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ORIGINAL RESEARCH ARTICLE

Circulating biomarker responses to medical management vs. mechanical circulatory support in severe inotrope-dependent acute heart failure

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Abstract

Background Severe inotrope-dependent acute heart failure (AHF) is associated with poor clinical outcomes. There are currently no well-defined blood biomarkers of response to treatment that can guide management or identify recovery in this patient population. In the present study, we characterized the levels of novel and emerging circulating biomarkers of heart failure in patients with AHF over the first 30 days of medical management or mechanical circulatory support (MCS). We hypothesized a shared a plasma proteomic treatment response would be identifiable in both patient groups, representing reversal of the AHF phenotype.

Methods and results Time course plasma samples of the first 30 days of therapy, obtained from patients managed medically ($n = 8$) or with implantable MCS ($n = 5$), underwent semi-targeted and candidate biomarker analyses, using multiple reaction monitoring (MRM) mass spectrometry, antibody arrays, and enzyme-linked immunosorbent assays. Differentially expressed proteins were identified using robust limma for MRM and antibody array data. Patients managed medically or with implantable MCS had a shared proteomic signature of six plasma proteins: circulating cardiotrophin 1, cardiac troponin T, clusterin, and dickopff 1 increased, while levels of C-reactive protein and growth differentiation factor 15 decreased in both groups over the 30 day time course.

Conclusions We have characterized the temporal proteomic signature of clinical recovery in AHF patients managed medically or with MCS, over the first 30 days of treatment. Changes in biomarker expression over the time course of treatment may provide a basis for understanding the biological basis of AHF, potentially identifying novel markers and pathophysiologic mechanisms of recovery.

Keywords Acute heart failure; Plasma proteomics; Biomarkers; Mechanical circulatory support; Ventricular assist device; Bioinformatics

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Introduction

Severe inotrope-dependent acute heart failure (AHF) is associated with poor clinical outcomes, and biomarkers to better guide therapy have the potential to improve clinical management of this condition. Unlike the situation in chronic heart failure (CHF), studies in severe AHF are confounded by rapid changes in clinical status of patients and the administration of multiple concurrent therapeutic interventions. Coupled with a rapid clinical course, these factors have resulted in a limited literature characterizing the pathophysiology of the development of, and recovery from, AHF syndromes. There are currently no well-defined biomarkers of response to

treatment that can be used to guide management or evaluate recovery in this patient population. Development of non-invasive monitoring strategies reflective of response to therapy would provide several advantages over current standard of care. At present, clinical observation and monitoring of laboratory variables form the mainstay of patient evaluation; however, these have demonstrated limited reliability.[1] Biomarkers representative of temporal treatment effects may have utility in informing treatment intensity and improve identification of responders and non-responders to therapy. Efforts to elucidate markers of clinical recovery would also have significant implications in guiding ventricular assist device (VAD) discontinuation in patients bridged to recovery. Protocols for device explantation continue to evolve[2] and the application of non-invasive biomarker-based stratification strategies could facilitate patient selection and reduce adverse events.

Existing data on circulating biomarker responses to heart failure (HF) treatment focus on natriuretic peptides in the setting of chronic disease,[1,3,4] and limited reports of plasma signatures in AHF exist. Few investigators have examined the plasma proteome in a non-targeted fashion. In the present study, we aimed to characterize the levels of novel and emerging circulating HF biomarkers in patients with severe inotrope-dependent AHF over the first 30 days of therapy. Time course samples from patients managed medically or with an implantable VAD underwent semi-targeted and candidate biomarker analyses to generate biomarker signatures characteristic of the response to each treatment modality. We hypothesized there would be in-common markers of recovery identifiable in these two treatment groups. Given the dramatic left ventricular (LV) unloading and restoration of circulation possible through mechanical circulatory support (MCS), plasma signatures obtained from this patient population may identify proteins reflective of clinical recovery from severe AHF. By investigating the temporal relationship between improvements in haemodynamics and perfusion, and circulating markers, we attempted to detect a proteomic signature representing recovery from inotrope-dependent AHF. Characterization of changes in biomarker expression over the time course of treatment may provide a basis for understanding the biological role of emergent and novel biomarkers of HF in the reversal of the AHF phenotype and potentially identify novel markers of pathophysiologic recovery that may also be applicable in the setting of chronic disease.

