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Hold or Fold – Proteins in Advanced Heart Failure and Myocardial Recovery

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Abstract

Advanced heart failure describes the subset of heart failure patients refractory to conventional medical therapy. For some advanced heart failure patients, the use of mechanical circulatory support provides an intermediary “bridge” step for transplant eligible patients or an alternative therapy for transplant ineligible patients. Over the past 20 years, clinical observations have revealed that approximately 1% of patients with mechanical circulatory support undergo significant reverse remodeling to the point where the device can be explanted. Unfortunately, it is unclear why some patients experience durable, sustained myocardial remission, while others re-develop heart failure (*i.e.* which hearts “hold” and which hearts “fold”). In this review, we outline unmet clinical needs related to treating patients with mechanical circulatory support, provide an overview of protein dynamics in the reverse remodeling process, and propose specific areas where we expect mass spectrometry and proteomic analyses will have significant impact on our understanding of disease progression, molecular mechanisms of recovery, and provide new markers with prognostic value that can positively impact patient care. Complimentary perspectives are provided with the goal of making this important topic accessible and relevant to both a clinical and basic science audience, as the intersection of these disciplines is required to advance the field.

Keywords

advanced heart failure; myocardial recovery; circulatory support; proteomics; pluripotent stem cells

1 Introduction

Heart failure is a complex clinical syndrome in which the heart's ability to pump blood becomes impaired to the point at which it eventually becomes insufficient to adequately meet physiologic demand. Advanced heart failure (AHF) comprises the subset of heart failure patients that are resistant to medical therapy, whose disease progression ultimately results in a tenuous clinical course culminating in either mechanical circulatory support (MCS), cardiac transplantation, or death [1, 2]. Though exact figures are unknown, it is

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estimated that approximately 300,000 – 350,000 patients in the United States suffer from AHF [3]. Cardiac transplantation remains the definitive therapy for AHF, though its' availability is limited by an inadequate pool of suitable donors, totaling a mere 2,200 usable organs in North America per year [3]. MCS in the form of ventricular assist devices (VAD), which in essence take over for the failing ventricle to maintain end-organ perfusion, has become an intermediary “bridge” step for transplant eligible patients, or at times, an alternative therapy for transplant ineligible patients, termed “destination therapy” [4]. By accepted clinical indication, all other therapeutic interventions (*e.g.* neurohormonal therapy, cardiac resynchronization therapy with bi-ventricular pacing) must have failed prior to proceeding with MCS [1]. Clinical observation has revealed that a small subset of VAD patients undergo significant reverse remodeling (*i.e.*, an improvement in myocardial systolic function and regression of ventricular enlargement while supported by a device) [5-7]. This improvement provides sufficient recovery to consider explanting the device for approximately 1% of all VAD patients [8, 9], though in selected patient cohorts treated with targeted therapeutic strategies aimed at myocardial recovery, higher rates have been observed [10]. While events occurring during this reverse remodeling process potentially hold important clues for how to stimulate the heart to self-recover and reveal novel therapeutic targets, the molecular mechanisms underlying reverse remodeling and myocardial recovery remain poorly understood. However, if these processes were to be elucidated on a molecular level, it would have the potential to revolutionize the care of AHF patients.

Already, the proteomics community has had an impact on our knowledge of biological mechanisms and biomarkers of heart failure and numerous well-written reviews on this topic are available [11-14]. However, reverse remodeling and myocardial recovery during MCS have received comparatively little attention in the research community and thus are the focus of this review. We begin with a clinical perspective on AHF, MCS and myocardial recovery, highlighting current challenges in patient care and where new information is urgently needed to improve clinical outcomes. Then, we review the current literature regarding myocardial protein dynamics during MCS. We conclude with perspectives from the bench regarding important considerations for future investigation, including the valuable role pluripotent stem cell technologies and proteomics can play in fundamental studies of the myocardium as they apply to AHF and myocardial recovery. Overall, while our current knowledge of processes involved in AHF and myocardial recovery is limited, we believe that applying state-of-the-art mass spectrometry analyses in well-designed studies will have a significant impact on our understanding of disease progression, molecular mechanisms of recovery, and provide new markers with prognostic value that can positively impact patient care. Ultimately, we hope this review will shed light on this underserved field and will inspire future proteomic studies aimed at tackling current clinical challenges.

