

ARTICLE TITLE

Proteomic biomarkers of heart failure

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KEY POINTS

- Heart failure is associated with significant morbidity and mortality
- Biomarkers are commonly used for diagnostic and prognostic purposes
- Protein-based biomarkers have been identified to aid clinicians for early diagnosis of heart failure and provide added information for prognosis
- Proteomics is an ever-expanding field that employs techniques to measure a wide-range of proteins and peptides in search to identify potential protein biomarkers

SYNOPSIS

Heart failure (HF) is associated with significant morbidity and mortality. Biomarkers are used to assist clinicians with timely diagnosis, prognosis and risk prediction of patients for personalized treatment. Using modern proteomic methods such as mass spectrometry, an increasing number of novel biomarkers have been identified that further aid clinicians in the early diagnosis and outcome prediction of HF. This review focuses on the array of common and novel protein-based biomarkers which provide diagnostic and prognostic information in HF.

Introduction

It is estimated that in excess of 20,000 protein-coding genes are responsible for the presence of more than one million proteins found in biological matrices (1). The measurement of these proteins, commonly in plasma, serum, urine, saliva and tissue samples (2), has provided critical advancements in medical science through the development of diagnostic and prognostic assays for patients presenting with, or at risk of, a multitude of diseases (3). The use of protein measurements has been particularly beneficial for the assessment of cardiovascular disease, with notable inclusion of natriuretic peptides and troponin isoforms in clinical decision-making for heart failure (HF) (4) and acute coronary syndromes (ACS) (5), respectively. Clinical measurements of endogenous biological substances, such as proteins, lipids and metabolites, are commonly referred to as biomarkers and provide pathophysiological information through an associative or direct mechanistic interaction with the diseased system, organ or tissue (6). The relationships of protein biomarkers with disease allow physicians to assess the presence, severity, and/or prognosis of a condition with improved precision and accuracy (7).

The progression in medical diagnosis and treatment of HF has been heavily influenced by the inclusion of protein biomarker analyses, with measurement of natriuretic peptides commonly employed in hospitals worldwide (8). HF is a major worldwide epidemic associated with high morbidity, mortality, and healthcare costs affecting more than 23 million, especially those aged ≥ 65 years (9); therefore, any improvements in diagnosis, prognosis and therapeutic monitoring using protein measurements provide direct improvements in patient care and outcome, as well as economic burden. Difficulties in HF diagnoses exist due to the multifactorial pathophysiology (such as cardiac stress and injury, neurohormonal activation

and endothelial congestion), and that the signs and symptoms may not arise during early stages of the disease (10, 11). Current guidelines suggest patients presenting with suspected HF should be referred measurement of circulating natriuretic peptides to aid in diagnosis of the condition (12).

The development and pathophysiology of HF is associated with changes in the expressions of an array of metabolic, signaling and structural proteins (13). Although there are a number of protein-based assays currently used in the clinical laboratories, extensive research is being performed to isolate and identify novel protein biomarkers associated with HF in a bid to improve sensitivity and/or specificity of biomarker information. Leading these discovery-led investigations are mass spectrometry (MS)-based assays which involve a non-targeted approach to protein measurement and fall under the remit of proteomics. These assays measure all detectable proteins that are expressed by a cell, tissue or organism, known as the proteome, and reflect levels present at the time of sample collection (14, 15).

Proteomic Biomarker Discovery

For discovery-led proteomics investigations, the initial phase employs methods using either a wide-span-targeted or non-targeted approach in order to measure a large number of proteins and/or peptides from various biological sample types. This generates a list of numerous proteins that are identified as associated with the condition being investigated and, therefore, selected as candidate proteins for subsequent verification experiments. Although many candidate protein biomarkers may be identified through these experimental workflows, very few survive the rigorous validation processes leading to the development of high-throughput assays for measurement (16). Mass spectrometry (MS) is the most widely used instrumentation for non-targeted discovery and identification of potential protein

