Identifying new Risk Markers and Potential Targets: The Value of the Proteome

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Abstract. Several protein biomarkers, including cardiac troponin T, cardiac troponin I, B-type natriuretic peptide, C-reactive protein and apolipoprotein A-I, are widely employed in the evaluation of cardiovascular disease. Several of such potential biomarkers, or their multiscores, have been assessed over the last years for the prediction of cardiovascular risk but only a few of them have been validated for clinical use. Substantial improvement in the cardiovascular risk prediction and reclassification relative to traditional models therefore remains a difficult task presently unresolved. Hence, a potential importance of alternative approaches which may rely on novel proteomic biomarkers among others. Plasma or serum concentrations of numerous proteins were measured using proteomic approaches to establish their relationships with cardiovascular disease; none of them was however evaluated for cardiovascular risk prediction and subject stratification in rigorous large-scale studies. Thus, further research is needed to identify novel candidates that can improve cardiovascular risk prediction, subject stratification and standard care. Proteomics will undoubtedly remain a key approach to address this major clinical and scientific challenge.

1. Introduction

The concept of proteomics aims at characterizing and quantifying the proteome - all proteins present in a biological fluid, biological tissue or a given cell type. The term “proteome” was coined by Mark Wilkins as early as 1994 to describe total protein composition of a biological tissue or cell type [1]. Key technical advancements, including developments of soft ionisation techniques and specialized algorithms to analyse the proteome, occurred over this time to allow quantitative assessment of the proteome. As a result, proteomics has emerged as a tool to evaluate metabolic alterations present in a disease state or pathological condition. Multiple applications of proteomic analyses to the clinics have been developed to contribute to our understanding of molecular mechanisms of diseases and to identify potential biomarkers of disease presence and progression.

Biomarkers are molecules whose levels provide information about disease. Biomarkers are typically determined in biological fluids that can be readily obtained under clinical conditions, primarily in plasma, serum and urine. Measurements of biomarkers can be valuable to diagnose the disease, to assess its progression, to monitor effects of a therapy, to discover novel therapeutic targets and to understand molecular mechanisms underlying the disease development. The importance of the biomarker development in the field of cardiovascular (CV) disease, which represents the leading cause of death in industrialized countries, is highlighted by the need of improved strategies for the primary prevention of CV disease. Indeed, predictive value of current risk-assessment models remains limited, with a large proportion of patients without traditional risk factors (up to 20%) [2, 3]. Thus, novel diagnostic and prognostic biomarkers of CV risk bear a potential to improve the selection of individuals for preventative strategies and to ameliorate disease management. This review

summarizes our current knowledge of the value of proteomics to discover new biomarkers and targets in the field of CV disease.

2. Novel protein biomarkers and CV risk prediction

Several protein biomarkers are widely employed in the evaluation of CV disease (Table 1). Thus, **cardiac troponin T** and **cardiac troponin I (cTnI)** are used for the diagnosis of acute coronary syndromes and myocardial infarction (MI), **B-type natriuretic peptide (BNP)** and its N-terminal form are employed to diagnose congestive heart failure, **C-reactive protein (CRP)** is a useful biomarker of inflammation in atherosclerosis, and **apolipoprotein A-I (apo A-I)** is an excellent risk predictor of CV risk related to the metabolism of high-density lipoprotein (HDL).

Troponin T represents the classical biomarker of MI possessing a high predictive value for the disease [4]; elevated plasma levels of this molecule constitute the gold standard approach to detect MI [5]. Circulating concentrations of BNP reveal strong associations with CV risk under a range of different clinical conditions [6]. Similarly, circulating levels of CRP are strongly associated with CV disease [7]. Finally, plasma levels of apoA-I, the major protein components of HDL, can be superior to HDL-cholesterol as a predictor of CV risk [8]. However, the guidelines of the American College of Cardiology Foundation and the American Heart Association (ACCF/AHA) released in 2010 largely failed to add protein-derived measurements to the list of clinically useful biomarkers of CV disease [9]. Indeed, only the evaluation of family history of CV disease received a class I recommendation, the highest possible level of a clinical importance. Remarkably, circulating levels of CRP and BNP firmly established to be strongly associated with CV disease were concluded to only