2 Advanced Heart Failure, Recovery, and Clinical Needs

From a clinical perspective, the transition in a patient's disease trajectory from heart failure to AHF is often gradual and insidious. Unfortunately, there is no singular test or imaging modality capable of differentiating these patient populations. Commonly, patients are characterized as having progressed to AHF when they remain symptomatic (NYHA

Functional Class IIIb and IV) despite “optimal medical therapy” [1]. Simplistically, patients who are unable to at least comfortably ambulate a city block or flight of stairs without symptoms, despite maximally tolerated dosages of neurohormonal therapy, fall into the category of AHF [1]. While numerous risk stratification schemes exist, none of them perfectly predict disease progression or risk of morbidity and mortality, particularly not for individual patients. Identifying clinical inflection points necessitating MCS and cardiac transplantation thus remains more of an art than a scientific decision, leading to tremendous practice pattern variation amongst physicians [15, 16]. Ideally, clinicians should be able to stratify patients with objective data, such as biomarker profiles predicting risk of progression or alternatively, the potential of reverse remodeling and myocardial recovery.

2.1 Remodeling, Mechanical Circulatory Support, Reverse Remodeling, and Recovery

In heart failure, the weakened heart attempts to maintain its function by an adaptive remodeling process that initially maintains cardiac output, but eventually leads to abnormal mass, volume, geometry and composition of the ventricle [4]. MCS, in the form of a contemporary VAD, involves a surgically implanted mechanical blood pump, continuously moving blood from the failing left ventricle to the aorta. MCS results in a decrease in intracardiac pressures and enhanced systemic perfusion and, in many patients, results in a gradual improvement of native left ventricular systolic function so that the ejection fraction increases (*i.e.* reverse remodeling [17, 18]). Reverse remodeling varies widely among MCS patients [17], but for a minority, a sustained and significant degree of reverse remodeling is achieved, whereby the previously failing heart shrinks and becomes stronger to the point where it is able to again meaningfully contribute to overall systemic perfusion [19]. In a small subset of patients (~1%), this reverse remodeling proceeds to the point of myocardial recovery, which is defined as sustained normalization of molecular, structural, and functional changes with sufficient contractile reserve that the VAD may be explanted [17, 19]. While myocardial recovery occurs on a phenotypic level, it is apparent on the cellular level that full recovery is not truly achieved (*i.e.* the heart does not restore to a pre-disease state), but rather, this recovery is more accurately a form of “remission” [18, 20]. Since the first clinical observations of myocardial recovery in MCS [5-7], research efforts have attempted to define the mechanistic underpinnings of this phenomenon, though causative and correlative processes underlying reverse remodeling and myocardial recovery remain elusive [18]. Better understanding of molecular events, especially those features that distinguish patients that recover from those that reverse remodel without recovery, could be exploited for development of novel therapies to stimulate self-recovery and reveal new clinical biomarkers for better patient stratification regarding predicted response to therapy and decisions regarding course of treatment.

2.2 A Clinical Perspective on Current Biomarker Opportunities

In modern clinical practice, establishing a diagnosis of heart failure has become relatively straightforward. However, in AHF there is a paucity of biomarkers correlating to disease severity, clinical trajectory and potential of recovery with therapy in this highly complex patient cohort. As outlined in Figure 1, opportunities for more informative biomarkers exist along the entire clinical spectrum related to disease diagnosis, decisions regarding therapy, and assessing final outcomes. First, specific markers of cardiac inflammation, an integral

part of the healing process, would allow for an assessment of active damage and recovery potential. Systemic markers of inflammation are not specific to myocardium and have limited clinical utility in this context [21]. Second, the degree of cardiac fibrosis is a correlative predictor of improvement during VAD therapy [22, 23]; thus, more accessible markers would avoid the need for invasive biopsy. Third, the ability to predict which patients will benefit from MCS, with the addition of pharmacological adjuvants, would present new opportunities to refine treatment strategies. Finally, the process of clinically quantifying myocardial recovery is not yet well-defined, but often includes cardiac catheterization, echocardiography, and metabolic stress testing [18]. Thus, it would be valuable to develop new, less-invasive strategies that can accurately provide a more quantitative measure of recovery and assist physicians in deciding if and when VAD explantation should proceed.