biomarkers. It allows for quantitative and qualitative analysis, peptide sequencing and identification with great accuracy and sensitivity (17). Proteomic workflows can vary greatly across investigations, including sample preparation, chromatographic gradients and inclusion of complementary analytical techniques such as ion mobility spectrometry. Furthermore, differences across studies in data processing and statistical testing can lead to misidentification or masking of candidate biomarkers. These widely varied approaches provide limitations in that biomarker identification may not be reproducible across multiple methods, complicating the validation process for novel protein biomarkers. Typically, MS method workflows include fractionation to crudely separate proteins in the sample, removal of highly abundant proteins such as albumin in plasma samples, further separation of each fraction using liquid chromatography, and MS using electrospray ionization (ESI) in positive ion mode coupled to accurate mass analyzers such as time-of-flight (ToF) and orbitrap (18). Alternatively, gel-based approaches are initially employed to separate proteins by their isoelectric point and then by mass using polyacrylamide gel (SDS-PAGE), followed by staining, excising, digesting using trypsin and analysis by MS (19). Following identification of candidate biomarkers, mass spectral data are cross-referenced with large-scale databases to confirm protein identification. Errors in protein quantitation in global discovery techniques can be associated throughout the analytical workflow from samples preparation to analysis. To assist in reducing these errors, isotopic labelling of internal protein standards can allow for relative quantitation of multiple proteins. Examples of these include metabolic labelling (^{15}N) and isotope-coded affinity tags (ICAT), however, they lack in accuracy and precision and more reliable approaches for sample-wide quantitation are required (20).

Traditional non-targeted MS based methods are important in candidate biomarker identification, however complex sample preparation and analysis steps create a time

consuming process that limits the throughput required for larger-scale validation studies. Once a list of candidate biomarkers is produced, a shift toward targeted MS approaches allows for improved specificity, reproducibility and quantitation of candidates, while also drastically reducing the analytical run time. A commonly employed approach for targeted MS is to develop assays using selective or multiple reaction monitoring (SRM/MRM), where a single ion (SRM) or up to five fragment ions (MRM) are monitored in association with a specific product ion, typically using a triple quadrupole MS system which is able to provide enhanced discriminating power, leading to increased sensitivity, absolute quantitation (21, 22) and improved cross-compatibility between instrumentation (23). Aside from ESI-MS, matrix-assisted laser desorption ionization (MALDI) ToF based MS is employed for targeted MS where proteins of interest can be isolated using immunoprecipitation or LC prior to spotting onto a target plate for analysis. Several targeted protein analyses using MALDI have been reported (24, 25), including an application in clinical studies (26, 27). Prior to commercialization, targeted protein experiments must replicate the results observed from the non-targeted investigations, as well as expanding to larger sample cohorts including diseased and non-diseased populations to validate as a biomarker of a condition and to understand normal ranges and potential disease cut-off values.

As protein expressions exhibit multifaceted temporospatial characteristics driven by responses to physical and/or biological stimuli, there are several complexities involved in the process of identifying a novel protein biomarker. In order to confirm the utility of a candidate biomarker for a clinical purpose, several steps must be achieved including discovery, qualification, verification, optimization and validation, followed by commercialization and distribution of assays (28) (Figure 1). Although the discovery of novel proteins is driven by mass spectrometric methods, validation and commercialization more frequently involve

traditional antibody-based techniques such as ELISA, Western Blotting and immunoblotting, with the less common use of MS-based methods in a clinical setting dictated by obstacles in regulatory approval and cross-site/equipment reproducibility (29). Regardless of the most suitable analytical method, a successful biomarker must be easily measured, low cost, patient-friendly, and show high levels of sensitivity and specificity for their purpose (e.g. diagnosis, prognosis) (30).

This review intends to highlight the current clinical uses of protein biomarkers in HF (Figure 2) and discuss the application of targeted and non-targeted proteomic investigations to discover and develop novel biomarkers centered on using a personalized medicine approach for improved prognostic information.

Markers of cardiac stress

BNP/NTproBNP

B-type natriuretic peptide (BNP) is perhaps the most widely used biomarker for cardiac stress. It is a central component in cardiovascular homeostasis and is released from the cardiomyocytes, primarily located in the ventricles, in response to stress and stretch of the cardiac muscle (31). After binding to specific receptors, BNP is activated and drives a reduction in systemic vascular resistance, antagonizes the actions of the renin-angiotensin-aldosterone system (RAAS), and promotes vasodilation and natriuresis (32). BNP has been studied extensively for its role as a diagnostic (33-35) and prognostic (36-38) biomarker in HF, including both chronic patients and acute decompensated admissions. However, an important limitation of BNP for HF diagnosis is that circulating levels may become elevated in response to alternative disorders such as renal dysfunction, left ventricular hypertrophy and right ventricular dysfunction (39). Furthermore, as factors such as sex, age and body mass

index are also associated with fluctuations in BNP levels, accurate interpretation of circulating concentrations is crucial (40).

In addition to uses in diagnosis and prognosis, BNP has shown utility for the monitoring of patients treated with diuretics and vasodilators such as ACE inhibitors (41), angiotensin-II receptor antagonists (42), and aldosterone inhibitors (43). Circulating BNP levels are known to fall rapidly following successful treatment strategies, therefore repeat measurements of BNP concentrations provide an observation of responses to medical interventions.

