, is used for proteomics analysis [30].

3.2 Sub-cellular proteomes

The majority of the sub-cellular studies in the field of cardiovascular proteomics focus on the analysis of the mitochondrial [31] and extracellular matrix (ECM) proteomes [32]. Other sub-cellular proteomes, including myofilaments [33], sacromere [34] and proteasomes [35] have been less extensively investigated. Isolation of the sub-cellular fractions in general involves application of differential centrifugation, immune-based isolation, and membrane protein enrichment [36] methods.

3.2.1 ECM

The ECM is involved in a broad spectrum of signaling events during growth, differentiation, injury, and remodeling [37]. Technical challenges are the insolubility and extensive PTMs of the ECM proteins, and their low abundance (~1% of proteome) compared to the highly abundant cellular or plasma proteins. ECM enrichment strategies via decellularization, sequential solubilization and extraction of ECM constituents have been applied to overcome these obstacles [38-41]. Of note, Didangelos et al, through the application of these procedures, analyzed the ECM proteome in human aortic samples [39] and identified 103 ECM proteins with the one third of them not previously described in the context of vascular

tissue proteomics. The same group characterized ECM remodeling in abdominal aortic aneurysms [42]. ECM remodeling has also been described in HF and cardiac fibrosis [32,43].

3.2.2 Mitochondria

In a healthy cardiomyocyte, mitochondria represent ~40% of the volume. Mitochondria are essential for providing energy (for the contraction of the muscle), but also for regulating programmed cell death. Accumulating evidence supports that CVD progression is associated with changes in mitochondria structure and function [44,45]. An increasing number of studies focus on the characterization of alterations of the mitochondrial proteome in CVD [31], including pressure overload-induced HF [46], atrial fibrillation (AF) [47] and type I [48] and type II diabetic heart [49]. Goudarzi et al., performed LC-MS/MS analysis of isolated mitochondrial enriched fractions from right atrial tissues and identified 32 differentially expressed proteins in patients with AF compared to non-AF patients [47]. Other studies have also tried to analyze the effect of oxidative stress on cardiac mitochondrial protein dynamics by using *in vivo* models [50].

3.3 Biological fluids

Biological fluids, such as serum, plasma and urine, represent the ideal specimen for biomarker detection because they are easily accessible. Since urine and plasma account for the vast majority of biomarker studies, they will be reviewed extensively in the section on biomarkers (below).

3.4 Circulating cells and extracellular vesicles

Proteome analysis of circulating cells such as monocytes, platelets and endothelial cells may provide a better understanding of the involvement of these cells in CVD and enable identification of disease associated biomarkers. Monocytes play important roles in inflammation and atherosclerosis and therefore their proteomic profiling may provide novel

insights into their function, as indicated from recent studies [51,52]. Additionally, proteomic analyses of platelets in CVD [60,61], as well as circulating endothelial cells (CECs) and the endothelial progenitor cells (EPCs) in patients with ACS have also been described [53].

Plasma microvesicles (including microparticles and exosomes) are advocated as source of vascular-specific disease biomarkers. Membrane microvesicles, mainly released from activated platelets into the circulation, represent an important mode of intercellular communication and their number is increased in patients with acute coronary syndromes [54]. Comprehensive proteomic analysis of the plaque microparticles derived from human atherosclerotic lesions revealed that they derived primarily from leukocytes and are implicated in inflammation [55].

4. PTMs

Protein PTMs, including phosphorylation, ubiquitylation, acetylation, *N*- and *O*-linked glycosylation, and methylation, are key regulators of the protein conformation, stability and activity [56].

Protein phosphorylation is critical to myocardial function since it underpins cellular processes associated with energy metabolism, signal transduction and contractile function of the myocyte [57]. Thus, phosphorylation is the most commonly described PTM in the cardiac proteome [58,59]. Changes in the abundance of 25 phosphoproteins have been previously identified in hypertensive cardiac remodeling through the application of 2D-DIGE on phosphoenriched proteins [60]. Among them, myofilament proteins such as Alpha-tropomyosin and Myosin; mitochondrial proteins such as Pyruvate dehydrogenase A; phosphatases such as Protein phosphatase 2A; other proteins including Proteasome subunits α-type 3 and β type 7 and Eukaryotic translation initiation factor 1A, were included. Employing top-down MS-based quantitative proteomics, the phosphorylation of cardiac

Troponin I has been identified as a biomarker for congestive heart failure (CHF) [61], as described below.

5. Biomarkers currently applied

Biomarkers must provide accurate and reliable information about disease in order to aid in prognosis, diagnosis, or therapy monitoring. Currently, there are four biomarkers recommended for clinical use in CVD (summarized in **Figure 3**): 1) Cardiac Troponin T and cardiac Troponin I [62] are used for the diagnosis of acute coronary syndrome (ACS) and MI, while elevated plasma levels of troponin T represent the gold standard approach to detect MI [63] with a high predictive value [64]. The development of high-sensitivity assays for Troponin I and T has improved the diagnostic sensitivity for acute myocardial infarction (AMI) [65]. 2) B-type natriuretic peptide (BNP) and its N-terminal form are employed to detect congestive heart failure [66]. Circulating concentrations of BNP reveal strong associations with CVD risk under a range of different clinical conditions. 3) C-reactive protein (CRP) [67,68] and D-dimer [69], are inflammatory markers associated with ischemic heart disease. 4) Apolipoprotein A-I , the major protein component of HDL, is an excellent risk predictor for CVD related to the metabolism of high-density lipoprotein (HDL) [70]. While these biomarkers have clearly demonstrated value in multiple studies, a significant limitation appears in that they detect mainly late stage of CVD.

In the following section we present an overview of potential biomarkers for CVD that have been recently identified using proteomic technologies, categorized by the biological source investigated, and summarized in the respective tables.

6. Proteomic biomarkers in CVD

6.1 Tissue

The affected tissue certainly holds the most relevant information on molecular pathology, enabling identifying potential therapeutic targets. Tissue is not easily accessible, and certainly cannot be assessed for routine diagnosis. However, results from tissue proteomics may enable generating hypotheses on circulating biomarkers, which can subsequently be tested [71]. Several studies therefore focused on tissue, as summarized in **Table 1**.

6.1.1 Arterial Tissue

Atherosclerotic plaque may hold proteomic information on molecular alterations that discriminate patients with adverse cardiovascular events from those that remain stable during follow-up. De Kleijn et al. investigated the carotid atherosclerotic plaque proteome by MS in relation to outcome, during a 3-year follow-up. With this approach, Osteopontin (OPN) was identified as a potential instable plaque biomarker and subsequently validated in femoral plaque samples, indicating predictive value independent of plaque localization. The authors concluded that high levels of OPN from atherosclerotic plaques could be prognostic of the occurrence of cardiovascular events [72]. Hao et al. fractionated proteins from human carotid atherosclerotic plaques using Electrostatic Repulsion-Hydrophilic Interaction Chromatography followed by offline LC-MS/MS. Several previously undetected low-abundant proteins with important functions in atherosclerosis, such as TGF-β, interleukins and other growth factors were identified. The authors highlighted three proteins; Myeloperoxidase (MPO), Fibrinogen gamma chain (FGG) and Fibrinogen beta chain (FGB), as potential CVD biomarkers [73].

To overcome the barrier of early studies using whole tissue proteome, LMD of the intimal layer from human atherosclerotic and pre-atherosclerotic coronaries was used in combination with a 2D-DIGE approach. This study identified 13 proteins, with three of them,

Annexin 4 (ANXA4), Myosin regulatory light 2 smooth muscle isoform (MYL9) and Ferritin light chain (FTL), representing novel findings in the atherosclerotic coronary intima [7].

6.1.2 Cardiac tissue

The number of proteomic studies using human heart tissue samples is low, due to obvious difficulties in sampling. One study performed by Kakimoto et al. included the proteomic analysis of human cardiac tissue obtained from cases of sudden death due to AMI. In this study, using LC-MS/MS, reduced levels of the sarcoplasmic protein, Sorbin and SH3 domain-containing protein 2 (SORBS2) was detected in the infarcted myocardia. SORBS2 levels were also found increased in serum of AMI patients, indicating its potential to be used as a biomarker [74].