modestly improve CV risk assessment when evaluated together with traditional CV risk factors [10, 6, 11]. In a more recent report, levels of high-sensitivity troponin T (hsTnT) were associated with CV mortality but only marginally imporved a traditional risk factor model in the Dallas Heart Study [12]. In a similar fashion, plasma levels of apoA-I are not always superior to HDL-cholesterol as a predictor of CV risk [13]. Finally, **growth-differentiation factor- 15 (GDF-15)** expressed by cardiomyocytes under conditions of ischaemia or pressure-related stress was a strong predictor of all-cause, CV and non-CV mortality but only moderately improved prediction of all-cause mortality in the Rancho Bernardo Study [14]. Such negative results may in part reflect the lack of causative relationships of a given biomarker with the disease. Thus, Mendelian randomization studies evaluating subjects with genetically determined CRP levels do not support a causative role of the protein in the development of CV, suggesting that CRP is a biomarker rather than a cause of atherosclerosis [15].

In order to improve risk predicition using individual protein biomarkers, they were proposed to be combined into **a multi-biomarker score ("multiscore")**. Such approach is based on the assumption that biomarkers to be combined reflect different pathophysiological pathways independently contributing to CV disease, which is not always valid.

Thus, in the Framingham Offspring Study, five biomarkers, and notably BNP, CRP, urinary albumin/creatinine ratio, homocysteine and renin, out of ten selected on the basis of known associations with CV risk, were retained as predictors of death [16]. In addition, two biomarkers (BNP and urinary albumin/creatinine ratio) were retained as predictors of CV events. A multiscore constructed using the ten biomarkers was significantly related to all-cause mortality and CV events; however, assessment of CV risk was only minimally improved relative to the model containing traditional CV risk factors. Similarly, two of six biomarkers, and notably BNP and mid-region pro-adrenomedullin, were retained as predictors

of CV events in the Swedish Malmo Diet and Cancer cohort [10]. A multiscore constructed using the biomarkers was significantly related to the risk of coronary events but the risk prediction was only marginally improved when the multiscore was added to traditional risk factors. Finally, four biomarkers (troponin I, N-terminal of the prohormone BNP (NT-proBNP), CRP and cystatin C) only modestly improved prediction of CV and all-cause mortality in elderly men without CV disease in the Uppsala Longitudinal Study of Adult Men when added to a traditional risk factor model [17]. An attempt to add a larger number of novel biomarkers to the traditional risk prediction model was similarly unsuccessful as revealed by the Women's Health Initiative cohort in which five (out of 18) biomarkers moderately improved CV risk prediction [18]. Simialr results were observed in the MORGAM study that evaluated 30 biomarkers, including NT-proBNP, CRP and troponin I, none of which alone was capable of improving discrimination in terms of CV risk [19]. A combination of NT-proBNP, CRP and troponin I only slightly improved the discrimination.

3. Potential novel proteomic biomarkers of CV disease

The data on the evaluation of CV risk with a help of circulating levels of individual proteins, or using a multiscore built of their combinations, reveal that substantial improvement in the risk prediction and reclassification relative to traditional models remains a difficult task presently unresolved. Hence, a potential importance of alternative approaches which may rely on novel proteomic biomarkers among others.

Plasma or serum concentrations of numerous proteins were measured using proteomic approaches to evaluate their relationships with CV disease (Table 1 and Figure 1); none of them was however evaluated for CV risk prediction and subject stratification in rigorous large-scale studies. Elevated plasma levels of several proteins were documented in MI (Figure

1). Thus, concentrations of **creatine kinase CK-MB** were increased in plasma following MI [20, 21], while concentrations of **fatty acid-binding protein (FABP)** were elevated in MI, stroke and coronary artery disease (CAD) [22, 21, 23]. Positive associations with MI were also observed for plasma levels of **cyclophilin A, cluster of differentiation 5 molecule [CD5] antigen-like, mucin cell surface associated protein 18 [MUC-18], a cell-surface glycoprotein, collagen α-1(XVIII) chain, salivary α-amylase 1, CRP** and **multimerin-2** [24]. In another study, plasma concentrations of **apoC-I**, **apoC-II** and **apoE** were found to be elevated in ST segment elevated MI as revealed by multiple reaction monitoring (MRM) proteomic measurements [25]. In a simalr fashion, levels of **myeloperoxidase (MPO)** were capable of predicting CV events in patients with CAD [26]. In addition, **cardiac myosin-binding protein C (cMyBP-C)** was strongly increased in patients with non-ST segment elevated MI [27].