Although widely used in clinical analysis, BNP exhibits a short half-life (approximately 20 minutes) when present in the circulation (44) and, therefore, care must be taken during the sampling and storing of blood samples. N-terminal proBNP (NTproBNP) is released in conjunction with BNP and is considered as a more stable alternative due to its longer half-life (45). NTproBNP has reported similar characteristics to BNP as a biomarker for diagnosis (46-48), prognosis (42, 46, 49) and guided treatment (50) in HF.

When studied in direct comparison, BNP and NTproBNP show comparable utility for diagnosis (42), prognosis (51, 52) and biomarker-guided therapy (53) in chronic HF, with reductions in all-cause mortality reported with titration of therapies based on repeat measurements. Circulating levels of these natriuretic peptide biomarkers are increased in HF and are strongly associated with disease severity and myocardial stretch (54). Studies, such as the Valsartan Heart Failure Trial (Val-HeFT), have also shown BNP and NTproBNP to provide superior prognostic information when compared to alternative neurohormonal markers of risk (55).

Molecular forms of BNP

There have been recent research efforts to further understand the degradation pathways of BNP and, with its short half-life in circulation, experiments have also reported the presence of its molecular forms. These molecular forms are truncated BNP peptide chains that are synthesized by the proteolysis of end-chain amino acids and have been identified in the circulation of HF patients (e.g. BNP 3-29, 3-30, 4-29, 5-29 etc.), with BNP 3-32, 4-32 and 5-32 reported as the most commonly present (56). These molecular forms have recently emerged as potential biomarkers for HF, with the major forms previously implicated in ischemic heart disease (57), a major risk factor for development of HF. Furthermore, molecular forms have been reported to more closely associate with clinically measured BNP levels in comparison to the parent BNP molecule (BNP 1-32). This suggests that the specificity of clinical BNP assays is not unique to BNP 1-32, and that the combination of intact and molecular forms of BNP is a more accurate representation of circulating BNP measurements (58). More recently, molecular forms of BNP have shown superior or comparable prognostic qualities to NTproBNP for risk stratification of acute HF patients. BNP 3-32, 4-32, and notably 5-32 were able to independently predict adverse outcome of patients at 6 months and 1 year, outlining its use as a biomarker to guide outpatient management (27). However, direct mechanistic actions of these truncated forms, along with the dynamics and kinetics of their degradation pathways are not currently understood and remain areas of current research.

ANP

Atrial natriuretic peptide (ANP) is primarily secreted from the atria and has similar physiological properties to BNP. It is thought to play a role in early HF by preserving the compensated state of left ventricular dysfunction (59). Although reported to be a prognostic indicator in HF, studies have reported ANP as inferior to BNP primarily due to its decreased

stability in circulation and its insensitivity to levels of HF severity (60, 61). Other forms of ANP, such as N-terminal ANP (N-ANP/NTproANP) and mid-regional pro-ANP (MRproANP), have also been shown to offer diagnostic and prognostic roles in HF (61-63). MRproANP has far greater stability in circulation and therefore could prove more suitable for use as a biomarker in comparison to ANP. An investigation using The Biomarkers in ACute Heart Failure (BACH) cohort reported that MRproANP was a suitable diagnostic and prognostic biomarker in dyspneic patients, with results comparable to BNP (63). Furthermore, additional studies have also indicated that MRproANP provides additive prognostic information to NTproBNP in chronic HF (64) and as a diagnostic marker of acute destabilized HF in patients with dyspnea, again reporting comparable results to the use of both BNP and NTproBNP (65).

ST2

ST2 is a member of the interleukin receptor (IL-1) family and has been identified as the target for IL-33 which is expressed under biochemical stress of the heart (66). ST2 is basally expressed by cardiomyocytes and is detectable in circulation in its soluble form which is elevated in response to mechanical stress of the heart (67). Its utility has been recognized in HF as an independent predictor of mortality or need for transplantation in severe chronic HF patients (NYHA class III/IV) (67), as well as providing prognostic information for acute HF patients when combined with natriuretic peptides (68), suggesting applicability as a biomarker in combination with current clinical testing strategies (e.g. BNP and NTproBNP). Furthermore, although NTproBNP showed improved diagnostic accuracy for patients with acute HF, the PRIDE (Pro-Brain Natriuretic Peptide Investigation of Dyspnea in the Emergency Department) study showed that ST2 was a suitable biomarker to predict 1-year mortality in dyspneic patients, irrespective of a positive or negative diagnosis of acute destabilizing HF

(69), offering a more generalized biomarker function that can be further refined when applying a multi-biomarker approach.