Aiming at identification of novel biomarkers for early detection of CHF, Zhang et al., characterized the PTMs associated with disease progression. Top-down quantitative proteomics using affinity chromatography and high resolution MS was applied onto human heart tissue samples. Phosphorylation of cardiac Troponin I was identified as a candidate biomarker for detection of CHF [61].

6.1.3 Atherosclerotic vulnerable plaques

Aiming at identifying circulating biomarkers derived from atherosclerotic vulnerable plaques that could predict adverse cardiovascular events, Malaud et al. performed protein enrichment followed by 2DE in protein extracts obtained from human fibrotic and hemorrhagic carotid atherosclerotic plaques. Several proteins were found to be differentially released by vulnerable hemorrhagic human carotid plaques when compared with stable fibrotic plaques. The authors suggest that combinations of the circulating biomarkers Calponin-1 (CNN1), IL-8, DJ-1 (PARK7), Vascular endothelial growth factor (VEGF) and PCPE-1 (PCOLCE) could be used for coronary patient stratification [75].

6.1.4 Thrombus

Intra-luminal thrombus (ILT) secretions from AAA patients were analyzed by LC–MS/MS and two thrombus associated proteins, serum Thrombospondin-1 (TSP-1) and Clusterin (CLU), were identified at reduced levels in circulation of patients with AAA compared to non aneurismal controls [76]. Targeting the identification of biomarkers in AAA progression, Martinez-Pinna et al. analyzed the proteins released by the different layers (luminal/abluminal) of the ILT by 2D-DIGE. This analysis identified Peroxiredoxin-1 (PRDX1) as prominently released by the luminal layer of the ILT. PRDX1 levels were further analyzed by ELISA in the serum of patients with AAA and were found increased compared to the healthy individuals. A significant positive correlation between PRDX1 serum levels with AAA size and growth rate had an additive predictive value in AAA, indicating PRDX1 as a potential biomarker for AAA progression [8]. The same group analyzed the tissue-conditioned media from patients with AAA by LC-MS/MS and identified that decreased levels and activity of systemic C3 in advanced AAA stages are associated with AAA evolution [77]. In another study, emphasis was given to the identification of peptides and low-molecular-weight proteins released by the different layers of abdominal aortic aneurysm thrombus. By employing SELDI-TOF mass spectrometry, LVV- Hemorphin7 (H7) and VV-H7, both generated from hemoglobin proteolysis, were found more abundantly released by recently formed luminal layer of AAA thrombus relative to intermediate and abluminal older layers. The levels of the H7 peptides were also found increased in the serum of AAA patients compared to controls and were positively correlated with the AAA diameter and thrombus volume. These peptides may be used as biological markers of pathological vascular remodeling [78].

The proteomic profiling of the coronary thrombus from MI patients has been recently performed using three different proteomic approaches: 2DE-MALDI MS/MS, 1DE- LC-MALDI-

MS/MS or 1DE-LC-ESI-MS/MS. Co-expression of 5 focal adhesion proteins [Fermitin homolog 3 (FERMT3), Thrombospondin-1 (THBS1), Myosin-9 (MYH9), Beta parvin (PARVB) and Ras-related protein Rap-1b (RAP1B)] with CD41 (ITGA2B) was found to be potentially implicated in platelet activation during thrombus formation. Additionally, Death-inducer obliterator 1a (DIDO1), was found up-regulated in the plasma of patients and was suggested as potential biomarker of thrombosis [28]. In an effort to better understand the characteristics of coronary thrombus, Ramaiola et al. employed 2DE-MS and found that the aged ischemic thrombi T6 (more than 6h of evolution) in STEMI patients are characterized by reduced levels of Profilin-1 (PFN-1) compared to T3 (thrombus of less than 3h of evolution) likely due to its release in the peripheral circulation since its levels were found by ELISA significantly increased in coronary and systemic blood in T6 patients compared to T3 [79].

6.1.5 Tissue secretome

Several proteomics approaches aimed at elucidating the molecular mechanisms involved in plaque formation to identify potential biomarkers for early diagnosis of disease. The advantage of using tissue secretome instead of whole tissue proteome is that secretome may reflect the *in vivo* condition with reduced complexity. Early studies revealed a significant reduction of soluble TNF-like weak inducer of apoptosis (sTWEAK) in carotid plaque supernatants compared to normal mammary endartery conditioned media [80]. The same trend was also described in chronic stable HF [81], peripheral artery disease (PAD) [82], AAAs [83] and CAD [84]. The potential of sTWEAK as prognostic biomarker in CVD has also been suggested [85,86].

The carotid plaque secretome has also been studied using LC-MS approaches. Among the 31 differentially secreted proteins from plaques, extracellular and intracellular proteins such as Thrombospondin-1 (THBS1), Vitamin D binding protein (GC), and Vinculin (VCL) were

identified. A significantly higher concentration of THBS1 and GC was confirmed in atherosclerotic subjects in comparison to controls [87].

In an attempt to identify novel circulating CAD biomarkers Reiser et al, analyzed the secretome derived from atherosclerotic plaques of carotid/iliac arteries and control arterial tissue by antibody phage display followed by MS. In parallel, gene expression analysis of the coronary thrombi versus peripheral blood mononuclear cells was performed. Integrating the data from the proteomic and gene expression studies enabled identification of Fatty acid-binding protein 4 (FABP4) as a candidate biomarker for CAD. FABP4 was found in the plaque but not in the control secretome and its expression was higher in thrombi than in peripheral blood mononuclear cells. FABP4 failed to serve either as a clinically relevant diagnostic marker in stable CAD and ACS or as prognostic biomarker in an asymptomatic population. However, FABP4 could identify ACS patients at risk for adverse cerebrovascular or cardiovascular events and therefore the authors concluded that circulating FABP4 may be utilized as a prognostic biomarker in risk stratification of ACS patients [88].

6.2 Plasma and Serum biomarkers

Plasma has been the main target specimen of biomarker research in cardiovascular disease, owed to its availability, and proximity to the affected organ (vessel or heart). The results obtained are summarized in **Table 2.** Darde et al. [89], investigated the plasma proteome from patients, healthy controls, and stable CAD patients using immunodepletion of the six most abundant proteins, and 2DE and 2D-DIGE analysis. Along with proteins previously associated with ACS, four novel proteins associated with the pathology [Alpha-1- B-glycoprotein (A1BG), Hakata antigen (FCN3), Tetranectin (CLEC3B) and Tropomyosin 4 (TPM4)] were identified [89]. More recently, Kristensen et al., employed plasma-based

quantitative proteomics for the analysis of four well-phenotyped patient cohorts: individuals 1) without cardiovascular symptoms and without the presence of coronary calcium, 2) without cardiovascular symptoms but with high amounts of coronary calcium, 3) operated because of atherosclerotic diseases, and 4) with ACS [90]. Through a 5-plex SRM-MS assay, statistically significant increased levels of the cytoskeletal protein Vinculin (VCL) were verified, along with other known risk markers of CVD such as CRP, Serum amyloid protein A (SAA1) and Apolipoprotein-A (APOA1) in the ACS group [90]. Lepedda et al., performed a comprehensive analysis of the differentially expressed Apolipoprotein component of plasma VLDL, LDL and HDL from patients undergoing carotid endarterectomy compared to healthy individuals. A panel of twenty three proteins was identified through the application of 2DE coupled with Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) MS analysis. Among them, Acute-phase serum amyloid A protein (SAA1) was over-expressed in all lipoprotein fractions indicating a potential association with the presence of advanced carotid lesions [91]. Recently, a novel targeted proteomics approach, based on the proximity extension assay proteomics chip, allowed the discovery of associations of Osteoprotegerin (TNFRSF11B), Growth hormone (GH1), Tumor necrosis factor ligand superfamily member 14 (TNFSF14) and Renin (REN) with plaque occurrence, independently of each other and traditional CVD risk factors [24]. Cheng et al. selected 13 candidate biomarkers possibly associated with plaque vulnerability and investigated their prognostic value in CAD patients in a nested-case-control study involving 768 patients undergoing coronary angiography. Increased plasma levels of circulating Osteoglycin (OGN) and Neutrophil gelatinase-associated lipocalin/matrix metalloproteinase 9 (NGAL/MMP9) were independently predictive for occurrence of major adverse cardiovascular event (MACE) within 1 year after

coronary angiography. Combining these two proteins with convential risk factors significantly improved risk reclassification [92].