Methods

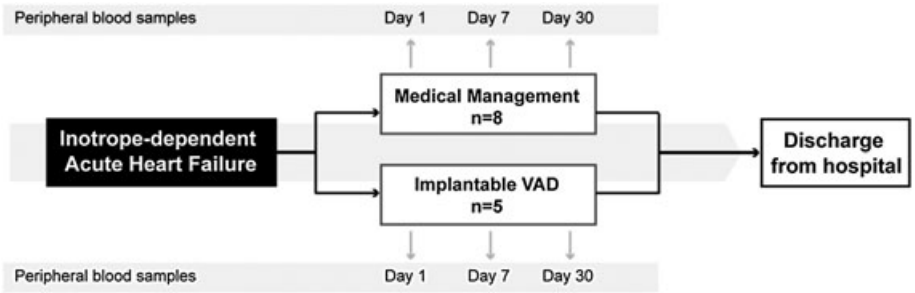
Study design

This prospective, longitudinal study was approved by the Human Research Ethics Boards of the University of British Columbia and Providence Health Care. All patients presenting to St Paul's Hospital (Providence Health Care, Vancouver, BC) with AHF in the intensive care unit, cardiac intensive care unit, or cardiac surgery intensive care unit were evaluated for inclusion criteria: (i) AHF; (ii) supported by at least one inotrope; and (iii) 19 years or older. All patients were INTERMACS Profile 1 or Profile 2. Patients providing informed written consent were enrolled in the study. Peripheral blood samples were collected at Days 1, 7, and 30 (*Figure 1*). Clinical outcomes were followed over a minimum of 1 year. All VAD patients were supported with a HeartWare device (Framingham, MA, USA). Baseline clinical history was collected at enrollment for all study subjects. Pharmacy records of all inotropic and renin–angiotensin–aldosterone system (RAAS) antagonist medications administered during hospitalization were tabulated.

Sample collection and processing

Blood samples from study subjects, taken at the scheduled time points, were drawn into EDTA tubes and stored on ice before processing. Plasma was centrifuged at 4°C within 2 h of collection, aliquoted, and stored at −80°C until further analysis.

Figure 1 Patients admitted to St Paul's Hospital for acute heart failure and supported on at least one inotrope in the intensive care unit, cardiac intensive care unit, or cardiac surgery intensive care unit were enrolled in the study and had serial blood samples collected over the first 30 days of treatment. Of these 13 patients, 5 underwent implantation of a left ventricular assist device, and 8 were managed medically; all were stabilized following treatment and ultimately discharged from hospital. Blood samples taken at days 1, 7, and 30 of treatment were analysed for differential protein expression to characterize reversal of the acute heart failure phenotype.



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Multiple reaction monitoring mass spectrometry

Patient plasma samples were prepared and analysed as previously described.[5,6] In brief, 5 μL of plasma was diluted 1:10 with 25 mM ammonium bicarbonate, denatured with sodium deoxycholate, reduced with tris(2-carboxyethyl) phosphine, alkylated with iodoacetamide, and quenched with dithiothreitol. Samples were digested with sequencing grade modified trypsin (Promega, Madison, WI, USA) overnight at 37°C and acidified with an equal volume of formic acid to stop digestion. A concentration-balanced mixture of isotopically labelled internal standard peptides was added to each sample in a 1:4 ratio. Injection of plasma digest samples onto reversed-phase capillary columns was performed using an Eksigent NanoLC-1Dplus HPLC (Eksigent, Redwood City, CA, USA). An Applied Biosystems/MDS Sciex 4000 QTRAP was used for liquid chromatography-tandem mass spectrometry with multiple reaction monitoring (MRM) analyses (Applied Biosystems, San Francisco, CA, USA). MRM data were processed using Agilent MassHunter Quantitative and Qualitative Analysis software (Agilent, Santa Clara, CA, USA). A table summarizing assayed proteins can be found in Supporting Information, Appendix S1.

Quantitative antibody arrays

Custom Quantibody® antibody arrays for quantitative detection of 20 proteins were obtained from RayBiotech (Norcross, GA, USA). Array slides were processed according to manufacturer's instructions. Thawed plasma samples were diluted 1:5 in assay diluent and 100 μL loaded per well. Data were acquired using an Axon GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA, USA) and quantified using ImaGene® 9.0 microarray analysis software (Bio Discovery, Hawthorne, CA, USA). Standard curves were generated for each analyte using a best fit line (linear or log-log), and unknown values were interpolated using manufacturer's software. Array scanning and quantification were performed by the Prostate Centre Microarray Facility, Vancouver, Canada. Analytes are summarized in Appendix S1.

Enzyme-linked immunosorbent assays

Plasma/serum samples were thawed on ice, diluted per protocol, and assayed in duplicate as per manufacturer's directions. Plates were read on a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA), and values were interpolated using SoftMax Pro microplate software (Molecular Devices). Analytes are summarized in Appendix S1.

Statistical analytical approaches

Multiple reaction monitoring mass spectrometry data

Statistical analysis was performed using R 2.15.3 software[7] and Bioconductor version 2.11 (www.bioconductor.org), as per our previously published procedures.[6,8–10] MRM data were evaluated for quality. Peptides with a median relative ratio <0.0005, median response <100, and >1 standard deviation beyond the 80–120 range were eliminated from further analyses. Peptides present in $<75\%$ of patients were removed from further analysis. For peptides not detected in a given sample, a value half the minimum peptide level detected in remaining patient samples was substituted. MRM data were Log2 transformed. For proteins with multiple peptides assayed, protein level was calculated from the peptide with the highest relative ratio in the majority of the samples analysed.

Biomarker analyses

Proteins differentially expressed over the 30 day time course, from MRM and array data were identified using robust limma with $P < 0.05$ and false discovery rate <0.05. Baseline and Day 30 ELISA were analysed using paired Student's *t*-test. Two-way analysis of variance (ANOVA) was used to calculate treatment effects between management groups.