MRproADM

Adrenomedullin (ADM) is a peptide hormone that has natriuretic, vasodilatory and hypotensive effects on the heart, with plasma concentrations elevated in response to these effects, such as seen in chronic HF (70). However, since ADM is unstable in circulation, its mid-regional pro-hormone fragment (MRproADM) is often measured to perform an indirect quantification (71). It has reported a varied role in prognosis, providing information for short- (30 days) and long-term (4 years) prognosis, but less success for mid-range predictions of outcome (1 year) (72). MRproADM has also been successfully demonstrated as a prognostic biomarker for acute HF patients presenting with dyspnea (73). In this study it was reported as a superior biomarker to both BNP and NTproBNP for the short-term prediction of mortality (90 days) as well as for subsequent patient rehospitalization (73). Data suggest it has a suitable role for use in HF prognosis, however, further investigations to confirm its superiority to current biomarkers are warranted.

Markers of inflammation or injury

Troponin

Troponin proteins are found in cardiac and skeletal muscle tissue and are involved in the regulation of actin and myosin interactions during muscle contraction. Troponin I (cTnI) and T (cTnT) have unique isoforms that exist only in cardiac muscle allowing the measurement of these specific isoforms to provide information in cardiovascular disease, notably as diagnostic biomarkers for ACS (74). However, development of high-sensitivity troponin assays have

further allowed the measurement of elevated cTnl/cTnT levels in HF patients (75, 76), with increased concentrations associated with poor outcome. High-sensitivity assays for cTnT have been applied for prognosis in chronic HF, with circulating concentrations able to predict mortality and hospitalization in stabilized patients (77). Furthermore, cTnT has been shown as a suitable marker to reflect myocardial damage in severe chronic HF (78), to risk-stratify patients on admission for subsequent mortality and morbidity (79), and to identify patients at high-risk of disease deterioration (80). For acute decompensated HF in the absence of ACS, cTnT is a prognostic marker for short- and long-term outcomes (81).

In addition, cTnl has been shown to reflect elevated BNP levels, impaired hemodynamics and worsening of left ventricular dysfunction (82). For acute admissions, serial changes in cTnl levels over 90 days were functional in predicting increased likelihood of mortality and rehospitalization (83). When used in combination with BNP measurements, cTnl measurements on admission were shown to predict in-hospital mortality, and increasing concentrations were associated with risk of death in a large-scale registry cohort (The Acute Decompensated Heart Failure National Registry {ADHERE}) (84, 85).

H-FABP

Heart-type fatty acid-binding protein (H-FABP) is a small cytosolic protein involved in transporting long-chain fatty acids in the myocardium and is released in response to myocardial damage (86), indicating its potential as a sensitive biomarker for acute myocardial infarction (MI) (87). H-FABP concentrations are known to be increased in congestive and chronic HF (88), and reported to be a more sensitive than troponin to detect myocardial damage and identify patients at high-risk (86). Initial investigations have been performed to

understand the role and/or association of H-FABP in HF, however further studies are required to assess its utility in comparison with current clinical measurements.

CRP

C-reactive protein (CRP) is a traditional marker of inflammation and high concentrations are commonly associated with mortality in acute MI patients. However, studies have also shown increased CRP levels in HF patients, reflecting myocardial damage (89) and associations with HF severity, mortality and morbidity (90), and rehospitalization (91). Data indicate that CRP can be used as a predictor for deterioration of heart function, however it has also been reported to show no statistical association with left ventricular ejection fraction, providing complications for its suitability and specificity as a biomarker in HF (92).

TNF- α

Tumor necrosis factor- α (TNF- α) is a cytokine involved in inflammation and has been reported to exhibit elevated levels in chronic HF (93). TNF- α has also been implicated in newly diagnosed HF patients, with elevated levels associated with abnormal left atrial dysfunction, and advanced left ventricular diastolic and systolic dysfunction (94). Further associations have been reported with NYHA class and disease severity (95), alongside the use of TNF- α as a predictor of mortality in advanced HF (96).

IL-6

Interleukin-6 (IL-6) is also a cytokine involved in inflammation, but has additional cardiovascular properties through regulation of cardiomyocyte hypertrophy and apoptosis (97). Cardiac IL-6 expression is reported to increase in advanced HF, providing suggestion for its potential role in prognosis (98). In addition, elevated IL-6 levels have been associated with

left ventricular dysfunction prior to HF diagnosis, highlighting its potential utility as a risk marker for the onset and progression of HF (99). This prognostic ability has been confirmed in acute HF for prediction of short- and long-term mortality, both as a sole biomarker and in a multi-marker approach when combined with NTproBNP (100). Furthermore, IL-6, along with CRP and IL-4, concentrations have been shown to increase during a coronary event, returning to pre-event levels as symptoms of HF subside over time. This observation indicates a potential role for IL-6 in differentiating between the decompensated and compensated state (101).