AAA is associated with inflammatory responses with cytokines as important mediators. By analyzing the cytokine profile in plasma of patients with AAA via a protein array approach, elevated Insulin Like Growth Factor Binding Protein 1 (IGFBP1) was identified as a potential novel disease biomarker [25]. Burillo et al. performed differential quantitative proteome analysis using iTRAQ labeling and nano-LC-MS/MS of plasma proteins from patients at different AAA stages of evolution [small (aortic size 3-5cm) vs large AAA (aortic size > 5cm)]. Among the differentially expressed proteins APOA1 was identified significantly reduced in patients with large AAA compared to small AAA. Furthermore, a strong correlation of APOA1 levels with HDL-Cholesterol (HDL-C) concentration as well as with aortic size and thrombus volume (negative correlations) were also observed. The authors concluded that the systemic levels of APOA1/HDL-C are negatively associated with AAA progression [93].

To identify novel plasma protein biomarkers that could improve risk assessment for MI and atherosclerotic cardiovascular disease (ASCVD), Yin et al., compared plasma proteins in patients and controls through the application of a discovery-validation pipeline. In the discovery phase, plasma proteins in MI case-control pairs were analyzed using iTRAQ with multidimensional LC-MS/MS (discovery MS). The most promising protein biomarkers were subsequently chosen for the validation phase where plasma proteins from ASCVD case-control pairs were analyzed using MRM. This approach enabled identification of single- and multiple-marker protein panels significantly associated with CVD, the latter including 4 proteins in combination [α-1-acid glycoprotein 1 (ORM1), Paraoxonase 1 (PON1), Tetranectin (CLEC3B), and CD5 antigen-like (CD5L)] [94].

Comparative proteome analysis was performed in depleted plasma protein samples from patients with AMI, unstable angina pectoris (UAP), stable angina pectoris (SAP) and control subjects, using 2D SDS-PAGE followed by nanoLC-MS/MS analysis. Although two of the identified proteins, Alpha-1 microglobulin (AMBP) and Vitamin D-binding protein (GC) failed as suitable CVD markers, Apolipoprotein-A1 (APOA1) isoforms detected by 1D and 2D SDS-PAGE only in patients groups were presented as possible biomarkers for CVD [95]. In an effort to identify prognostic biomarkers for AMI, proteome analysis of depleted plasma samples from AMI patients was performed by 2D-DIGE. A 1-year follow-up was used to identify patients with heart failure and these data were used for the hierarchical clustering of the proteomic findings. Among the 22 differentially expressed proteins were isoforms of Haptoglobin (HP). Their distinct patterns were associated with stratification of the disease and HP plasma levels predicted the occurrence of HF at 1-year [96].

Identification of plasma biomarkers for early detection of myocardial injury is an active research field; one study applied integrated proteomic technologies in a human model that faithfully reproduces clinical aspects of spontaneous MI [patients undergoing a therapeutic, planned myocardial infarction (PMI) for treatment of hypertrophic cardiomyopathy]. Coherent, MS-intensive pipeline, including high-performance LC-MS/MS, accurate inclusion mass screening (AIMS), stable isotope dilution (SID)-MRM-MS and immunoassays were employed which collectively revealed a panel of candidate biomarkers that may be specific to MI. This included previously known cardiovascular biomarkers such as Creatine kinase MB, Fatty Acid Binding Protein (FABP) and Myeloperoxidase (MPO) and novel candidates such as Aortic Carboxypeptidase-Like Protein ACLP1, Four And A Half LIM Domains 1 (FHL1), Myosin Light Chain 3 (MYL3) and Tropomyosin 1 (TPM1) [97]. Applying an aptamer-based platform in patients with planned and spontaneous MI, 79 candidate infarct markers were

detected, including previously known markers (eg. Troponin I and Creatinine kinase MB) and novel candidates [eg. Fibroblast growth factor 18 (FGF18) and Interleukin-11 (IL11)]. This study allowed also the identification of low abundance cytokines and cell-surface proteins in association to disease, not previously reported [27].

Analyzing plasma samples from 90 CVD patients with a highly multiplexed MRM-based assay, Domanski et al assessed 67 putative CVD plasma biomarkers involved in coagulation and thrombolysis pathways, acute-phase reaction, inflammation as well as lipoprotein formation [19]. An MRM assay panel was also developed for the detection of ischemic heart disease and MI, based on 11 proteins with association to CVDs. Comparative analysis of plasma samples obtained from patients with ST-segment elevation MI and chest pain due to other causes identified increased levels of Apolipoprotein C1 (APOC1), Apolipoprotein C2 (APOC2) and Apolipoprotein E (APOE) in the former, rising the interest for further studying the role of these apolipoproteins in the pathophysiology of AMI [98].

Plasma proteomics also enables identification of potential biomarkers for acute decompensated heart failure (ADHF). Employing a discovery platform where depleted plasma protein samples from ADHF patients were analyzed by nano LC-MALDI-TOF/TOF and a verification platform based on nano LC-SRM analysis, Mebazaa et al. identified increased levels of Quiescin Q6 (QSOX1) as a novel biomarker further validated in human studies and also in animal models. Combination of QSOX1 to the gold standard biomarker BNP improved diagnostic accuracy and specificity for ADHF diagnosis [99].

To gain insight into the pathology of valvular aortic stenosis (AS), plasma proteins from AS patients and controls were analyzed by 2D-DIGE followed by MS. In this analysis, crude and pre-fractionated plasma either depleted or equalized was analyzed leading to the identification of 36 differentially expressed proteins in AS. These were further clustered into

functional groups including protease inhibitors and proteases, blood homeostasis and coagulation, inflammation and immune response, lipid metabolism and transport and others [100].

Degraba et al. investigated the proteomic signature of serum of patients with carotid artery disease compared to healthy individuals in order to identify protein biomarkers associated with increased atherosclerotic risk. Using 2D-DIGE in fractionated serum, decreased levels of α(1)-antitrypsin (A1AT), Haptoglobin (HP) and Vitamin D binding protein (GC) and increased levels of α(2)-glycoprotein precursor (AZGP1) in the subset of patients with symptomatic carotid atherosclerosis were detected [101]. Targeting to correlate CAD imaging findings with circulating biomarker expression, proteome analysis of the serum of 66 patients with stable or unstable angina and AMI after 3 and 6 months follow up was performed. Pro-inflammatory markers [6C Kine (CCL21), CTAK (CCL27), MIG (CXCL9) and Platelet Factor 4 (PF4)], pro-coagulable markers [D-dimer (FDP), Platelet Factor 4 (PF4) and Hepatocyte Growth Factor (HGF)] and marker of shear stress and remodeling [Follistatin (FST)] all decreased over time whereas anti-apoptotic markers [PAI-10 (CXCL10) and I-309 (CCL1) chemokine] were up-regulated [26]. Han et al. employed 2DE coupled with MS to characterize protein expression patterns in the serum of CAD patients. Serum cyclin-dependent kinase 9 (CDK9) was found elevated in the serum with a concomitant increase in monocytes and artery plaque samples of CAD patients. Further analysis indicated CDK9 as a potential biomarker of atherosclerotic inflammation [102].

In an effort to identify biomarkers for ventricular dysfunction and HF, Watson et al. analyzed the coronary sinus serum from asymptomatic, hypertensive patients. By employing 2DE, they detected increased levels of Leucine-rich α2-glycoprotein (LRG) in the serum of the

patients with increased BNP. Further investigation in an independent cohort demonstrated significant association of LRG with HF, independent of BNP [103].