In an ideal world, a patient with *de novo*, therapy-naïve heart failure would undergo a comprehensive evaluation that includes a biochemical “barcode” to stratify them prospectively in terms of disease trajectory and recovery potential. In most cases, circulating panels of diagnostic or prognostic markers would be most clinically relevant due to their non-invasive nature, assuming they can be developed with adequate sensitivity and specificity, and would complement existing physiological measurements such as invasive hemodynamics, echocardiography, and exercise testing. Additionally, it is expected that new agents for real-time imaging of the myocardium would be well-suited for questions requiring more direct measures of cardiac function, as it is unclear whether the complexity of multi-cellular interactions within the myocardium could be fully reflected by measuring circulating biologics. The availability of such a barcode would allow for tailored therapy, earlier intervention, and more sophisticated decision-making to proceed with AHF therapy, MCS and transplantation for patients failing other therapy. Ultimately, if clinicians could predict which patients will recover with medical therapy alone, which will require MCS as a bridge to recovery, and which will have no potential of recovery, the decision to allocate resource-limited therapy like cardiac transplantation would become much more objective.

As outlined below, many described changes to the myocardium occurring in reverse remodeling are post-translational [24-32], thus it is unlikely that genetic determinants will be fully predictive with respect to the aforementioned clinical needs [33]. Consequently, the examination of protein-level changes is expected to offer mechanistic insights into complex disease processes and provide biochemical metrics that will serve as empirical indicators in the clinic. With the above stated clinical goals in mind, we next present a summary of current knowledge regarding protein dynamics in myocardial recovery with MCS, highlighting where our knowledge is limited and why new studies are needed.

3 Hold or Fold? Our Current View of Protein Dynamics in Myocardial Recovery

Our current knowledge of cellular, structural, and functional changes occurring during reverse remodeling with MCS has been the topic of several excellent reviews [17, 34-36]. To date, the majority of studies describing molecular changes in AHF and myocardial recovery have used transcriptomic approaches [17, 33, 37-40]. However, transcriptomic

approaches are unable to capture important information regarding post-translational processes such as protein localization, modifications, interactions, and degradation - each of which directly relate to a protein's function. Moreover, mRNA expression levels do not always directly correlate to protein abundance levels [41, 42] and are unable to precisely assess various molecular products of a single gene (*i.e.*, proteoforms [43]), which collectively describes the amino acid sequence plus all post-translational modifications). Thus, this section focuses specifically on those protein abundance levels, subcellular localization, and proteoform changes within the myocardium that have been described in AHF as altered in MCS and, when data are available, myocardial recovery.

3.1 Changes in Protein Abundance, Modifications and Localization

The majority of protein abundance changes described thus far in AHF and myocardial recovery include those related to maintenance of cardiomyocyte structure, metabolism, and protein homeostasis. In one study of left ventricle samples taken at time of VAD implantation and subsequent explantation in patients with myocardial recovery, a decrease in talin and vinculin and an increase in sarcomeric proteins were correlated with recovery [44]. These changes may indicate that there is partial normalization of the cytoskeletal environment during myocardial recovery. Periostin, an extracellular matrix protein increased in the failing heart [45] is decreased after MCS [46], suggesting extracellular matrix components are also normalized in reverse remodeling. The decrease in creatine kinase (CK) observed in the failing heart is reversed upon MCS, specifically via increases in protein levels of CK-M and CK-Mt [47]. Also, representative genes in the mitochondrial electron transport chain can be restored with mechanical unloading [48]. Altogether, these changes suggest that alterations to mitochondria and cellular metabolism occurring during heart failure can be restored. Transverse tubules, which are reorganized in heart failure [49], are reverse remodeled upon mechanical unloading in a rodent model [50], though it is yet unclear whether this occurs in humans during MCS. In AHF, the 20S proteasome abundance and activity are decreased commensurate with accumulation of ubiquitinated proteins, suggesting impairment of proteasome-dependent degradation, and reversal of this has been observed with mechanical unloading [51, 52]. Autophagy markers including Beclin-1, Atg5-Atg12 conjugate, and LC3-II, which are increased in hypertrophy, are reduced with mechanical unloading in humans and rodents [52, 53]. Collectively, these studies indicate that protein and cellular homeostasis mechanisms maybe normalized at the protein level by MCS.