Results

Sample selection and cohort characteristics

Clinical data are summarized in *Table 1*. There were no significant differences in baseline parameters between treatment groups. All patients receiving VAD underwent cardiac transplantation within 1 year post-discharge. Of patients managed medically, one died after the 30 day study time point and one underwent cardiac transplantation. Inotropic support was discontinued over time in all VAD patients, one patient in the medical management group continued to have inotropes administered over the full 30 days of treatment (*Table 2*). Use of RAAS inhibitors was initiated by Day 7 of treatment in both groups. By Day 30, all medically managed patients were receiving angiotensin-converting-enzyme inhibitor as compared with 40% in the VAD group.

Effect of treatment on clinical and laboratory parameters

Changes in haemodynamic and laboratory variables between baseline (prior to initiation of therapy) and at Day 30 of treatment were evaluated (*Table 3*). Patients in both groups demonstrated improvements in systolic blood pressure and blood urea nitrogen over the time course of treatment; however,

Table 1 Cohort characteristics

	Implantable ventricular assist device	Medical management	*P*-value
N	5	8	
Age (years)	58.2 (5.8)	54.4 (8.9)	0.418
Female (%)	0 (0)	4 (50)	0.105
Body mass index (kg/m^2)	27.4 (3.1)	26.2 (4.9)	0.637
Cardiac history			
Left ventricular ejection fraction	21.2 (7.5)	22.0 (7.7)	0.858
Myocardial infarction	3 (60)	2 (25)	0.293
Chronic heart failure	2 (40)	4 (50)	1.000
Percutaneous coronary intervention	2 (40)	2 (25)	1.000
Implantable cardioverter defibrillator	1 (20)	2 (25)	1.000
Coronary artery bypass graft	1 (20)	1 (13)	1.000
Medical comorbidities and risk factors			
Current or former smoker	3 (60)	2 (25)	0.293
Hypertension	5 (100)	6 (75)	0.487
Diabetes mellitus	2 (40)	1 (13)	0.511
Cerebrovascular accident	2 (40)	0 (0)	0.128
Chronic obstructive pulmonary disease	3 (60)	1 (13)	0.217
Malignancy	0 (0)	1 (13)	1.000
Liver disease	2 (40)	1 (13)	0.511
Aetiology of heart failure			
De novo	2 (40)	5 (63)	0.592
Acute decompensated chronic heart failure	3 (60)	3 (38)	0.592
Acute coronary syndromes	4 (80)	4 (50)	0.266
Non-ischaemic dilated cardiomyopathy	1 (20)	3 (38)	1.000
Myocarditis	0 (0)	0 (0)	—
Valvular disease	0 (0)	1 (13)	1.000
Arrhythmia	1 (20)	3 (38)	1.000
1 year outcomes			
Death	0 (0)	1 (13)	1.000
Cardiac transplant	5 (0)	1 (13)	**0.005**

Clinical variables for treatment groups were evaluated using Fisher's exact test for categorical variables and unpaired Student's *t*-test for continuous variables. Bold text highlights significantly different variables.

these were not statistically significant. There were significant differences between groups at the Day 30 time point for serum creatinine, systolic blood pressure, and pulse pressure. Platelet counts increased in both groups over the 30 days of treatment.

Effect of treatment on circulating proteins

Proteomic analysis of the 30 day time course demonstrated a significant alteration in circulating levels of eight proteins in each treatment group. Six of these proteins were differentially regulated in both treatment groups (*Figure 2*). The identified proteins are summarized in *Table 4*. Patients managed both medically and with VAD had increases in circulating cardiotrophin 1 (CT1), cardiac troponin T (cTnT), clusterin (CLU), and dickopff 1 (DKK1). Levels of C-reactive protein and growth differentiation factor 15 (GDF15) decreased in both groups over the 30 day time course (*Figure 3*). GDF15 was differentially abundant between treatment groups as evaluated by two-way ANOVA ($P < 0.001$). Higher levels of GDF15 were observed in VAD managed patients at all time points following initiation of treatment. Each treatment group had two unique proteins differentially regulated over the time course of AHF treatment: medically managed patients demonstrated increased circulating interleukin (IL) 6 and N-terminal pro-brain natriuretic peptide over 30 days (*Figure 4*); patients managed with an implantable VAD had

Table 2 Medication use over the first 30 days of treatment in medically vs. ventricular assist device managed patients

	Implantable ventricular assist device (*n* = 5)			Medical management (*n* = 8)		
	Day 1	Day 7	Day 30	Day 1	Day 7	Day 30
Inotrope/vasopressor	5 (100)	2 (40)	0 (0)	8 (100)	2 (25)	1 (13)
Angiotensin receptor blocker (candesartan)	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)
Angiotensin-converting-enzyme inhibitor (ramipril)	0 (0)	1 (20)	2 (40)	0 (0)	5 (63)	8 (100)

Summary of major medication classes administered to the management groups in our study. Only medication regimens initiated prior to the study time point were included in each time point tabulation. All values expressed as *n* (%).