Cubedo et al. compared the serum proteome of new-onset AMI patients with healthy individuals to identify biomarkers of early stages of AMI. Applying 2DE followed by MALDI-TOF, decreased levels of Apolipoprotein J/clusterin (APOJ) within the first 6h after the onset of AMI accompanied by alterations in its glycosylation pattern were detected [104]. Following the same approach, the same group compared the serum proteome from early and late phase AMI patients. Late phase AMI was associated with a decrease in immune response-inflammation proteins [alpha-1B-glycoprotein (A1BG), Fetuin-A (AHSG), Complex-forming glycoprotein HC (AMBP), Complement C1r Subcomponent (C1R), Complement Component 3 (C3) and Factor-B (CFB)] and an increase in the levels of Serum amyloid P-component (SAP) in comparison to early AMI [105]. In a more recent study, the same group also found that decreased serum Retinol Binding Protein 4 (RBP4) levels are implicated in acute new-onset MI in male patients [106].

The detection of novel serum biomarkers could be also very helpful for both diagnosis and development of novel treatment modalities for transient ischemic attacks (TIA). To this end, George et al., attempted to identify novel serum TIA biomarkers through the application of mass spectrometry-based proteomics. The study highlighted Platelet basic protein (PBP) as a candidate TIA/minor stroke serum biomarker [107].

To identify biomarkers for acute aortic dissection (AAD), Gu et al., compared serum samples from AAD patients, AMI patients and healthy individuals using iTRAQ MS proteomics. Analysis of the differentially expressed proteins identified increased levels of Lumican (LUM) as a potential AAD-related serum marker [108].

6.3 Urine

Urinary proteomics benefits from the considerable stability of the urinary proteome and the establishment of large scientific networks [109], and hence has been applied in multiple studies also in the context of CVD (**Table 3**). Hou et al. applied 2D-DIGE for the analysis of urinary protein extracts of CHF patients and healthy donors. Among the twenty detected differentially expressed proteins, Orosomucoid 1 (ORM1) was selected based on bioinformatics analysis for further verification by Western blot analysis and ELISA. The authors conclude that increased levels of ORM1 may be used as potential novel urinary biomarker for the early detection of CHF [110].

Applying 1DE-LC-MS/MS, Lee et al. detected increased levels of monocyte antigen CD14 in the urine samples from CAD patients compared to controls. Furthermore, the proportion of CD14+ monocytes was found elevated in CAD patients compared to controls. The authors suggested that the increased release of CD14 in urine coupled with the elevated number of CD14+ monocytes in CAD patients may be associated to CAD severity [111].

Recently, efforts have been made towards employing multiple proteomic biomarkers, in order to more comprehensively depict complex disease pathophysiology. To this end, Delles et al., analyzed urine samples from 623 individuals with and without CAD by CE-MS. A panel of 238 CAD-specific urinary polypeptides with good sensitivity and specificity for CAD diagnosis was reported. The identified discriminatory polypeptides included fragments of alpha-1-antitrypsin (SERPINA1), Collagen types 1 and 3, Granin-like neuroendocrine peptide precursor (PCSK1N), Membrane-associated progesterone receptor component 1 (PGRMC1), Sodium/potassium-transporting ATPase gamma chain (FXYD2) and Fibrinogen-alpha chain (FGA) [13]. The same group, by following the same approach, recently demonstrated the value of the CAD238 panel as predictor of coronary events in asymptomatic subjects with

hypertensive atherosclerotic cardiovascular disease (HACVD) [112] and in the diagnosis of stable angina [113].

The development of urinary biomarker panels has also been applied targeting early detection of ischemic stroke specifically reliable detection of minor ischemic stroke or TIA, especially in cases of inconclusive brain imaging [14]. To this end, the urinary proteome of patients with acute stroke or TIA and controls with elevated cardiovascular risk was explored through the application of CE-MS. Candidate biomarkers were further identified following LC-MS/MS. A biomarker-based classifiers was developed that enabled differentiating between patients with acute stroke or TIA and controls [14]. Peptides of the discriminatory panels included FXYD domain-containing ion transport regulator 4 (FXYD4), Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), Uromodulin (UMOD), Polymeric-immunoglobulin receptor (PIGR) and Collagen fragments.

A pilot study, using CE-MS based urine proteome analysis, enabled identification of a vast array (103) of HFrEF-related urinary peptide biomarkers which mainly included fragments of fibrillar type I and III Collagen but also Fibrinogen beta (FGB) and alpha-1-antitrypsin (SERPINA1) peptides [15]. This opens the possibility of early diagnosis of HFrEF even before the disease progresses to an overt symptomatic stage. Application of the same technology identified a set of HFrEF-specific urinary peptide biomarkers on a background of chronic kidney disease (CKD). Consistent with the previous study, the majority of sequenced peptides were fragments of Collagen type I and III [114].

Urinary proteome-based classifiers have been assessed for their value in the early detection of asymptomatic left ventricular diastolic dysfunction (LVDD) in hypertensive patients. In one study, a set of urinary polypeptides was identified by CE-MS to distinguish hypertensive patients with overt HF from healthy controls: HF1 and HF2 are classifiers based on 85 and

671 urinary peptides, respectively, and were generated to distinguish between patients with LVDD and controls [115]. These classifiers were further assessed in a population study by analyzing the urinary proteome by CE-MS [116]. The same group, in a subsequent study investigated whether HF1 classifier predicts cardiovascular end points in a population of 791 randomly recruited normal subjects over a 5 year follow-up period. The urinary proteomic signature was found to predict CVD incidence at higher accuracy than systolic pressure [117]. While the advantage of CE-MS in the management of CVD is very promising and attributed not only to the robustness of the technique (i.e. inter-laboratory variability, stability, interference with drugs) [118], but also to its multiple applications in patient management and drug development [119], it has a low urine loading capacity and is only suitable for small proteins (< 20 kDa) [119]. CE-MS identifies naturally occurring peptides with distinct c- and n-terminal that enables a specific evaluation of the disease pathology. Frequently a highly significant change in abundance of specific collagen fragments is detected. The most likely hypothesis today is that these specific peptides reflect altered protease activity which has a specific impact on collagen (as well as likely multiple other proteins), and which is associated with several pathologies [120].

6.4 Circulating Cells

Circulating cells and vesicles may play a major role in CVD pathophysiology. Several proteomics studies targeting these specimens have been reported, summarized in **Table 4**.

Polymorphonuclear neutrophils (PMNs) play a key role in the pathophysiology of AAA progression [121,122], and as such, Ramos-Mozo et al., comparatively analyzed their proteome in AAA patients and controls. Using 2D-DIGE-MALDI MS, they identified decreased

levels of Catalase (CAT) further confirmed by an apparent decrease activity of Catalase in circulating PMNs and plasma in AAA patients [123].

To gain insight into the association of circulating cells with coronary atherosclerosis, Bleijerveld et al. performed in-depth proteomic profiling of granulocytes in a cohort of patients suffering from chronic (sub)total coronary occlusion in comparison to matched control patients. Using stable isotope peptide labeling and 2DE LC-MS/MS, 57 candidate biomarker proteins were identified including Bactericidal/permeability-increasing protein (BPI), Ficolin-1 (FCN1), Charcot–Leyden crystal protein (CLC), Eosinophil granule major basic protein (MBP) and Eosinophil-derived neurotoxin (EDN). Following further verification in an independent cohort by label-free proteome analysis, the down-regulation of Bactericidal/permeability-increasing protein (BPI) in circulating granulocytes was proposed as a promising biomarker for severe atherosclerotic coronary stenosis [124].

To investigate the profiles of macrophages from AAA patients and peripheral arterial occlusion (PAO) patients without AAA, transcriptomics and proteomics approaches were followed. Differentially expressed proteins were identified through the application of 2D-DIGE followed by MALDI TOF. An antibody protein array was used to validate selected proteins found (or predicted) to be differentially expressed in macrophages and plasma based on the transcriptomic and proteomic analysis. TIMP-3, ADAMTS5, and ADAMTS8 were found differentially expressed between the macrophages and plasma of AAA and PAO patients [125].