Markers of neurohormonal activation

Copeptin

Pre-pro-vasopressin is preteolytically cleaved into copeptin, neurophysin II and vasopressin, with the latter also known as antidiuretic hormone that has a prominent role in fluid homeostasis and shown to be related to the severity of HF (102). However, vasopressin is known to exhibit instability in circulation and therefore is troublesome for clinical measurements. On the other hand, copeptin is considered to have a high stability and is released in equimolar concentrations to vasopressin, allowing a more reliable and reproducible alternative for indirect measurement of vasopressin (103). Research into the clinical role of copeptin as a prognostic biomarker has been compared to measurements of natriuretic peptides and initial indications suggest it offers a superiority in prediction for 14- and 90-day mortality in acute admissions (73, 104) and longer-term prediction at 24 months for patients across various stages of disease (105), as well as for those with advanced HF (106). Although a relatively contemporary biomarker for HF, data highlight copeptin measurements as a potential clinical tool for risk-stratification, particularly in acute cases.

MMPs

Matrix metalloproteinases (MMPs) are a family of zinc-dependent protease enzymes required for normal tissue remodeling, and mediating collagen metabolism and extracellular matrix (ECM) homeostasis (107). Four common classes of MMP's have been identified: gelatinases (MMP-2 and MMP-9), collagenases (MMP-1 and MMP-8), stromelysin (MMP-3) and matrilysin (MMP-7), all of which are regulated by tissue inhibitors (TIMPs) (108).

Elevated MMP-9 and MMP-2 concentrations have been reported in HF patients (105), with the latter associated with mortality (109). Conversely, MMP-8 has been shown to exhibit a decreased concentration in chronic HF patients (110). Although there are variable alterations of MMPs in patients and more extensive research is required to identify their individual suitability for prognostic investigation, data do suggest that they offer additional information when included within a multi-biomarker panel (111). These panel risk scores can improve the information available to identify disease process and HF risk in-line with changes to ECM collagen homeostasis and activity of enzymes of remodeling.

Markers of remodeling

Galectin-3

Galectin-3, a member of the lectin family, has shown to be implicated in multiple aspects of HF physiology, including inflammation and ventricular remodeling. It is secreted by activated macrophages which proliferate and cause cardiac fibrosis (112, 113), and has been demonstrated to provide a positive role as a prognostic marker of HF (114, 115). Elevated galectin-3 levels have been associated with an increased risk of HF and mortality (116), with a two-fold increase in levels associated with a two-fold increase in risk of death or

rehospitalization over an 18-month period (114). Furthermore, increased concentrations have also been associated with adverse outcome in patients with preserved ejection fraction (HFpEF) (117). As with many proteomic biomarkers of HF, study into the role of gelactin-3 requires further research, but initial data offer potential as a marker to stratify patients for HF with or without remodeling.

GDF-15

Growth differentiation factor-15 (GDF-15) is a stress-responsive, transforming growth factor beta-cytokine involved in inflammatory and apoptotic pathways of tissue injury. It is an emerging marker of cardiac dysfunction and elevated levels of GDF-15 have been shown to identify high-risk cardiovascular disease patients (118, 119). Elevated GDF-15 has demonstrated mortality prediction in patients with chronic HF, with increased expression of GDF-15 linked to the promotion of protective mechanisms for inhibition of apoptosis, hypertrophy and adverse remodeling (120). Elevated levels have also reported prognostic utility in both patients with reduced (HFrEF) and preserved (HFpEF) ejection fraction, adding to current markers such as troponin and NTproBNP (121, 122). For acute decompensated HF, elevated GDF-15 concentrations have shown prognostic value for predicting mortality and HF rehospitalization at 1-year, supported by twenty-one original studies (123, 124).

Markers of associated comorbidities

Cystatin C

Cystatin C is a small protein molecule that is involved in the extracellular inhibition of cathepsins. It is removed from circulation through the kidneys, thus providing biomarker information for renal dysfunction and therefore an interest within cardiovascular disease

(125). Cystatin C has shown prognostic capabilities in chronic HF patients as well as those with reduced ejection fraction (126-128). These positive relationships with adverse outcome in HF, as well as providing information on dysfunction in the renal system, signify cystatin C as a useful biomarker for a combinatory view of cardiovascular disease and its comorbidities.

NGAL

Neutrophil gelatinase-associated lipocalin (NGAL) is an innate antibacterial factor protein of the lipocalin family, initially found to be expressed in neutrophils and later in kidney tubular cells. In kidney dysfunction, NGAL has been shown to be an early marker of injury in animal models and detectable in blood and urine following acute kidney injury (129, 130).