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Table 3 Laboratory and haemodynamic variables at baseline and after 30 days treatment with ventricular assist device or medical therapy

	Implantable mechanical circulatory support		Medical management		
	Baseline (pre-implant)	Day 30	Baseline (at admission)	Day 30	*P*-value
Laboratory variables					
White blood cell count ($\times 10^9$/L)	9.88 (0.8)	8.50 (1.2)	9.84 (4.29)	8.26 (4.7)	0.399
Haemoglobin (g/L)	95.6 (13.0)	99.2 (15.4)	107.6 (17.0)	110.1 (19.0)	0.773
Platelet count ($\times 10^9$/L)	164.4 (98.9)	264.8 (102.3)	91.0 (42.5)	210.75 (104.8)	**0.012**
Haematocrit (proportion)	0.29 (0.04)	0.30 (0.05)	0.32 (0.05)	0.32 (0.06)	0.684
Blood urea nitrogen (mmol/L)	11.9 (4.4)	9.3 (4.1)	18.9 (19.7)	8.3 (3.9)	0.227
Sodium (mmol/L)	137.0 (5.1)	136.8 (2.3)	140.9 (5.2)	141.0 (4.9)	0.238
Potassium (mmol/L)	3.9 (0.5)	4.3 (0.5)	4.0 (0.3)	4.2 (0.3)	0.250
Creatinine (μmol/L)	134.6 (26.8)	153.4 (73.4)†	114.1 (46.7)	82.4 (26.1)†	0.056
Haemodynamic parameters					
Left ventricular ejection fraction (%)	21.3 (7.5)	N/A	22.0 (7.6)	N/A	
Systolic blood pressure (mmHg)	80.6 (13.0)*	105.0 (16.0)†	84.2 (8.7)*	107.0 (12.8)†	**0.003**
Diastolic blood pressure (mmHg)	63.6 (6.8)	62.2 (8.7)	57.5 (9.3)	57.5 (6.7)	0.429
Mean arterial pressure (mmHg)	71.8 (9.0)	83.8 (8.0)	72.4 (11.2)	N/A	0.113
Heart rate (bpm)	83.6 (25.8)	83.8 (25.9)	105.5 (20.5)	86.6 (24.2)	0.269
Pulse pressure (mmHg)	17 (14.1)*	22 (16.6)†	47.5 (14.6)*	46.8 (13.4)†	0.001

N/A, insufficient data to report.
P-values calculated using one way analysis of variance with a post hoc Bonferroni multiple comparison test to determine differences between groups. Bold text highlights significantly different variables.
*<0.05 at baseline between groups;
†<0.05 at Day 30 between groups.

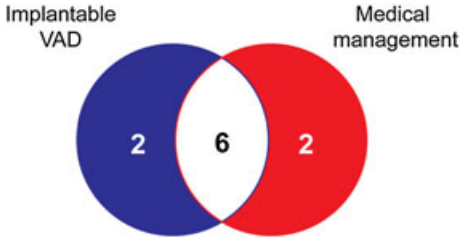
reduced circulating soluble ST2 and vitamin K-dependent protein Z (PROZ) over the same time frame (*Figure 5*). The time course of these protein levels is shown for comparison between groups.

Discussion

In this study, we characterized the temporal proteomic signature of clinical recovery in AHF patients managed medically, or with VAD. Our analysis evaluated 148 proteins and identified a common set of six that were differentially expressed in the circulation of both management groups over the first 30 days of treatment and were associated with symptomatic recovery from AHF.

Previous work on biomarkers of response to HF therapy has largely derived from CHF cohorts, with a longer time frame of analysis appropriate for this chronic condition. Reports on plasma responses to MCS are limited. Recent data have examined biomarker responses within the first week of temporary MCS[11] on a limited set of markers but did not compare these biomarker fluctuations with medical management. Conversely, data on responses to medical management of AHF have focused on early risk-stratification time points (<72 h post-admission),[12] or longer-term outcomes. Consequently, any therapy-specific differences in biomarker responses and the contribution of mechanical unloading to plasma signatures have remained unclear. We have generated a novel data set illustrating a complete time course of AHF treatment and have evaluated many of the emerging and established HF markers reflective of the major pathophysiologic pathways thought to be important in AHF. As noted by Shah *et al.*,[11] reversal of the pathophysiology of severe inotrope-dependent AHF has the potential to provide much needed insight into active processes associated with recovery.

Figure 2 Patients managed medically or with ventricular assist device had eight differentially expressed proteins identified in our analysis over the first 30 days post-admission for acute heart failure. Six of these proteins were shared by both management groups.



Evaluation of proteomic responses in the setting of VAD implantation is complicated by the inherent invasiveness of surgical implantation, and further confounded by blood products received by patients receiving this treatment modality. It is therefore perhaps surprising that a larger dichotomy was not observed between differential proteins in the two treatment groups, particularly given the distinct mechanisms characterizing each intervention. However, within this context, the overlap in biomarkers suggests that similar mechanisms are initiated in the setting of augmented cardiac output and improved systemic perfusion. Our data support the hypothesis that a common pathway of recovery occurs in the resolution of AHF, irrespective of aetiology or treatment. In the same way that the final pathologic remodelling processes

Table 4 Differentially expressed proteins over the time course of treatment in patients managed medically vs. those receiving an implantable ventricular assist device