In addition to these protein abundance changes, there is also evidence to suggest reverse remodeling is, at least partially, regulated at the post-translational level. The disruption of the N-terminus of dystrophin accompanying heart failure has been shown to reverse somewhat after mechanical unloading [32, 54] and phosphorylation of cardiac troponin I decreases after VAD support [24, 30]. VAD support increases overall caveolin-1 protein abundance [31], and, while total levels of caveolin-3 remain unchanged, caveolin-3 within the sarcolemma is increased [31]. In a study of the failing heart after VAD support, vinculin quantity is unchanged, but localization to the intercalated disc is diminished in the failing heart and restored during MCS [25]. Also, the increase in desmin protein in Z-lines and hyperpolymerization of β -tubulin observed in heart failure is reversed with VAD support

[25]. We Mechanical unloading has also been found to reverse β -adrenergic receptor density loss observed in heart failure as well as to improve localization of the receptors to a more homogenous distribution [26]. Finally, while changes in collagen levels and the extent of cross-linking vary among studies of VAD recipients [24, 27-29, 55], these processes are regulated by matrix metalloproteinases and tissue inhibitors of metalloproteinases, which quantitatively change in the failing heart and during MCS [29, 55]. The degree of collagen cross-linking is directly linked to myocardial stiffness and contraction [56] which supports measurement of fibrosis as an indicator of myocardial recovery with MCS [22, 23].

3.2 Study Limitations and New Opportunities

Altogether, these studies demonstrate that at least some of the molecular changes occurring during remodeling in AHF can be reversed or normalized with MCS. However, to assert any of these proteome-level changes as necessary, causative, or correlative of myocardial recovery is premature for several reasons. First, most studies have been performed on bridge-to-transplantation samples and thereby do not directly measure functional improvement, but rather unloading. Second, few studies directly compare patients who recover to those who remodel without recovery; of those that do, sample sizes of patients exhibiting myocardial recovery have been limited (e.g. typically 6 recovery patients/study). Consequently, we currently lack a comprehensive understanding of molecular events that are required for recovery, how they are coordinated, and what distinguish espartial reverse remodeling from full myocardial remission [18]. Despite these limitations, these data suggest that post-translational processes play a role in reverse remodeling, and therefore, new studies that incorporate direct measurement of proteoform content and distribution within myocardium are expected to have a significant impact on our understanding of molecular events underlying physiological changes ultimately culminating in myocardial recovery associated with MCS. Specifically, the use of highly sensitive mass spectrometry approaches to determine which protein changes are “holding” during reverse remodeling (*i.e.* which changes sustain myocardial remission and ultimately, recovery) and which are “folding” (*i.e.* which changes are associated with the re-emergence of heart failure) should yield new mechanistic insight that could potentially be exploited for development of new treatments and markers with diagnostic and prognostic value.

4 The Future of Proteomics in Myocardial Recovery

At the time of this review, combining the terms “myocardial recovery” and “proteomics” in a PubMed search yields no publications. Considering the various sophisticated types of mass spectrometry instrumentation available, sample preparation strategies, biochemical tools, and bioinformatic workflows that can be combined in any number of ways, our proteomic toolbox provides a virtually limitless number of potential approaches for assessing protein content within a sample. While it is not possible to provide detailed descriptions of all strategies here, we include a discussion of sample source considerations and highlight proteomic approaches we believe are especially well-suited to address several key mechanistic questions and clinical needs described above. Undoubtedly, the first logical step to be addressed in future studies involves an experimental design that must carefully consider whether the study goals are to address a mechanistic question and/or develop new

informative biomarkers. Thus, we direct readers towards a recent in-depth review discussing a “roadmap” for development of clinically-relevant biomarkers of heart failure [57].

4.1 *In vivo* Sample Sources

In the heart failure literature, serum and plasma have been samples of choice for the majority of biomarker discovery efforts, largely due to their accessibility. However, with respect to identifying and characterizing proteins with direct relevance to mechanism, diagnosis, and prognosis involved in the care of patients with AHF, the myocardium offers several advantages as a sample source for proteomic studies (Figure 2A). First, direct analysis of tissue allows for characterization of proteins as they exist in their native context of disease, which is critical for interpretation of their mechanistic role. Second, proteins are at their highest concentration in tissue, which makes this a practical sample source for discovery of new biological molecules that can subsequently be probed for in urine or blood using targeted strategies that are more sensitive than those used for discovery. Direct analysis of the tissue also avoids the notorious challenges of identifying cardiomyocyte-specific proteins within a highly complex environment containing abundant circulating proteins, those originating from non-cardiac sources, and those that are a result of cellular necrosis unrelated to the heart [58]. As the number of VAD patients increases, so does the opportunity to study tissue at two timepoints within the same patient (*i.e.* implantation and explantation), which will be critical for deciphering successive events favoring myocardial reverse remodeling to the point of clinical recovery. When considering the appropriate experimental model, it will be important to consider that merging samples from patients of different ages, sex, and different etiologies together within the failure group may preclude identification of significant changes correlating to disease state [59] and this clinical reality may complicate data interpretation, including the varied use of pharmacological intervention among patients and inability to control for patient age, clinical presentation, and duration of MCS.