By contrast, onset of MI was associated with diminished concentrations of **retinol-binding protein 4 (RBP4)** [28, 29]. Another plasma protein whose levels can be negatively related to CV disease is exemplified by **heat shock protein-27 (HSP27)** that appears to be reduced in myocardial ischemia [30, 31]. Indeed, HSP27 concentrations are decreased in the blood of patients with carotid stenosis relative to healthy subjects [30]. In addition, low circulating levels of HSP27 are associated with the presence of CAD and prognostic of future adverse clinical events [31]. In a prospective study of initially healthy women, baseline HSP27 concentrations in plasma were however not associated with incident CV events [32].

Plasma levels of **soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK)** are equally decreased in patients with CAD [33], abdominal aortic aneurysm [34] and chronic heart failure [35]. Furthermore, sTWEAK concentrations were capable of predicting CV disease in renal transplant patients [36].

When proteomic analysis was applied to pooled plasma samples obtained from CAD patients and controls in order to identify low-abundance proteins, 95 differential protein signals were identified, including those of **CA11, CD59, CHFR-1, collagen α-3(VI) chain, complement C1s, defensin 5, emilin 3/multimerin-2, fibrinogen γ chain, GST-omega-1, IGF binding complex acid labile chain, secreted phosphoprotein 24** and some other proteins involved in natural defenses, inflammation, growth and coagulation [23].

While a vast majority of the proteomic studies were performed in plasma or serum, urine was occasionally employed (Table 1). Thus, urine levels of **collagen α-1(I)** and **collagen α-1(III)** were augmented in CAD patients relative to controls [37]. In some studies, proteins were measured in isolated lipoproteins rather than in whole plasma; the presence of **apoC-III** in HDL [38] and LDL [39] was thereby identified as a factor associated with CV disease. In addition, other proteins, including serum amyloid A and complement C3, were reported to be elevated in HDL from CAD patients relative to controls [40, 41].

Proteomic approaches have equally been applied to discover new biomarkers of cardiomyopathy and heart failure to improve risk assessment in these conditions (Table 1), which is presently based on the measurement of BNP firmly established as a gold-standard biomarker for this pathological condition. Thus, **myosin heavy chain 7 (MYH7), desmin, insulin-like growth factor-binding protein 7 (IGFBP7)** and **annexin A2** were initially proposed to represent circulating biomarkers of cardiomyopathy-induced heaft failure in a transgenic mouse model to be subsequently validated in humans [42]. In another study, **quiescin Q6 (QSOX1)** was reported to represent a biomarker for acute heart failure [43].

A strength of proteomic analyses involves their capacity to quantitatively assay proteins displaying **post-translational modifications,** which are typically present in the circulation at low abundance and whose levels can be altered by CV disease. Major post-translational protein modifications include phosphorylation, glycation and oxidation. Altered

phosphorylation of numerous proteins is observed in CV diseases; phosphorylation of cardiac Troponin I was documented as a potential biomarker of chronic heart failure [44]. Advanced glycation end products (AGEs) can accumulate on several proteins in patients with CV disorder [45]. In addition, key plasma proteins, including apoA-I, α-1-antitrypsin and fibrinogen, can also become oxidized, reflecting elevated oxidative stress in CV disease [46, 47]. It remains however unclear as to whether post-translational protein modifications can serve as biomarkers of the disease.

4. Technical challenges

Despite generating wide-spread enthusiasm and providing first promising data, proteomic methods face considerable technical challenges [48, 49]. Thus, proteomic quantification of circulating protein concentrations is complicated by their wide dynamic range. This question can be addressed by protein fractionation and depletion before LC/MS analysis. High inter-individual variability represents another issue, which can be resolved by enlarging study cohorts. Proteomics of post-translational modifications requires high-resolution and high-sensitivity mass-spectrometrical methodology.

Next, there still remains a gap between candidate biomarkers and their clinical applications. Numerous candidate proteins discovered in original proteomic studies often do not survive rigorous validation in follow-up investigations, implying a risk of false-positive reports and calling for stringent verification approaches.