Protein		Platform	Limma *P*-value	Limma false discovery rate	Fold change	Day 30 vs. baseline
Ventricular assist device treatment group						
C-reactive protein	**CRP**	Multiple reaction monitoring	<0.001	0.036	5.92	Down
Growth differentiation factor 15	**GDF15**	Antibody array	0.001	0.024	2.94	Down
Cardiotrophin-1	**CT1**	Antibody array	0.005	0.028	3.51	Up
Cardiac troponin T	**cTnT**	Antibody array	0.004	0.028	2.18	Up
Clusterin	**CLU**	ELISA	0.007*	—	2.27	Up
Dickopff 1	**DKK1**	Antibody array	0.009	0.039	2.07	Up
Soluble ST2/interleukin 33 receptor	ST2	Antibody array	0.013	0.043	4.52	Down
Vitamin K-dependent protein Z	PROZ	Multiple reaction monitoring	0.001	0.041	2.84	Down
Medical management group						
C-reactive protein	**CRP**	Multiple reaction monitoring	<0.001	0.026	10.9	Down
Cardiotrophin-1	**CT1**	Antibody array	<0.001	<0.001	4.19	Up
Clusterin	**CLU**	ELISA	0.001*	—	2.67	Up
Dickopff 1	**DKK1**	Antibody array	<0.001	<0.001	5.30	Up
Cardiac troponin T	**cTnT**	Antibody array	<0.001	<0.001	3.41	Up
Growth differentiation factor 15	**GDF15**	Antibody array	<0.001	0.002	2.57	Down
Interleukin 6	IL6	Antibody array	0.012	0.027	2.05	Up
N-terminal pro-brain natriuretic peptide	NTproBNP	Antibody array	0.022	0.040	2.26	Up

Common proteins identified in both groups of patients over the time course of treatment are highlighted using bold font.
*ELISA data were analysed using paired Student's *t*-test.

characterizing HF development are shared, so too may be the processes governing recovery.

Fibrotic and hypertrophic remodelling: cardiotrophin 1 and dickopff 1

Cardiotrophin 1 is a member of the IL6 cytokine family and has hypertrophic[13] and cardioprotective[14] properties. CT1 binding to its receptor, leukaemia inhibitory factor receptor β/glycoprotein 130 (gp130), prevents apoptosis and resultant myocardial chamber dilatation, promoting myocyte survival and hypertrophic responses to increased wall strain. It has been suggested that desensitization to CT1 signalling, via down-regulation of gp130, may be a contributory factor in HF development.[15,16] In normal rat hearts, CT1 treatment inhibited apoptotic and non-apoptotic cell death induced by angiotensin II or hydrogen peroxide; however, this effect was absent in failing hypertrophic cardiomyocytes.[16] It is possible therefore that the elevated CT1 levels we observed in our cohort represent a response to myocardial injury but have diminished protective effects in this setting. We observed increased CT1 over the first 30 days of AHF treatment in both management groups (*Figure 3*). Whether the effects of these elevations could be beneficial for cardiac viability would need to be investigated further, particularly in the context of myocardial gp130 receptor density. In terms of CT1 levels in response to HF therapy, a study on patients treated with cardiac resynchronization therapy reported diminished CT1 levels in non-responders.[17] This finding conflicts with the elevations in CT1 we observed in our cohort as they correlated with symptomatic recovery from AHF. The response of CT1 levels to MCS has not been characterized thus far, and it is possible that the mechanistic differences between cardiac resynchronization therapy and MCS may explain this apparent disparity in treatment response. In the context of medical therapy, *in vivo* models of isoprotenerol-induced cardiac hypertrophy demonstrated significant increases in cardiac CT1 mRNA and protein levels that could be abrogated with valsartan and spironolactone treatment via inhibition of angiotensin II-mediated CT1 induction.[18] We did not observe any reductions in CT1 in medically managed patients, all of whom were receiving RAAS antagonist pharmacotherapy by Day 30 (*Table 2*).

Dickopff 1 is an endogenous Wnt pathway inhibitor and has not been well described in the setting of HF. The Wnt pathway is one of a number of signalling pathways originally thought to be expressed exclusively during development but has come to be recognized for its contribution to cardiac remodelling in the setting of disease.[19] Activation of Wnt signalling is necessary for myocardial hypertrophy,[20] and agents inhibiting the canonical Wnt pathway have been shown to attenuate LV remodelling by inhibiting fibrosis, hypertrophy, and apoptosis.[19] DKK1 may therefore play a role in preventing myocardial remodelling, and the increases in circulating DKK1 levels observed in our cohort (*Figure 3*) could be reflective of therapeutic attenuation of progressive myocardial remodelling.

Figure 3 Significantly differentially regulated circulating proteins in patients managed medically or with ventricular assist device. C-reactive protein data were not available for Day 7 in medical management group. All points represent mean ± SEM.