While analysis of primary human myocardial tissue is undeniably valuable, it may not be feasible for all types of discovery-based proteomic analyses. First, access to valuable patient tissue is limited both in number of donors as well as sample size. Although limitations in tissue quantity are partially addressed by technical advancements in mass spectrometry instrumentation permitting detection of low levels (pmol/L) of protein in complex mixtures [60], the limited availability of patients undergoing myocardial recovery and non-failing controls is likely to pose a challenge for the foreseeable future. A second factor to consider is that the heart is comprised of multiple cell types, including fibroblasts, cardiomyocytes, endothelial cells, and connective tissue. Therefore, approaches like transcriptomics and proteomics will produce an average of all cell types present within the tissue section used for analysis. This can complicate data interpretation in cases where the contents of a single cell type are desired (*e.g.* cardiomyocyte), especially for lower abundance proteins. For these reasons, we envision proteomic strategies that begin with analysis of the tissue when available, or cell culture models as discussed below, will provide the most direct impact on our understanding of important biological processes involved in disease with respect to events within the myocardium. Of course, blood and urine are the most practical sources for monitoring disease progression over time in a single patient. Thus, in cases where

myocardium is used for discovery, proteins of interest can subsequently be assayed in blood and urine using highly sensitive targeted mass spectrometry strategies, either with or without antibodies, to test their utility as circulating biomarkers [61, 62].

4.2 *In vitro* Cell Models

The challenges of limited sample availability and cell type heterogeneity encountered when using primary tissue make *in vitro* culture models a practical consideration for the discovery stage, at least for certain questions where a relatively pure population of the desired cell type is advantageous. For example, outstanding questions in the field of AHF and myocardial recovery that could benefit from the use of simplified culture models include examining the effects of mechanical stress on cardiomyocytes, studying interactions in co-culture models, identifying receptors for biologically relevant ligands, and determining the physiological effects of ligands, drugs and small molecules on cardiomyocytes. Explanted primary cardiac myocytes and fibroblasts offer one possibility for analysis of relatively homogeneous populations of cells. However, these typically have limited potential for *ex-vivo* expansion in culture and some physiological changes may occur with culturing [63].

Another valuable cell culture model includes cardiomyocytes derived from human pluripotent stem cells (hPSC; which includes embryonic (hESC) and induced (hiPSC)). Although early protocols for cardiomyogenic differentiation of hPSC suffered from relatively low rates of efficiency, recently developed strategies routinely generate ~100 cells for every one input hPSC, with typically >95% of the population identified as cardiomyocytes based on staining for signature markers such as troponin I and T, albeit variation in cardiomyogenic efficiency remains among protocols [64-68]. Considering that patient-derived hiPSC can now be routinely generated, these cells are well-suited to the study of non-diseased controls as well as cardiomyocytes from patients with inherited cardiomyopathies [69]. Thus, the hPSC system offers the advantage of generating a relatively pure culture of cardiomyocytes useful for discovery-based proteomic studies aimed at characterizing the molecular content of the human cardiomyocyte, which remains relatively poorly defined [70].