Changes in the proteome profile of circulating endothelial cells (CECs) and EPCs have also been described in ACS. CECs and EPCs were isolated by flow cytometry from blood obtained from ACS patients and control subjects. Proteome analysis by LC-MS/MS identified differences in EPC and CEC proteins between control and ACS patients, predicted to

segregate into 6 molecular pathways (5HT4 type receptor mediated signaling pathway, 5HT3 type receptor mediated signaling pathway, 5HT1 type receptor mediated signaling pathway, Adrenaline and noradrenaline biosynthesis and Heterotrimeric G-protein signaling pathway-rod outer segment phototransduction exclusively represented in ACS). Although this study indicated the potential of CEC and EPC to reflect CVD, the development of a ''gold standard'' protocol for their isolation is imperative for their further implementation in biomarker development and diagnostics [53].

6.5 Extracellular vesicles

Membrane microvesicles (MVs) are released into the circulation from activated cells, such as platelets, they mediate intercellular communication, and their number is increased in patients with ACS [126]. To develop a unique panel of proteins discriminating patients with STEMI from stable CAD controls, Velez et al. performed comparative proteome analysis in plasma MVs based on 2D-DIGE MS. An up-regulation of α2-macroglobulin isoforms, Fibrinogen, and Viperin (RSAD2) was detected in MVs from STEMI patients [54].

In a recent study by Cheow et al. the proteome profile of plasma EVs derived from patients with MI in comparison to EVs from patients with stable angina was analyzed by LC-MS/MS. A biomarker panel for MI detection was developed, comprising of six up-regulated proteins: Complement C1q subcomponent subunit A (C1QA) and Complement C5 (C5), both associated with complement activation; Apoliporotein D (APOD) and Apolipoprotein C-III (APOCC3), implicated in lipoprotein metabolism; and Platelet glycoprotein Ib alpha chain (GP1BA) and Platelet basic protein (PPBP), both related to platelet activation [127].

Martinez-Pinna et al. employed label-free LC-MS for the quantitative proteome analysis of plasma-derived microvesicles (exosomes and microparticles) from AAA patients and control

627 subjects. Differentially expressed proteins not previously associated with AAA were
628 detected. Among the proteins upregulated in exosomes from AAA patients were Ferritin
629 light chain (FTL), C-reactive protein (CRP) and Platelet factor 4 (PLF4) whereas Dermcidin
630 (DCD), Annexin A2 (ANXA2) and Oncoprotein-induced transcript 3 protein (OIT3) increased in
631 microparticles of AAA patients [128].
632 The proteome of plasma-microparticles has also been investigated in DVT through the
633 application of 2D MALDI MS/MS. Galectin-3 binding protein precursor (LGALS3BP) and
634 Alpha-2 macroglobulin (A2M] were significantly enriched in DVT patients whereas among
635 others Alpha-1-antitrypsin precursor (SERPINA1), Histidine-rich glycoprotein precursor
636 (HRG), Hemopexin precursor (HPX), Fibrinogen beta chain precursor (FGB), Isoform Gamma-
637 B of Fibrinogen gamma chain precursor (FGG), Fibronectin 1 isoform 4 preproprotein (FN1),
638 and Rheumatoid factor RF-IP16 (RF) were reduced in DVT versus controls [129].
639
640 **6.6 Platelets**
641 Banfi et al. compared the proteome of isolated platelets from patients with stable angina or
642 non-ST elevation ACS compared to control subjects with no history of CAD, using 2DE-MS. Six
643 differentially expressed proteins were identified associated with energy metabolism [2-
644 oxoglutarate dehydrogenase (OGDH), and Lactate dehydrogenase (LDH)]; cytoskeleton-
645 based processes [Gamma-actin (ACTG1), Coronin 1B (CORO1B), and Pleckstrin (PLEK)]; or
646 involved in protein degradation [Proteasome subunit type 8 (PSMB8)] [130]. Comparing the
647 platelet proteome from patients with ACS to that of patients with stable CAD with 2DE-MS,
648 the levels of proteins involved in cellular cytoskeleton [F-actin capping (CAPZA2), β-tubulin
649 (TUBB), α-tubulin isotypes 1 and 2, Vinculin (VCL), Vimentin (VIM) and two Ras-related
650 protein Rab-7b isotypes (RAB7B)], glycolysis [Glyceraldehyde-3-phosphate dehydrogenase

(GAPDH), Lactate dehydrogenase and two pPruvate kinase isotypes]; cellular-related antioxidant system (Manganese superoxide dismutase), cell survival [Proteasome subunit β type 1 (PSMB1)] and the expression and activity of Glutathione-S-transferase were significantly reduced in platelets from ACS patients compared to CAD patients [131]. Platelets from patients with non-ST segment elevation ACS versus stable CAD controls were analyzed by 2DE-MS. The identified differentially expressed proteins were involved in cellular cytoskeleton [Actin Cytoplasmatic-1 (ACTB), Alpha-actinin-1 (ACTN1), Caldesmon (CALD1), F-actin-capping protein subunit beta (CAPZB), Filamin-A (FLNA), Myosin-9 (MYH9), Talin-1 (TLN1), Tropomyosin alpha chain 3 (TPM3), Zyxin (ZYX)], signaling [Adenylyl cyclase-associated protein 1 (CAP1), FYN-binding protein (FYB), Integrin-linked protein kinase (ILK), Proto-oncogene tyrosine-protein kinase Src (SRC), Rho GDP-dissociation inhibitor 2 (ARHGDIB)] and ECM [Serum Albumin, Secreted protein acidic and rich in cysteine (SPARC)], vesicles/secretory trafficking pathway [Dynamin-1-like protein (DNM1L), Ras-related protein Rab-27B (RAB27B), -6B (RAB6B), -11A (RAB11A)] [132].

Alterations in the platelet proteome have also been associated with HFpEF. Raphael et al. analyzed the proteome of platelets from three groups: 1) patients hospitalized with symptoms of HFpEF, 2) the same subjects several weeks later without symptoms and 3) control subjects. Among the 6102 proteins identified by mass spectrometry, S100A8 was found to be consistently expressed in HFpEF patients compared to controls. The levels of this protein were also found increased in the plasma of subjects with HFpEF in an external cohort. To investigate whether S100A8 is causal or only associated with the disease, its effects were further assessed in human induced pluripotent stem cell-derived cardiomyocytes. The results indicated direct effects of S100A8 on the electrophysiological

and calcium handling profile and suggest that this protein may be causally contributing to HFpEF [133].

7. Factors with an impact on cardiovascular biomarkers

7.1 Sex and ethnicity

Alterations in the plasma levels of CVD biomarkers are also associated with sex and ethnic differences and may have value in individualized CVD risk assessment. In a Mayo Clinic study, uniplex and multiplex assays were used to measure the circulating levels of 47 candidate CVD markers in men and women of African-American (AA) and non-Hispanic White (NHW) ethnicity. Female gender was associated with higher levels of inflammatory markers, adipokines, lipoproteins, natriuretic peptides, vasoconstrictor peptides and markers of calcification and thrombosis. AA ethnicity was associated with higher levels of inflammatory markers, leptin, vasoconstrictor-antidiuretic peptides and markers of calcification and thrombosis and with lower levels of adiponectin and vasodilator-natriuretic peptides [134].

7.2 Diet

Polyphenol rich diets are associated with reduced risk of CVD, hence their potential effects on CVD biomarkers may be relevant. A pilot study was conducted to detect quantitative differences in the urinary CAD biomarkers (CAD238 [13]) caused by short-term consumption of a polyphenol rich (P-R) drink. A group of overweight healthy subjects were randomized to P-R drink or placebo for two weeks. Following urinary analysis by CE-MS, 27 polypeptides that displayed more than 4-fold difference between the two groups were detected. Among these, seven were included in CAD238, five of which [Fibrinogen alpha chain (FGA) [607-622], Collagen alpha-1(I) chain (COL1A1) [543-588], Collagen alpha-2(V) chain (COL1A2) [1209-1225], Xylosyltransferase 1 (XYLT1) [51-66], Ig kappa chain C region (IGKC) [9-23]]

changing towards the healthy profile while two of them [Alpha-1-antitrypsin (SERPINA1) [276-295] and Collagen alpha-1(I) chain (COL1A1)[1095-1106]] changing towards CAD. Based on these results the authors suggested that P-R drink may have beneficial effects in CAD prevention [135]. More recently, the same group, in an effort to evaluate the impact of olive oil (OO) consumption in CVD prevention, investigated the impact of diet supplementation with OO, either low or high in phenolics, on urinary proteomic biomarkers (CAD238 panel). A significant improvement in the scoring of the CAD238 panel after supplementation with OO for 6 weeks was demonstrated, indicating a benefit of OO in preventing CVD [136].