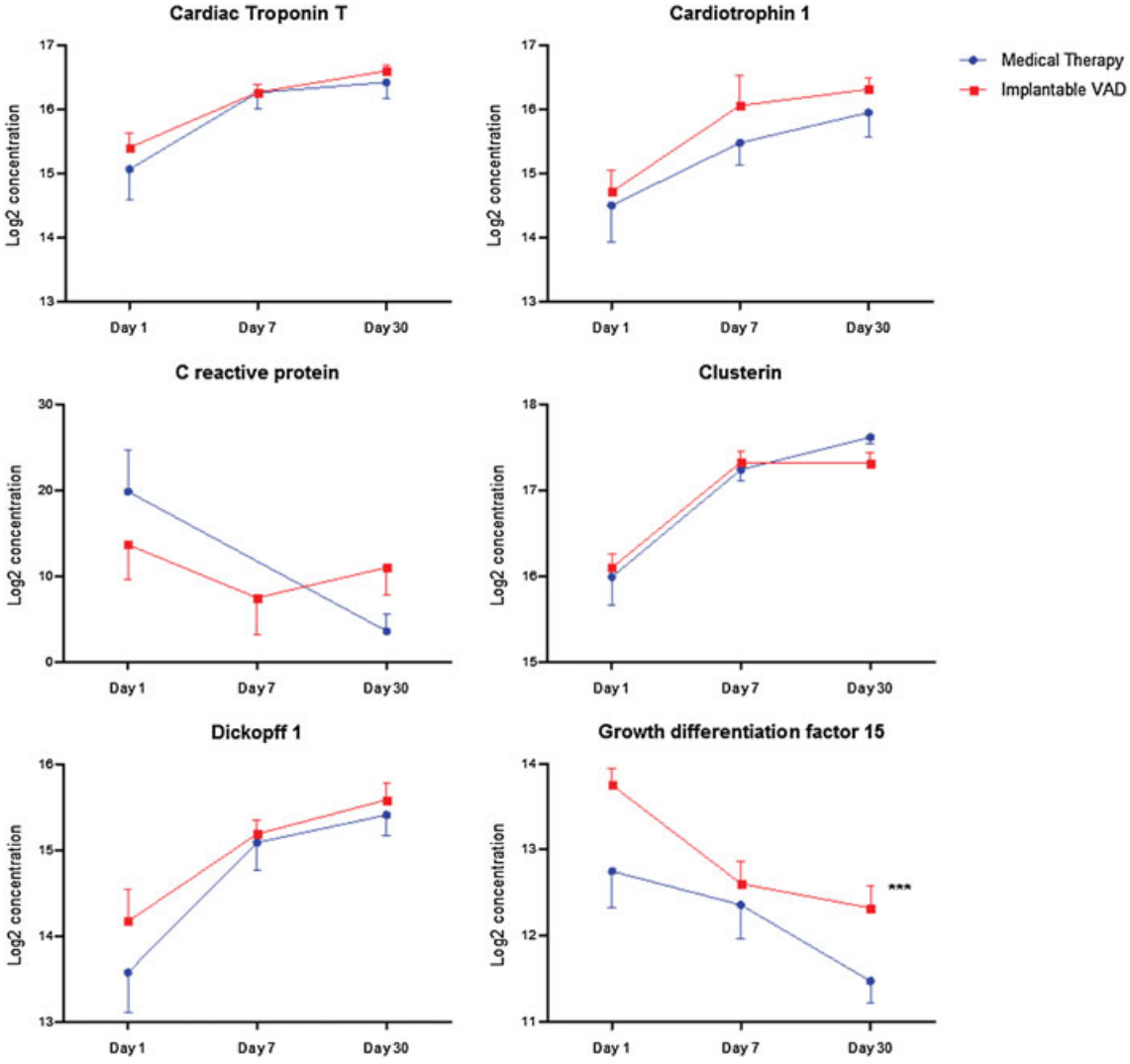


Figure 4 Medically managed patients had significantly increased circulating levels of interleukin 6 and N-terminal pro-brain natriuretic peptide over 30 days of therapy; however, ventricular assist device patients' levels (included for comparison) did not change significantly. All points represent mean ± SEM.