Of course, use of hPSC-derived cardiomyocytes for fundamental proteomic studies is not without caveats. Currently, hPSC *in vitro* differentiation protocols generate heterogeneous mixtures of cells reminiscent of multiple subtypes (e.g. ventricular, atrial, nodal), and the percentage of each subtype within the culture varies among protocols [71, 72]. Until more robust strategies to guide *in vitro* differentiation towards a specific subtype are developed, or new reagents are available for selecting homogeneous populations of a specific subtype are identified, this will remain an important consideration for research applications focused on chamber restricted pathologies. The developmental stage of the cells generated is also an important consideration. Current protocols generate hPSC-derived cardiomyocytes most closely resembling embryonic or fetal staged heart in terms of electrophysiological signals, gene expression patterns and lack of fully formed transverse tubules [73, 74]. While this should not preclude the utility of hPSC-derived cardiomyocytes for AHF and myocardial recovery research, the developmental stage of the hPSC-derived cardiomyocytes used for *in vitro* studies is an important consideration for data interpretation. Now that differentiation

protocols have become robust, the value of an unlimited supply of human cardiomyocytes in simplified cell culture models for proteomic discovery efforts is only beginning to be exploited [75-78]. Although it may never be possible to faithfully model complex cardiovascular diseases like AHF in culture, *in vitro* studies are invaluable for defining normal cellular phenotypes and providing adequate source material for many fundamental protein biochemistry studies (Figure 2B). Importantly, in cases where cell culture models are used, efficient validation of these observations can be made in the tissue using immunohistochemistry, imaging mass spectrometry [21], and targeted, quantitative mass spectrometry strategies [79-82] in a manner that conserves these limited resources.

4.3 High Impact Proteomic Strategies

Considering the needs for new clinical biomarkers (Figure 1) and currently unanswered questions regarding mechanisms of disease, many different proteomic strategies will benefit this field, some of which are listed in Figure 3. We cannot possibly enumerate all potential applications of proteomics for AHF research nor is our goal to provide an in-depth discussion of each approach. Rather, we direct readers to comprehensive reviews to learn details, benefits and caveats of the strategies outlined below. In this section, we highlight three broad categories of proteomic strategies that are expected to have a significant impact on our knowledge and treatment of AHF and myocardial recovery with the goal of introducing clinicians and other non-proteomic experts to state-of-the-art strategies that can be used to interrogate the proteome.

First, in reviewing the literature in this field it becomes clear that at least one of the reasons knowledge regarding protein dynamics in myocardial recovery remains limited is that protein quantity changes examined have largely been restricted to targets identified through transcriptomic approaches and nearly all studies reporting protein abundance changes in AHF and myocardial recovery use immunoblotting techniques. These studies are therefore limited to targets for which suitable and specific antibodies are available. Consequently, there is a clear opportunity for implementation of state-of-the-art quantitative mass spectrometry approaches to expand our understanding of protein abundance changes in AHF and myocardial recovery. Multiple well-established strategies for accurately measuring protein abundance are available and can be incorporated at nearly every stage of proteomic analysis, from discovery to validation, independent of whether the study is geared towards addressing a mechanistic question or developing a new biomarker and these methods have been extensively reviewed [83, 84]. Stable isotopic labeling strategies are well suited to multiplex comparisons and label-free strategies are also increasingly informative in discovery efforts. Both types of quantitative strategies can be especially informative when coupled with subcellular and/or post-translational modification enrichment approaches [11, 85]. When appropriate, higher throughput targeted quantitation by selected/multiple reaction monitoring can be used for multiplex quantitation of proteome changes across hundreds, if not thousands, of patient samples [79-81]. These targeted quantitation strategies are especially advantageous for those analytes for which highquality antibodies are not commercially available, or as an intermediate verification step before proceeding with development of new affinity reagents (*e.g.* new ELISA assays). Moreover, these strategies can be combined with affinity enrichment for even more sensitive analyses of very low

abundance analytes [82]. Altogether, it is possible to use quantitative mass spectrometry strategies to discover previously unknown protein quantitation changes in diseased tissue, validate protein-level changes predicted by transcriptomics, provide relative or absolute quantitative values, and to perform quantitation independent of availability of an antibody specifically recognizing a particular epitope, modification, or proteoform. Importantly, these mass spectrometry-based proteomic strategies are appropriate for pre-clinical testing and are progressing towards the clinic [86] where they are ultimately expected to join the ranks of already established clinical mass spectrometry based analyses of small molecules and metabolites [87].