7.3 CKD

CVD could also be a secondary endpoint in patients with CKD disease. To address this issue, Schiffer et al., performed a diagnostic phase I/II study in which CE-MS was applied to analyze plasma specimens from CKD stage 5D patients suffering from vascular disease. Comparative analysis identified 13 novel biomarkers for CVD of which four were identified by tandem MS as fragments of Collagen alpha-1 type I and III and one as fragment of Apolipoprotein C3 (APOC3). The markers were validated in an independent blinded cohort and enabled distinguishing mild and severe CVD with good sensitivity and specificity, suggesting that this specific polypeptide pattern in the plasma of CKD patients reflects CVD [137].

7.4 Ageing

Urinary proteomics was also employed to investigate the proteomic transition from normal ageing to age-related pathological complications including CVD. Using CE-MS in a study including over 10000 subjects, 112 age-correlated peptides (mainly originated from collagen, uromodulin and fibrinogen) were identified. Pathway analysis revealed perturbations in collagen homeostasis, trafficking of toll-like receptors and endosomal pathways being

associated with ageing in general, whereas increased degradation of insulin-like growth factor-binding proteins (IGFBPs) was observed in pathological ageing only [138].

7.5 Hemodialysis

Plasma proteomic analysis of the HDL fraction may enable identification of candidate biomarkers associated with cardiovascular risk in end-stage renal disease (ESRD) patients undergoing hemodialysis. To this end, Mange et al. investigated the proteome of HDL isolated from plasma of hemodialysis patients at high risk for CVDs and healthy volunteers. Using iTRAQ labeling and nano-LC/MS/MS analysis, forty proteins differentially expressed in the two groups were identified. These proteins were primarily involved in lipid metabolism, inflammation, complement activation and metal cation homeostasis. Among them, Apolipoprotein C2 (APOC2) and Apolipoprotein C3 (APOC3) were found elevated in hemodialysis patients whereas Serotransferrin was reduced after validation in an independent population set. The authors conclude that the identified proteins are linked to HDL dysfunction in chronic hemodialysis patients [139].

8. Data integration in CVD

The advantage of integrating different omics approaches has previously been shown in the management of chronic kidney disease [140]. Not only is the identification of a broader range of biomarkers possible, but also the systemic understanding of related molecular mechanisms is achievable, both contributing to improving disease management [3,4]. Towards this end, we performed gene ontology analysis of all the biomarkers summarized in the tables of this review through Cytoscape. Inflammation (acute, humoral, complement

activation etc.), wound healing and coagulation (platelet activation, degranulation, fibrin clot formation), proteolysis and extracellular matrix organization, and handling of cholesterol and LDL were among the most significantly pathways and biological functions reflected by the proteomic changes observed in CVD (data provided in supplementary table 2).

First reports on combining different omics traits were published recently. The integration of genomics and metobolomics has been explored in CVD and findings have shown an added value in the approach [141]. An integrated metabolomics and genome-wide association study (GWAS) has revealed that single nucleotide polymorphisms can explain variances observed with some metabolite levels [142]. Through the integration of the transcriptomic with proteomic findings from myocardial tissues global changes in dilated cardiomyopathy (DCM) in genes, proteins and pathways involved in cardiac function were unraveled [143]. A network biology approach that combined genomic and proteomic data was developed and distinguished pathological from physiological left ventricular hypertrophy (LVH) [144]. In another study, genetic, transcriptome and protein analyses of titin truncations provided evidence into the mechanisms of end-stage DCM [145]. A strategy combining proteomics with metabolomics was applied in human cardiac tissues from individuals with normal heart or patients with ischaemic (ISCM) and idiopathic dilated cardiomyopathies (IDCM). This study enabled the characterization of the differential regulation of molecular pathways and metabolism in ISCM and IDCM for the first time [146]. Apart from the potential to elucidate mechanisms of disease pathogenesis, the integrative omics approaches also hold promise for personalised medicine [147]. Combined, the published reports suggest that systems biology approaches underpinned by omics strategies in animal models and patients have the potential to aid in the dissection of the complicated networks interfering in cardiovascular pathophyshiology and lead to the development of novel diagnostic and therapeutic

769 modalities [119,148-150]. However, although the benefits of omics integration are quite
770 obvious, it is very challenging to achieve a successful integration. A major challenge
771 associated with data integration is the heterogeneity of data generated by omics
772 technologies. There is clearly an urgent need to develop robust algorithms that will facilitate
773 this process.

774 **9. Expert commentary**

775 Significant progress towards the development of state of the art proteomic technologies
776 applicable in the context of CVD has been made. These advancements include improvement
777 of sample preparation methods for detection even of the low-abundance proteins as well as
778 the development of high-throughput proteomic profiling techniques that enable the
779 detection of multiple candidate biomarkers rapidly and with high accuracy. Although early
780 studies focused mainly on tissue and plasma proteomics, recent studies also targeted other
781 biological sources such as urine, extracellular vesicles and sub-cellular proteomes for
782 biomarker discovery. The number of potential biomarkers that have arisen through the
783 application of proteomic technologies has substantially increased in the past few years. In a
784 considerable number of studies, efforts have been put on unraveling panels of proteomic
785 biomarkers. These appear to be of higher stability and accuracy than single biomarkers, since
786 panels are better suited to display the complex disease pathophysiology [151].
787 However, we also see substantial shortcomings in unfortunately many studies. As outlined
788 by us and others in detail (e.g. [152-154]), the mere detection of the association of a protein
789 with disease is insufficient to support claims for biomarkers. As a minimum a potential
790 biomarker must have a defined context-of-use and demonstrate a significant improvement
791 of the current state-of-the-art, in the population it is intended to be used. Unfortunately,
792 many studies fall short of this minimal requirement, and consequently the biomarker value

and reliability of findings cannot be readily attributed. In addition, although other factors and diseases, like diabetes type 2 and CKD, have significant impact on cardiovascular biomarkers and should be taken into consideration when performing such studies, this is frequently not attempted. Progress has been made in recent years in the development of proteomics technologies. Application of these novel approaches in the discovery process may result in additional, novel findings. In addition, major efforts should be directed towards actual assessment of the value of existing biomarkers for a specific context of use, in the relevant population, and in the light of the current state-of-the-art (currently routinely used biomarkers and pertinent demographic information). This has been outlined several years ago for bladder cancer [155], but the exact same considerations hold true for cardiovascular disease.

10. Five-year view

Summarizing the recent progress in the discovery of proteomic biomarkers for CVD, it is obvious that despite the plethora of studies performed in this field, only few of them appear close to clinical implementation. A significant number of studies stops at the discovery phase without subsequent validation of the findings. Since many of the studies cited in this review are very recent, we hope for a rapid progress in biomarker validation the next years.

Nevertheless, some biomarker panels have arisen the recent years holding promise for better management of CVD based on reported validation studies. It appears that the most relevant context of use for proteomics biomarkers in CVD is enabling early detection and prognosis.

The next step for these panels is to be validated in blinded studies and subsequently approved by the regulatory authorities for clinical application. An example is the application

of a proteomic biomarker panel for CKD detection: the panel, CKD273, was developed based on over 600 samples [156], and subsequently evaluated and validated in multiple studies [157-159]. As a result, this urinary proteome-based panel is now being used in patient stratification in PRIORITY, a large multicentric randomized controlled trial [160,161] and has also received a letter-of-support from the US-FDA (http://www.fda.gov/downloads/Drugs/DevelopmentApprovalProcess/UCM518268.pdf).