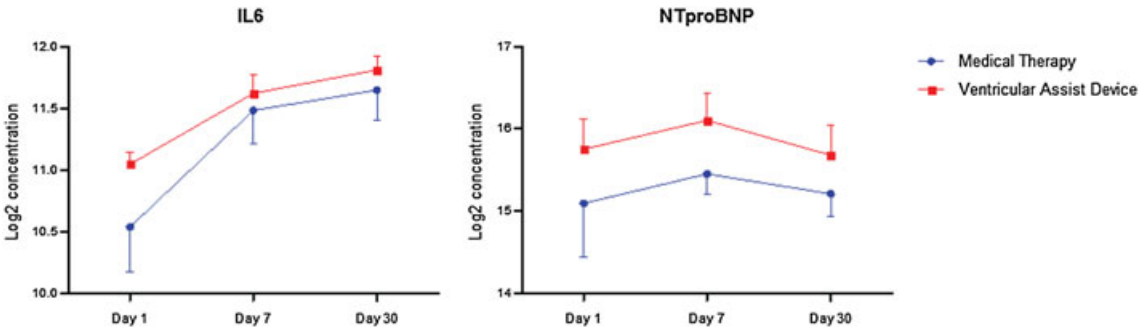


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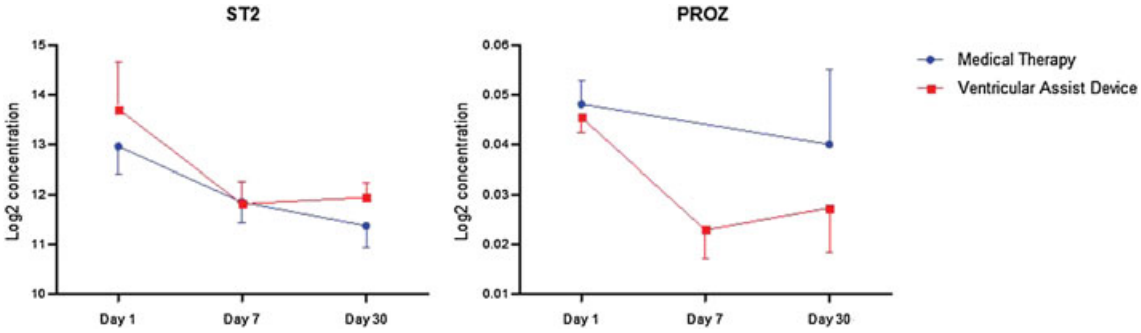
Figure 5 Patients undergoing ventricular assist device implantation had significant reductions in circulating levels of ST2 and vitamin K-dependent protein Z. Data from medically managed patients were included for comparison but were not significant in this group (vitamin K-dependent protein Z Day 7 data not available for medical therapy group). All points represent mean ± SEM.



Inflammation: interleukin 6 and C-reactive protein

Sustained elevations of IL6 observed in medical management may reflect ongoing inflammatory processes, an idea supported by observations that hospitalization for AHF results in a protracted vulnerable period of months following an index event.[21] The temporal changes in circulating IL6 seen in the medical treatment group are paralleled by patients receiving a VAD (*Figure 4*). AHF is characterized by a slow resolution of the pro-inflammatory state,[12] and this may help explain the sustained levels of IL6 observed. The significant proteins identified in this analysis were primarily derived from our candidate marker analysis. Of the 124 targets analysed by MRM, only one protein was found to significantly change in both groups of patients (C-reactive protein) (*Figure 3*). The biological pathways interrogated by the MRM assay were primarily inflammation and innate and acquired immune responses. Consequently, our data suggest immune responses do not play a major role in reversing the AHF phenotype. The observed reductions in circulating C-reactive protein support previous reports in the setting of AHF, where C-reactive protein levels declined 30 days post-admission and were reduced in response to treatment.[12] We observed a contradictory increase in IL6 coupled with a decrease in C-reactive protein levels in our cohort, giving mixed indications as to the short-term effects of therapy on inflammation. C-reactive protein may be more dynamically regulated, while IL6 elevations persist longer. There are data to indicate a role in myocardial hypertrophy and fibrosis for IL6;[22] thus, the paradoxical increase in IL6 may not be related to inflammation as much as to cardiac remodelling in response to injury and AHF therapy.

Myocardial injury: cardiac troponin T, growth differentiation factor 15, and clusterin

Cardiac troponin T is a well-described cardiac biomarker.[23] Troponin levels peak 18–24 h post-myocardial infarction (MI) but can remain elevated for up to 14 days. The presence of elevated cTnT in HF[24] may reflect ongoing myocyte injury associated with remodelling rather than infarction.[25] Our data demonstrate patients in both treatment groups had increased circulating cTnT over the first 30 days following AHF hospitalization (*Figure 3*). This observation suggests continuing troponin release in response to ongoing myocyte injury and appears inconsistent with the idea of myocardial recovery. Such contradictory findings have previously been reported in AHF: post-hospitalization survivors had higher circulating levels of cTnT than non-survivors, in patients with both ischaemic and dilated disease, and cTnT levels increased significantly over treatment time course.[26] There are data to suggest cTnT release is associated with cardiac remodelling[27] and hypertrophy,[28] and this may explain our observations and provide evidence of the effect of therapy on myocardial remodelling.

Growth differentiation factor 15 is a member of the transforming growth factor beta superfamily and its expression is limited in healthy subjects. Levels of GDF15 rise in many organs, including the heart, in response to injury, hypoxia, or cytokine exposure, and a wealth of data support its role as a cardioprotective agent.[29] Patients with CHF have increased circulating GDF15, levels of which correlate with New York Heart Association class and mortality.[30] *In vivo* and *in vitro* data on ischaemia-reperfusion injury have demonstrated protective effects of GDF15 in limiting myocardial damage and reducing cardiomyocyte apoptosis, respectively.[31] In the context of MCS, a recent report has demonstrated reductions in circulating GDF15 levels after 30 days of VAD support in patients with non-ischaemic dilated cardiomyopathy; furthermore, GDF15 blood levels were correlated with myocardial fibrosis.[32] Interestingly, in this study, GDF15 expression was not found in the heart, suggesting that systemic organs may be primarily involved in its production and release. The authors proposed that improvements in systemic perfusion reduce end organ injury levels, and circulating GDF15 was reduced

as a consequence. Our data demonstrate reductions in GDF15 by Day 7 of treatment in both VAD and medically managed patients (*Figure 3*), suggesting rapid regulation of growth differentiation factor expression in the setting of restored circulation. However, we also observed a significant treatment effect of VAD, with persistently higher levels of circulating GDF15 in this treatment group, perhaps reflective of the proposed pro-fibrotic effect of implantable MCS. The current understanding of GDF15's role in HF indicates that levels of this protein increase in the setting of injury and may have a protective effect on the myocardium, and AHF therapy, both medical and VAD, can reduce its levels in the circulation, reflecting reductions in myocardial and systemic injury.