In chronic HF patients, NGAL concentrations were found to be increased when compared to healthy subjects (131, 132). However, their applicability as a prognostic marker was proven to be inferior to currently established protocols (e.g. NTproBNP) (133). In acute cases, the GALLANT [NGAL Evaluation Along with B-type Natriuretic Peptide (BNP) in acutely Decompensated Heart Failure] trial indicated that NGAL was a strong short-term (30 days) prognostic predictor of HF-related outcomes (134).

Procalcitonin

Procalcitonin is a precursor peptide of calcitonin and a diagnostic marker of bacterial infections, such as in pneumonia (135), with elevated levels also measured in HF patients (136). Elevated procalcitonin concentrations were able to predict the risk of long-term death and rehospitalization in acute admissions, irrespective of bacterial infections (137), and were observed to be in-line with disease severity for chronic patients (138). Additionally, serum

procalcitonin concentrations provided diagnostic information for HF with high sensitivity and specificity (139).

Contemporary proteomic biomarkers

Recent research has led to an increased number of protein biomarkers that show promise in diagnosis and prognosis of HF conditions. For example, Proenkephalin A (PENK) and Chromogranin A (CgA) are proteins measured in the circulation that have shown utility as biomarkers in HF, and efforts to validate these for transition into a clinical setting are underway. PENK is a small endogenous opioid peptide which is cleaved to produce enkephalin. Studies have shown that enkephalins are released from non-neuronal tissues, including the kidneys and heart, in response to ischemia (140). In chronic and acute HF, PENK is associated with glomerular function, but does not offer significantly additive prognostic information in addition to current biomarkers of renal function (141). However, it has demonstrated useful prognostic information for hospitalization or mortality in stable HF patients (142). In addition, PENK concentrations have shown predictive capabilities for in-hospital mortality in acute HF patients, as well as indicating those at risk of worsening renal function (143). CgA is a prohormone produced in various tissues including the heart. Hyperglycosylations of CgA lead to its impaired conversion to catestatin, an action found to be associated with acute HF outcomes (144). Mixed prognostic quality has been reported in the literature with studies showing CgA to be associated with the severity of chronic HF and a prognostic marker for mortality (145), but providing no additive information for prognosis when compared to established protocols and biomarkers (146). In addition to CgA, chromogranin B (CgB), which is co-localized with CgA, has also shown an increase in concentrations to follow severity and development of HF (147).

Although PENK and CgA have been taken forward to extended validation studies, discovery-focused proteomics experiments have identified a number of circulating proteins as potential novel biomarkers for HF. The use of urine sampling as a less-invasive alternative to the traditional blood draw has led to an interest in highlighting urinary proteins for diagnostic testing in HF. Two proteins, insulin-like growth factor binding protein 2 (IGFBP-2) and orosomucoid 1 (ORM1), have been reported to possess diagnostic potential providing additive information to current biomarkers such as BNP for IGFBP-2 (148), as well as increasing concentrations in-line with severity of chronic HF and good diagnostic sensitivity (95%) and selectivity (85%) for ORM1 (149). These are examples of proteomic biomarkers that have provided initially positive associations, but further validation in extended experiments is required.

Other novel protein discoveries have been supported with initial mechanistic and/or clinical investigations and therefore are following the required pathway for translation into a clinical setting. Leucine-rich α 2-glycoprotein (LRG) was reported to have an exaggerated expression in patients with a measured BNP of ≥ 100 pg/mL, and provided similar diagnostic statistics to BNP (150). In addition to this, the authors demonstrated cardiac myocytes to be the origin of LRG release and more recently it was observed that LRG was active in suppressing adverse remodeling post-myocardial infarction (151), and that LRG release in HF may be in response to pressure overload. Calcium-binding proteins A8/9 (S100A8/9) have also been reported to exhibit an upregulated expression as a protective mechanism in HF development, with an observed contribution to the anti-HF effect of hypertrophic preconditioning (152). In addition to their mechanistic interactions, S100A8/9 have also been shown to provide predictive qualities for mortality in elder patients with severe HF (153). Similarly, circulating heat shock protein 70 (HSP70) has been shown to rise in concurrence with cardiac expression (154) and

HF severity (HF) (155), and has been implicated as a potential biomarker for early diagnosis of HF (155).

Although the prior examples of novel biomarkers have undergone initial stages of mechanistic and clinical validation, more recent experiments have indicated further potential biomarkers that remain at the discovery phase. For example, quiescin Q6 (QSOX1) has shown promising signs of a biomarker that is specific to acute decompensated HF with dyspnea, with reduced and comparable levels measured in chronic HF patients and healthy volunteers (156). QSOX1 has been shown to increase in the left ventricle in an animal HF model (157) and provides diagnostic qualities that are equal to BNP and NT-proBNP (156), with increased specificity over natriuretic peptides for diagnosis of acute decompensated HF in patients, irrespective of the presence of previous stable HF.