As depicted in **Figure 4**, the current state-of-the-art allows assessment of CVD with high confidence at late stage, when significant and frequently irreversible organ damage has occurred. Hence it seems biomarkers enabling detecting established disease are of very little, if any value. The potential of proteomic biomarkers appears in the early detection of disease, when disease-initiating molecular processes are already ongoing, but clinical symptoms are not detectable. This is also supported by the thoughts outlined in **Figure 1**: the hypothesis that proteins are the cause of CVD onset and progression. At an early stage also intervention will be most effective, ideally even preventing onset of disease.

Such approaches also depend on a holistic understanding of CVD processes, which will be one of the biggest challenges in forthcoming studies. In the near future, the development of systems biology approaches through the integration of omics technologies will provide a more systematic insight into CVD [3,4]. Integration of the proteomics data with findings from other omics approaches and bioinformatics analysis will pave the way for the development of novel biomarker panels detecting early stages of the disease leading to CVD prevention. A new era of proteomics towards personalized cardiovascular medicine will hopefully begin soon.

11. Key issues

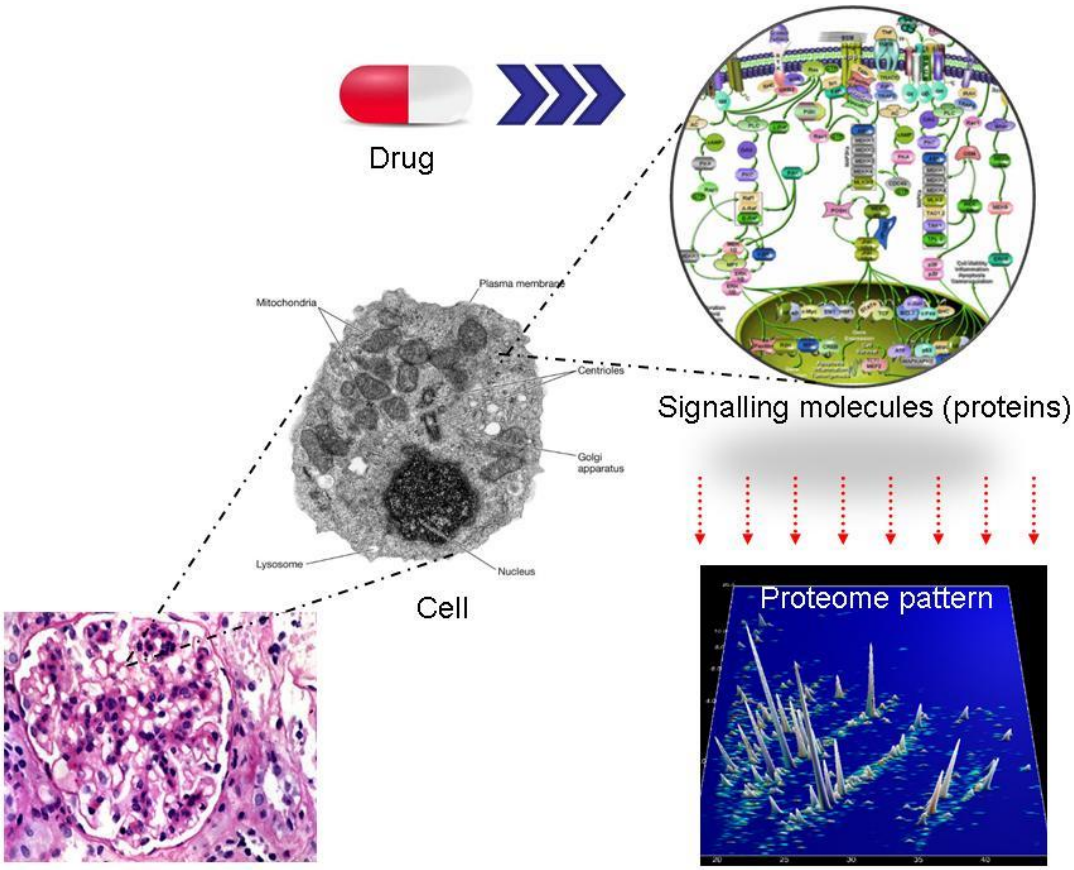
- CVD remains the major cause of morbidity and mortality worldwide, knowledge on molecular mechanisms and potential drug targets as well as validated biomarkers guiding intervention are urgently required.
- Proteomics has the ability to allow insights into the mechanisms underlying cardiovascular disease, should enable defining disease on a molecular level.
- Advancements and development of state of the art proteomics technologies have facilitated the high-throughput analysis of many and different samples.
- Progress has been also made the recent years towards the development of biomarker panels, demonstrating superiority over single biomarkers.
- A list of promising results and multiple potential biomarkers exists, but has to be investigated and validated in detail to have an actual impact, improve patient care in the near future.
- Other factors and systemic disorders have an impact on cardiovascular biomarkers and therefore should be considered when analyzing data from discovery and validation phases.
- Verification of the proteomic findings in appropriately powered studies appears imperative now, to receive approval from the regulatory authorities and enable implementation.

Figure 1: Graphic depiction of the information obtained from the different approaches. Histology can give information on microscopic structural changes. Subcellular structures, and even more the molecules involved in the molecular pathology can generally not be assessed. In contrast, proteome analysis does not give information on morphological changes, but gives information on global protein changes, which can be associated with the molecular changes in disease. Proteins represent the most appropriate targets of therapeutic drugs, hence, proteome analysis can give guidance on the molecular structures to be targeted in therapy, an information that can generally not be obtained from histology. Adapted with permission from [162].

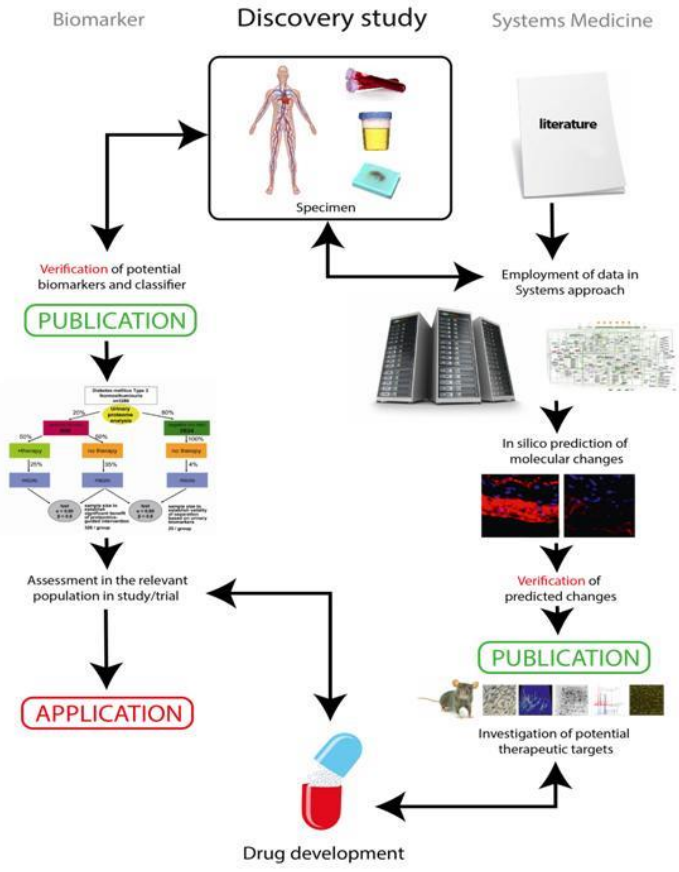
Figure 2: Graphical depiction of the main two different routes clinical proteomics approaches can take: towards biomarker discovery, or a Systems Medicine approach towards "modelling disease": The biomarker approach (left) requires identification of distinct potential biomarkers that are subsequently verified, and then applied in the appropriate clinical setting. If benefit can be demonstrated, they should be implemented/routinely applied. The Systems medicine approach takes advantage of the breadth of data, including literature sources, aims at molecular modelling of disease and predicting key structures. These are then verified (first valid result), and subsequently their value as potential targets is investigated in appropriate interference studies. If positive, drugs can be developed, tested, and, if beneficial, should be applied. Starting with the same or similar data, the downstream utilization (and the associated issues) is quite different, but ultimately in both cases we aim at investigating proteomics to improve patient care.