While protein abundance has been one of the most exploited parameters in the development of clinically relevant biomarkers, it is only one determinant of a protein's biological activity. Post-translational modifications [88] and isoforms [89] are emerging as important indicators of cardiac disease. Therefore, the second major type of proteomic strategy we expect will yield important new mechanistic insights and biomarkers is top-down proteomics, wherein proteoforms are analyzed in their intact state to reveal the amino acid sequence (*e.g.* is protein in the intact, isoform, splice variant form?) and the site and identity of post-translational modifications [90], including stoichiometry of modifications. Although analysis of intact proteins by tandem mass spectrometry has been challenging in the past, development of new mass spectrometers, data analysis platforms, and efficient sample preparation strategies have facilitated implementation of top-down analyses as routine in many laboratories [91]. Top-down proteomics is a valuable complement to peptide-centric approaches and relevant examples of this approach include monitoring proteoforms in diseased tissue and blood [92, 93].

The third major category includes analysis of cell surface proteins, namely identification and quantitation of proteins localized to the plasma membrane and extracellular matrix as well as characterization of ligand-receptor interactions. Historically, analysis of transmembrane proteins by mass spectrometry has been challenging due to their low abundance and hydrophobicity. However, with the recent development of new strategies allowing for specific enrichment of peptides from the extracellular domain of surface proteins, we can now overcome challenges in relying on database annotations for predicting whether proteins are localized to the plasma membrane or extracellular matrix [94-99]. Clinical applications of cell surface proteins include cell-type specific proteins that may be exploited for use in real-time patient imaging for tracking myocardial mass and structure and predicting patient outcomes in recovery. For example, if myocyte-specific markers suitable for imaging could be developed, this could help overcome limitations of current markers (*e.g.* gadolinium) merely indicative of perfusion or fibrosis, but not specific to metabolically-healthy cardiomyocytes. Moreover, given the importance of fibrosis in predicting patient trajectory [17], more elegant markers of extracellular matrix structure in fibrosis that could be used independently of MRI would be especially beneficial for those patients with contraindications to MRI scanning, as many AHF patients have implanted devices such as defibrillators and bi-ventricular pace makers preclusive to MRI. Cell-type specific surface proteins could also be used in affinity-reagent based sorting of primary cells to develop better explanted culture models, and, if surface exposed epitopes are released and found in

circulation, they could be especially useful as clinical biomarkers [100]. Beyond their utility as markers, cell surface proteins are the gateway through which cells send and receive signals. Therefore, new reagents [101, 102] for identifying cell surface receptors for ligands of currently unknown receptors (e.g. biologics, small molecules, circulating secreted factors) will be very relevant. For example, it may be possible to use such techniques to discover unknown or off-target receptors of common pharmacotherapies which may help explain their highly varied response among AHF patients [103].

Clearly, modern proteomic technologies are poised to revolutionize our understanding and treatment of AHF with the ultimate end-goal of myocardial remission and recovery. Of course, value will also come from approaches we did not have space to discuss. However, as with any -omic approach generating large scale data, perhaps one of the most challenging aspects will be to define which molecular processes are most important or required to preserve myocardial function longterm. Ultimately, synergistic efforts by clinicians and basic scientists will be required to decipher mechanisms of myocardial recovery, knowledge that could ultimately be exploited to meet clinical needs and improve patient care. Given the stark imbalance of donor supply and demand in AHF, the definitive biologic therapy of cardiac transplantation remains severely resource-limited and thus epidemiologically insignificant [16]. As such, we feel it is morally imperative for the translational community to collaborate to maximize scientific inquiry into viable therapeutic alternatives for treating AHF.

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Abbreviations

AHF	advanced heart failure
VAD	ventricular assist device
MCS	mechanical circulatory support
hPSC	human pluripotent stem cell
hiPSC	human induced pluripotent stem cell

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Figure 1. The clinical workflow for the treatment of advanced heart failure with ventricular assist device (VAD)

Within the context of advanced heart failure and mechanical circulatory support, the current major clinical questions are indicated. For each question, the types of protein biomarkers that would be especially beneficial are indicated.

Figure 2. Sample sources relevant for the proteomic analysis of advanced heart failure and myocardial recovery

A. Advantages and considerations of various sample sources for proteomic-based discovery and validation studies. B. Applications of hPSC-derived cardiomyocytes in the context of mechanistic and translational advanced heart failure research.

Figure 3. Summary of the types of mass spectrometry-based proteomic strategies that are especially relevant for the study of advanced heart failure and myocardial recovery
The inner circle (grey) indicates major categories of proteomic strategies and the outer circle (blue) provides examples of the types of research questions relevant for advanced heart failure and myocardial recovery that can be addressed by the appropriate strategy, including those that are expected to reveal mechanistic insights as well as those that can be exploited for new biomarker development.