In addition, biological importance of proteomic data obtained is frequently difficult to deduce from extensive numerical information reported, complicating data interpretation. Quantitative alterations discovered by proteomics need to be linked to biological function and integrated

with other “omics” data; modern computational and visualization tools, including network models and protein interaction networks, can provide a major progress in this regard. Finally, “classical” issues of LC/MS/MS analyses need to be addressed to ameliorate resolution, sensitivity, specificity, throughput, precision and accuracy of the assays, to standardize procedures for sample collection, preparation and analysis, and to lower the assay costs.

5. Conclusions and perspectives

Proteomic studies have revealed that plasma concentrations of numerous proteins are associated with CV disease. Several of such potential biomarkers for the prediction of CV risk have been evaluated over the last years but only a few of them have been validated for clinical use. Such predominantly negative findings highlight inherent difficulties of the biomarker discovery and validation, calling for further research to identify novel candidates that can improve CV risk prediction, subject stratification and standard care. Assuming major technical issues successfully resolved, new and powerful high-throughput proteomic platforms can ensure translation of technical advancements into clinical practice to further reduce the burden of CV disease [50].

In addition to clinical biomarkers, proteomic strategies can provide a valuable tool to identify proteins involved in CV disease, which may become therapeutic targets. Computational approaches which combine protein identity with biological pathways and activities are critical to uncover the role of proteins as functional mediators of disease [51]. Alternative sources of biological material, such as atherosclerotic plaques, circulating cells and plasma extracellular vesicles, can deliver novel information on physiopathological mechanisms underlying associations discovered by proteomics of biological fluids [52]. These considerations leave

little doubt that proteomics will both open wide diagnostic perspectives and drive basic science research in the field of CV disease in the near future.

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Figure Legend

Figure 1. Alterations in circulating concentrations of proteomic biomarkers in patients with CV disease relative to controls. Biomarkers with reduced concentrations are shown on the left, while those with elevated concentrations are listed on the right. For references see Table 1.

Table 1. Circulating proteomic biomarkers of CV disease

Protein	Disease	Ref.
Annexin A2	HF	[42]
ApoA-I	CAD	[8]
ApoC-I	MI	[25]
ApoC-II	MI	[25]
ApoE	MI	[25]
BNP	CAD, MI, HF	[10, 6, 11]
CA11	CAD	[23]
Cardiac Tn I	MI	[4, 5]
CD59	CAD	[23]
CD5L	MI	[24]
CHFR-1	CAD	[23]
CK-MB	MI	[20, 21]

cMyBP-C	MI	[27]
Collagen α-3(VI) chain	CAD	[23]
Collagen α-1(I) chain*	CAD	[37]
Collagen α-1(III) chain*	CAD	[37]
Collagen α-1(XVIII) chain	MI	[24]
Complement C1s	CAD	[23]
CRP	MI	[10, 6, 11, 7, 24]
Cyclophilin A	MI	[24]
Defensin 5	CAD	[23]
Desmin	HF	[42]
Emilin 3/Multimerin-2	CAD	[23, 24]
FABP	MI	[22, 21, 23]
Fibrinogen γ chain	CAD	[23]
GDF-15	CAD	[14]
GST-omega-1	CAD	[23]
HSP27	CAD	[30, 31]

IGF binding complex acid labile chain	CAD	[23]
IGFBP7	HF	[42]
MPO	CAD	[26]
MUC-18	MI	[24]
Multimerin-2	MI	[24]
MYH7	HF	[42]
QSOX1	HF	[43]
RBP4	MI	[28, 29]
Salivary α-amylase-1	MI	[24]
Secreted phosphoprotein 24	CAD	[23]
sTWEAK	CAD, HF	[33, 35]

* Determined in urine; all other proteins measured in plasma or serum. HF, heart failure.