Figure 3. Schematic representation of the workflow followed in cardiovascular proteomic studies. A plethora of biological sources including arterial tissue, cardiac tissue, tissue secretome, blood, urine, thrombus, circulating cells and platelets have already been used to investigate the proteome alterations in cardiovascular disease (CVD). Analysis of these proteomes through global proteomics (discovery phase) and targeted proteomics (validation phase) approaches will make possible the identification of novel candidate biomarkers for better management of CVD. Of note, there are four biomarkers recommended for clinical use: 1) Cardiac Troponin T and Troponin I, 2) B-type natriuretic peptide (BNP), 3) C-reactive protein (CRP) and D-dimer, 4) Apolipoprotein A-I.

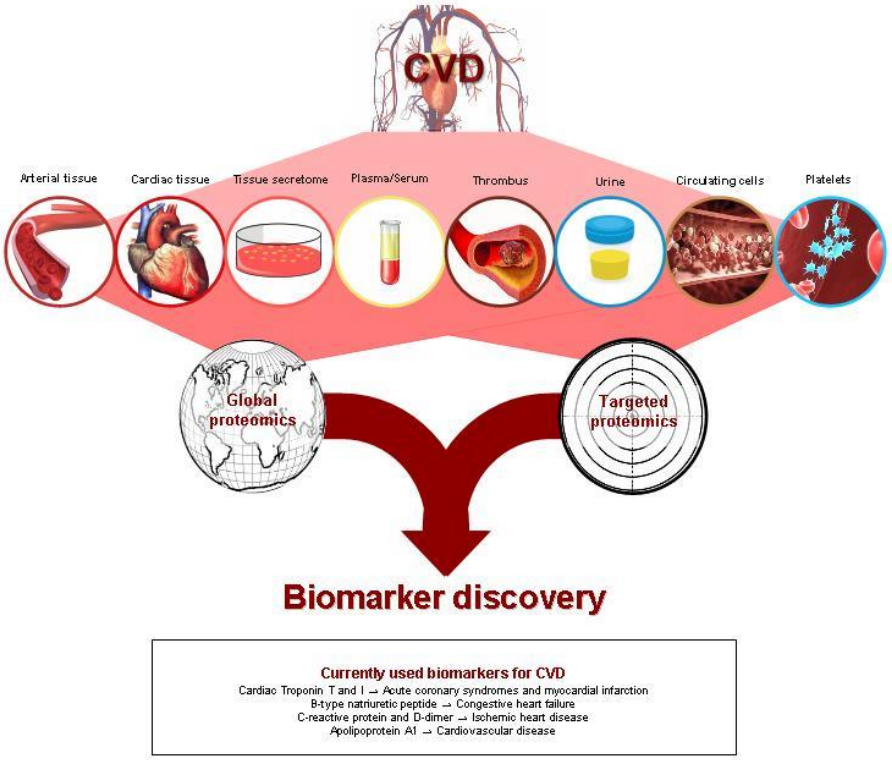
Figure 4. Early diagnosis and/or prognosis of diseases improves chances for a better outcome for the patients. The initiation of molecular processes that result in (chronic) diseases can be detected based on the decisive molecular changes, using proteomic technologies, substantially prior to advanced organ damage. In this early stage, molecular changes could be reverted, and onset of clinically relevant disease could be prevented. As a minimum, further progression can be slowed down by applying of appropriate therapeutic interventions and/or changes to the lifestyle. At a later stage, the disease becomes clinically evident, as a result of the organ damage. However, at this point in time curative treatment is not possible anymore, only disease progression can possibly be delayed to some extend (although with moderate success, as also evident by the multiple trials that failed). Reprinted with permission from [163].


Drug
Signalling molecules (proteins)
Mitochondria
Plasma membrane
Centrioles
Golgi apparatus
Lysosome
Nucleus
Cell
Proteome pattern
Tissue

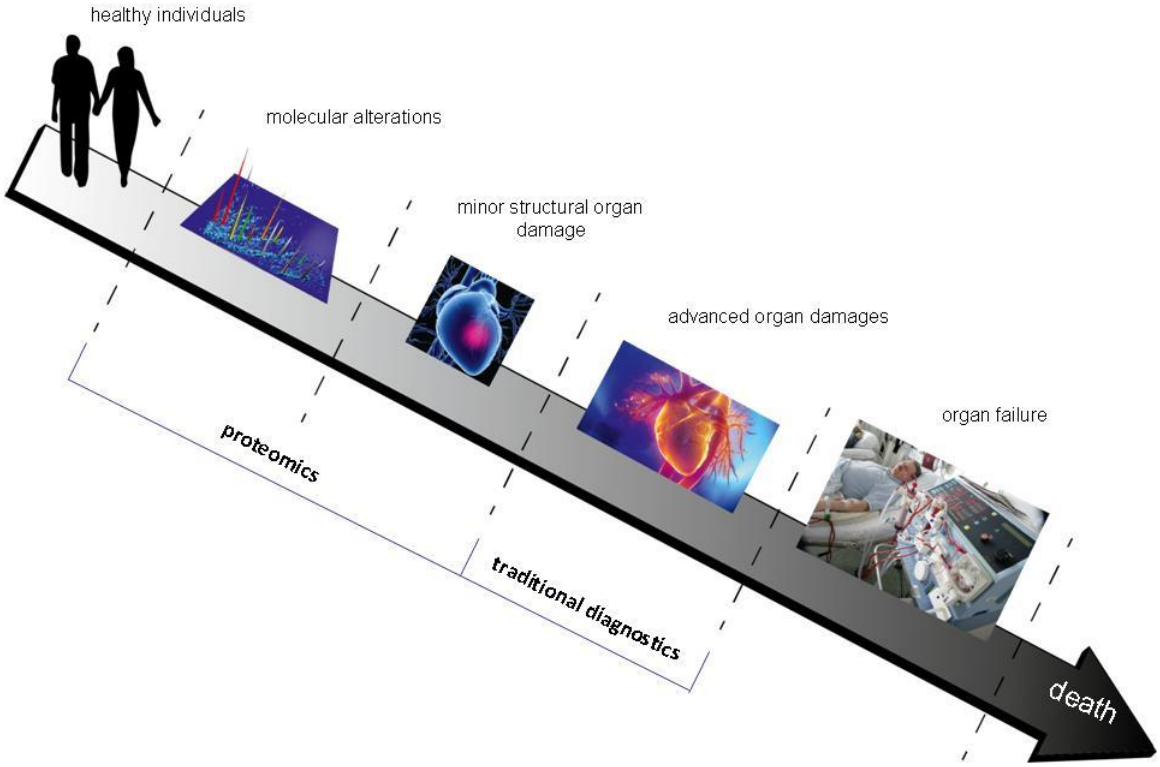
905 •


Biomarker
Discovery study
Systems Medicine
Specimen
literature
Verification of potential biomarkers and classifier
PUBLICATION
Employment of data in Systems approach
In silico prediction of molecular changes
Assessment in the relevant population in study/trial
Verification of predicted changes
PUBLICATION
APPLICATION
Investigation of potential therapeutic targets
Drug development

906 •


CVD
Arterial tissue
Cardiac tissue
Tissue secretome
Plasma/Serum
Thrombus
Urine
Circulating cells
Platelets
Global proteomics
Targeted proteomics
Biomarker discovery
Currently used biomarkers for CVD
Cardiac Troponin T and I → Acute coronary syndromes and myocardial infarction
B-type natriuretic peptide → Congestive heart failure
C-reactive protein and D-dimer → Ischemic heart disease
Apolipoprotein A1 → Cardiovascular disease

907


healthy individuals
molecular alterations
minor structural organ damage
advanced organ damages
organ failure
proteomics
traditional diagnostics
death

908

909

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Reference annotations

**** Of interest***

***** Of considerable interest***

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