Many candidate proteomic biomarkers for HF have been discovered and are still in the research phase to determine their roles in HF. Limited information regarding their additive role as biomarkers in HF is available and further research for their capacity is required. Extending from single biomarker analyses, proteome-wide investigations have provided insight into multi-biomarker models to predict future disease developments. A notable example was presented by Hollander *et al.* who identified a list of 17 candidate protein biomarkers that, when combined with BNP measurements, were able to provide 97% sensitivity and 100% specificity for classifying patients on recovery from cardiac transplants (158). This provides an exciting opportunity to provide outpatient screening to monitor response to HF treatments but requires extensive additional testing to validate its applicability for everyday clinical use.

Summary

A wide range of protein-based cardiac biomarkers have shown success in diagnostic and prognostic applications, with several already established as routine measurements in clinical laboratories. Extensive research efforts are currently underway to enhance our knowledge of protein-cardiovascular disease interactions, led by proteomic-based organizations such as Human Proteome Organization (HUPO). The flagship venture has been The Human Proteome Project (HPP) that is looking to map the entire human proteome to further our understanding in the localized and systems biology of proteins and protein-protein interactions for diagnostic, prognostic and therapeutic roles in disease (159). In particular, HUPO has emphasized the need to develop open-access databases that allow the sharing of proteomics research data across equipment and institutions to detail the human proteome library (160).

The advancement of technologies, such as mass spectrometry, which complement traditional enzyme-based assays have allowed the development of highly sensitive and selective methods with the ability to measure multiple relevant biomarkers in a high-throughput manner. Although at a stage of infancy for translation to functional clinical laboratories, these methods offer potential for future advancements in breadth and depth of clinically-relevant biomarkers. In addition, the use of multiple 'omics-based investigatory pathways, including proteomics, metabolomics, lipidomics and genomics, allow for the discovery and validation of novel biomarkers that provide improved clinical information to patients in cardiovascular disease and beyond. An example of this approach includes the focus of implementing the combination of protein and metabolite biomarkers to improve risk-stratification, with recent demonstration of enhanced prognostic capabilities in chronic and acute HF when combining BNP/NTproBNP with trimethylamine *N*-oxide, a metabolite biomarker linked to gut microbial

breakdown of dietary molecules (161, 162). Continued efforts for biomarker discovery and validation offer the promise to unearth novel and contemporary molecules for application in personalized and precision medicine, with the potential to lead to improved prognosis, treatment and early diagnosis of conditions at the center of public health concerns.