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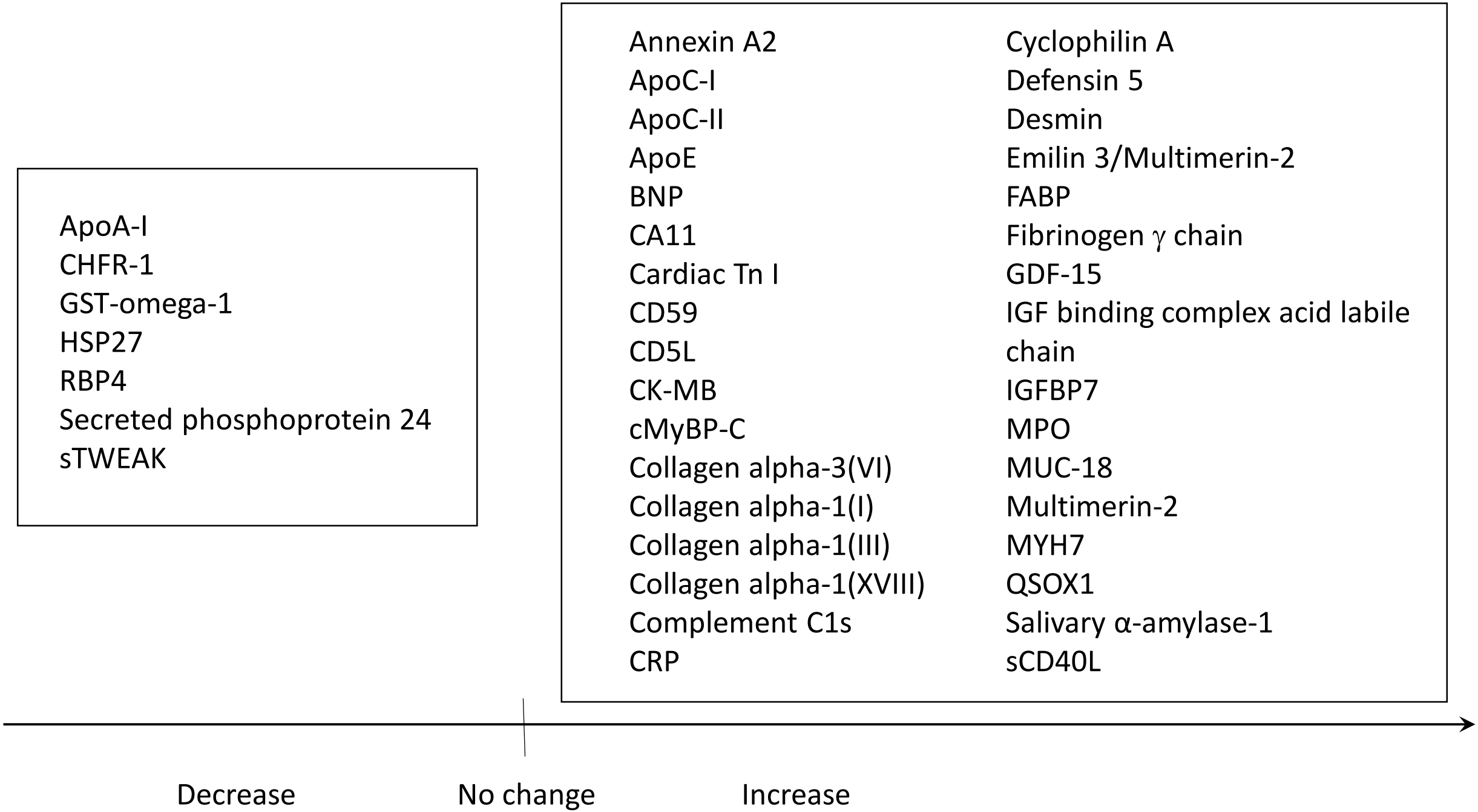
ApoA-I
CHFR-1
GST-omega-1
HSP27
RBP4
Secreted phosphoprotein 24
sTWEAK
Annexin A2
ApoC-I
ApoC-II
ApoE
BNP
CA11
Cardiac Tn I
CD59
CD5L
CK-MB
cMyBP-C
Collagen alpha-3(VI)
Collagen alpha-1(I)
Collagen alpha-1(III)
Collagen alpha-1(XVIII)
Complement C1s
CRP
Cyclophilin A
Defensin 5
Desmin
Emilin 3/Multimerin-2
FABP
Fibrinogen γ chain
GDF-15
IGF binding complex acid labile chain
IGFBP7
MPO
MUC-18
Multimerin-2
MYH7
QSOX1
Salivary α-amylase-1
sCD40L
Decrease
No change
Increase