Clusterin is a chaperone protein up-regulated after injury and may have a protective role in cardiovascular disease. CLU expression in experimental viral myocarditis has been suggested to limit damage to surrounding viable tissue, and CLU may function to protect against inflammatory injury.[33] However, the converse has also been proposed; increased CLU gene expression was linked to restenosis after angioplasty and promoted smooth muscle cell proliferation.[34] A recent study by Cubedo *et al.* profiling the proteomic signature of CLU following acute MI demonstrated significant reductions in CLU levels 6 h post-MI, which increased to control levels by Day 4.[35] Our data also demonstrated elevations in circulating CLU levels within 1 week of therapy, although our cohort contained patients with both ischaemic and non-ischaemic disease. CLU continued to increase over 30 days of treatment in our cohort (*Figure 3*), which may reflect either ongoing myocardial injury or progressive repair. Further work will be needed to determine the mechanistic involvement of CLU in cardiac remodelling, but our data indicate it may have potential as a marker of recovery following AHF.

Myocardial strain: ST2 and N-terminal pro-brain natriuretic peptide

Soluble ST2 is known to be up-regulated in settings of myocardial strain, as well as post-MI. It has been proposed as a valuable serial marker of progressive decongestion in AHF.[36] In the setting of MCS treatment, circulating ST2 levels were found to decrease after 1 month of left ventricular assist device support and remained constant thereafter in end-stage CHF patients.[37] Our data not only support this observation but also indicate that stabilization of ST2 levels occurs within 7 days of MCS unloading (*Figure 5*). ST2 levels were found to be higher in non-survivors of acute decompensated heart failure when measured at emergency department presentation, and changes in ST2 over the first 48 h of treatment were significantly associated with long-term survival.[38] ST2 levels did not change significantly in the medical management group, confirming the superior LV unloading provided by MCS. This disparity in unloading could also explain the increased levels of N-terminal pro-brain natriuretic peptide in the medical management group (*Figure 4*).

Therapy-specific mechanisms are also likely in the case of circulating PROZ being significantly reduced in the VAD treatment group. Decreased PROZ post-VAD implantation (*Figure 5*) likely reflects the effects of surgical and maintenance antithrombotic therapy used in MCS. As a part of the coagulation cascade, PROZ functions as a cofactor to inhibit coagulation. Little is known of its clinical relevance beyond the alterations measured in patients receiving anticoagulant treatment; however, lower levels are associated with bleeding tendency,[39] which is a common adverse event in VAD patients.

Our study is limited by sample size and would be greatly strengthened if our cohort comprised responders and non-responders to therapy. The patients we selected to characterize the plasma responses to clinically meaningful 'recovery' shared proteomic signatures, indicative of the potential role of the identified markers to reflect reversal of the AHF phenotype. It cannot be said that myocardial recovery was achieved in either treatment group; all but one of the VAD patients in our AHF study went on to cardiac transplantation within 1 year of VAD implantation. At 1 year post-admission, of the patients managed medically, one received a heart transplant and one died later than 30 days post-admission. The remainder continues to be managed for CHF. However, given the absence of major adverse events during the span of our biomarker study, the markers we have characterized may be reflective of positive outcomes and symptomatic improvement, and the temporal changes observed in their circulating levels could correlate with functional clinical improvements observed over the duration of therapy. Our data are lacking certain clinical outcome measures of cardiac recovery such as ejection fraction, or structural data (LV mass index, end diastolic volume, etc.); thus, our results cannot be correlated with established measures of reverse remodelling or myocardial recovery.

A biomarker panel reflective of myocardial recovery would be of particular significance in the application of MCS in bridged to recovery cases. Decisions regarding weaning from device support are fraught with difficulty, and there is a paucity of data to support current weaning or explantation protocols. Although this study was not designed to identify reverse remodelling or cardiac recovery, but rather clinical recovery sufficient to allow discharge from hospital, our results could indicate potential candidate markers relevant in this patient population.

Characterizing the temporal plasma responses to therapy is a critical first step in the process of identifying

clinically relevant markers of recovery and response to therapy. Further studies are urgently needed to develop and validate AHF prognostic tools to guide management of this complex condition. A number of studies have attempted to improve clinical risk models through integration with proteomic biomarkers. However, there is currently no well-defined algorithm to predict clinical outcome or stratify treatment for the wide range of patient presentations encountered in AHF. Attempts to improve predictive performance through proteomic approaches have had little success thus far, with identified proteins not performing with sufficiently high specificity and sensitivity to add clinical value. By applying a broad semi-targeted approach, our data have provided evidence for the key biological processes involved in clinical recovery from AHF and may guide future in-depth studies on these pathways. The complex nature of AHF pathophysiology implies the need for multi-marker strategies reflective of complementary remodelling pathways, and the dynamic responses of our biomarkers to therapeutic intervention suggest these markers may reflect multiple rapidly regulated recovery mechanisms. The proteins identified in this study may represent an in-common proteomic signature of myocardial recovery and response to treatment, and if validated, may help guide clinical care by identifying responders vs. non-responders to AHF therapy.

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Conflict of interest

The authors have no conflicts of interest to declare. Dr Cheung has served as a consultant for HeartWare Inc.

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Supporting information

Supporting information may be found in the online version of this article.

Appendix S1. Supplementary materials.

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