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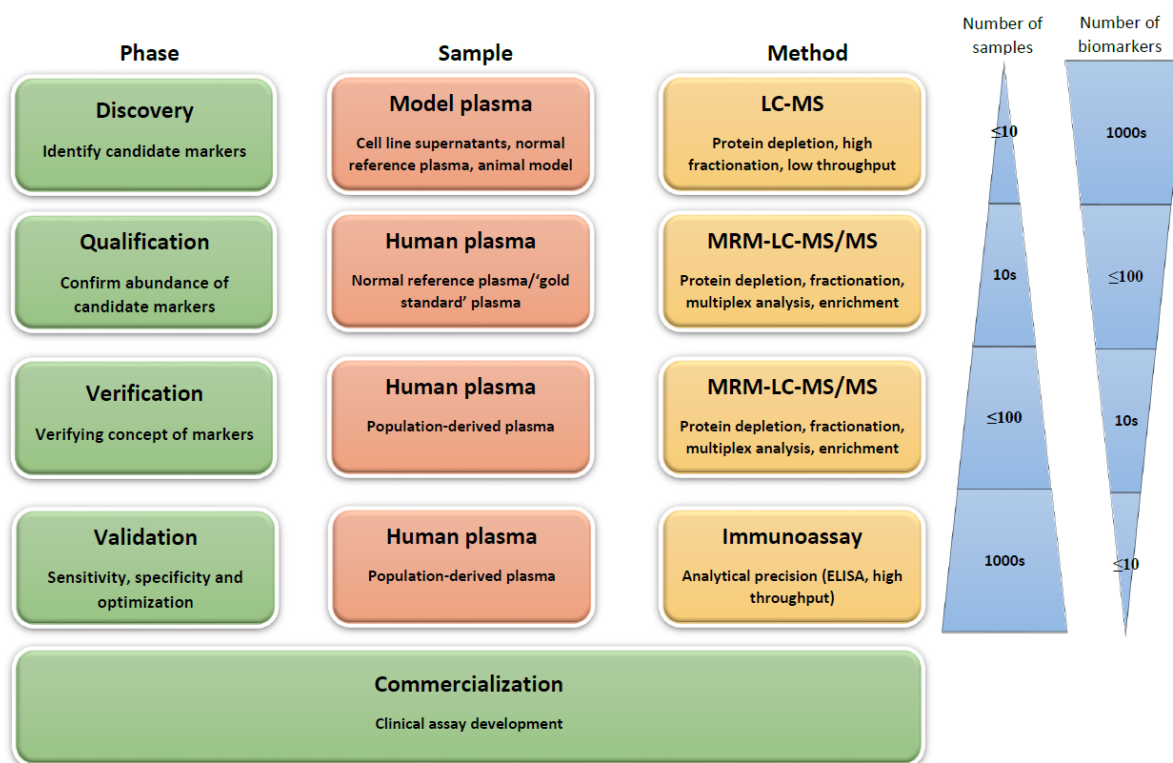
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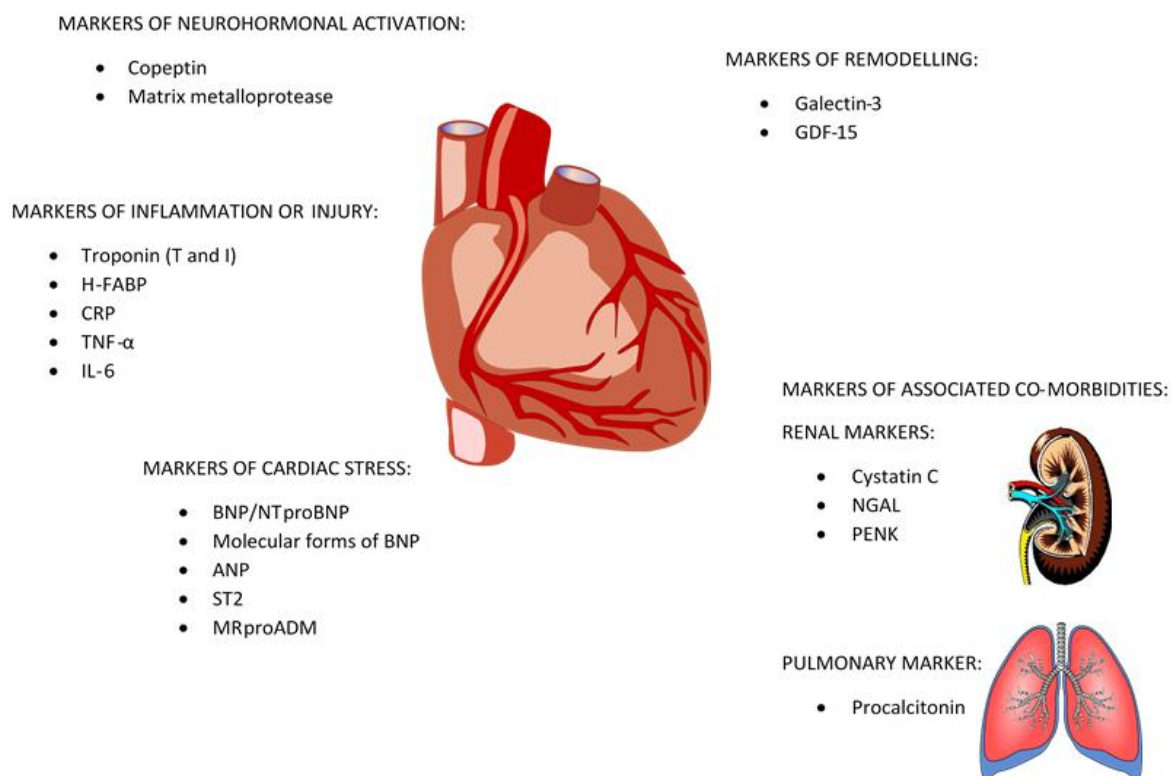
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Figure 1 Protein biomarker discovery pipeline of novel candidate biomarkers



ELISA, enzyme-linked immune-sorbent assay; LC-MS, liquid chromatography mass spectrometry; LC-MS/MS, liquid chromatography tandem mass spectrometry; MRM, multiple reaction monitoring

Figure 2 Protein biomarkers of heart failure and their various pathophysiological associations



ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; CRP, C-reactive protein; GDF-15, growth differentiation factor-15; H-FABP, heart-type fatty-acid binding protein; IL-6, interleukin-6; MRproADM, mid-regional pro-adrenomedullin; NGAL, neutrophil gelatinase-associated lipocalin; NTproBNP, N-terminal pro B-type natriuretic peptide; PENK, proenkephalin; TNF- α , tumour necrosis factor- α