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RESEARCH ARTICLE

A network analysis to identify pathophysiological pathways distinguishing ischaemic from non-ischaemic heart failure

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Aims Heart failure (HF) is frequently caused by an ischaemic event (e.g. myocardial infarction) but might also be caused by a primary disease of the myocardium (cardiomyopathy). In order to identify targeted therapies specific for either ischaemic or non-ischaemic HF, it is important to better understand differences in underlying molecular mechanisms.

Methods and results We performed a biological physical protein–protein interaction network analysis to identify pathophysiological pathways distinguishing ischaemic from non-ischaemic HF. First, differentially expressed plasma protein biomarkers were identified in 1160 patients enrolled in the BIOSTAT-CHF study, 715 of whom had ischaemic HF and 445 had non-ischaemic HF. Second, we constructed an enriched physical protein–protein interaction network, followed by a pathway over-representation analysis. Finally, we identified key network proteins. Data were validated in an independent HF cohort comprised of 765 ischaemic and 100 non-ischaemic HF patients. We found 21/92 proteins to be up-regulated and 2/92 down-regulated in ischaemic relative to non-ischaemic HF patients. An enriched network of 18 proteins that were specific for ischaemic heart disease yielded six pathways, which are related to inflammation, endothelial dysfunction superoxide production, coagulation, and atherosclerosis. We identified five key network proteins: acid phosphatase 5, epidermal growth factor receptor, insulin-like growth factor binding protein-1, plasminogen activator urokinase receptor, and secreted phosphoprotein 1. Similar results were observed in the independent validation cohort.

Conclusions Pathophysiological pathways distinguishing patients with ischaemic HF from those with non-ischaemic HF were related to inflammation, endothelial dysfunction superoxide production, coagulation, and atherosclerosis. The five key pathway proteins identified are potential treatment targets specifically for patients with ischaemic HF.

Keywords Ischaemic heart failure • Heart disease • Physical protein–protein interaction • Pathway • Cardiomyopathy

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Introduction

Heart failure (HF) is a clinical syndrome with multiple underlying causes, which are broadly classified as ischaemic vs. non-ischaemic. Myocardial dysfunction subsequent to ischaemic heart disease and impaired blood supply due to atherosclerosis and the presence of scar formation and remodelling are considered to be main drivers of ischaemic HF. Non-ischaemic HF is considered as a primary disease of the myocardial cell and interstitium. Guideline-directed therapies targeting HF are the same regardless of aetiology.[1]

In contrast to morphological studies, clinical data on differences in molecular mechanisms between ischaemic and non-ischaemic HF are scarce. Pathway analysis using multiple circulating biomarkers is a well-validated method to elucidate pathophysiological pathways that are typically related to a specific phenotype. We recently performed such a network analysis to distinguish HF patients with a preserved vs. reduced left ventricular ejection fraction (LVEF).[2]

Incremental and distinct from our previous work, in the present study, we performed network analyses based on differential protein expression coupled with biological physical protein–protein interaction, and clinical outcome, to identify pathophysiological pathways distinguishing ischaemic from non-ischaemic HF.

Methods

Patient population and study design

We performed our analyses on the BIOlogy Study to TAilored Treatment in Chronic Heart Failure (BIOSTAT-CHF).[3] Briefly, BIOSTAT-CHF consists of two independent cohorts. The index cohort included 2516 patients with worsening signs and/or symptoms of HF from 11 European countries. The validation cohort is an independent cohort of 1738 HF patients from Scotland. The design and baseline characteristics of both cohorts of BIOSTAT-CHF have been published elsewhere.[3] Inclusion criteria were the same in the index and validation cohorts, except that in case LVEF was more than 40%, there was a B-type natriuretic peptide (BNP)/N-terminal pro BNP (NT-proBNP) threshold of respectively >400 pg/mL or > 2000 pg/mL in the index cohort, but not in the validation cohort. The study complied with the Declaration of Helsinki and was approved by the medical ethics committees of participating centres. Herein, we classified patients into two groups; ischaemic HF or non-ischaemic HF based on their medical and clinical histories and availability of study biomarker data (*Figure 1)*.

Study group definition

From both BIOSTAT index and validation cohorts, we classified ischaemic HF patients as (i) those who were considered by the recruiting investigators to have an ischaemic aetiology (BIOSTAT-CHF case report form required investigators to fill in the primary aetiology of HF), and (ii) those who had a history of documented myocardial infarction. The non-ischaemic group of HF patients are (i) those who were considered by the recruiting investigator to have a cardiomyopathy; and (ii) those who had no evidence for any form of ischaemic heart disease (i.e. did not have a previous myocardial infarction, percutaneous coronary intervention and/or coronary artery bypass surgery). All patients, irrespective of LVEF, that met the study group definitions were eligible.

Protein biomarker data

The protein biomarker data used for this study have been described in recent papers.[4,5] In brief, we used the Cardiovascular III (CVDIII) panel of 92 cardiovascular disease-related proteins provided by Olink Bioscience analysis service (Uppsala, Sweden). The proteins were profiled using the Olink Proseek® Multiplex Inflammatory[96*96] platform (details in online supplementary material and supplementary *Table S1*).

Differential protein expression analyses

Differential protein expression analysis was done by simply tethering the fold change of the 92 protein biomarkers in ischaemic vs. non-ischaemic patients for the index and validation cohorts. A minimal fold change of 1.15 yielded consistent results between patients in the index and validation cohort (online supplementary *Figure S1*). This threshold was subsequently applied to a more complicated differential expression analysis using the Linear Models for Microarray data analysis (Limma)[6] software (version 3.34.9). Analyses were done at a fold change threshold of 1.15, *P*-value and false discovery rate (FDR) <0.05. Proteins that met these cutoffs were considered differentially expressed in ischaemic relative to non-ischaemic HF patients. In practice, the *P*-value is lower than the FDR, so effective differential expression hits are determined by the fold change and FDR.

Construction of enriched physical protein–protein interaction networks

We constructed a comprehensive biological network of human physical protein–protein interactions (NR_HsapiensPPI; details in online supplementary material), consisting of 17 625 unique nodes with 330 157 interactions. These protein–protein interactions are not based on in-silico predictions, but are rather biologically determined and manually curated and stored in secondary databases used in this manuscript. We have rendered these into current terms to enable integration with contemporary data analyses to ensure minimal information loss. The biological methods used to detect physical interactions are presented in online supplementary *Figure S6*, and are mainly based on two-hybrid, and/or affinity purification followed by mass spectrometry methodologies.[7–12]

From the general 'NR_HsapiensPPI' network, we harvested an 'Olink PPI network' as the subnetwork that contains nodes (and associated interactions) belonging to any of the 92 Olink protein biomarkers. We checked the cohesiveness of the 'Olink PPI network' using the physical interaction enrichment (PIE) algorithm,[13] which corrects for inquisitional bias in biological networks for proteins that are often studied. The 'Olink PPI network' was found to be a significant physically cohesive network (PIE score = 1.24, $P = 0.004$) (online supplementary *Figure S2*). This 'Olink PPI network' was used for subsequent network enrichment analyses.

Pathway over-representation analyses

We assessed over-representation with ClueGO[14] (in Gene Ontology biological processes, KEGG and Reactome pathways) using the hypergeometric test and the default Bonferroni step down method for multiple testing corrections (family-wise error rate). We used the whole

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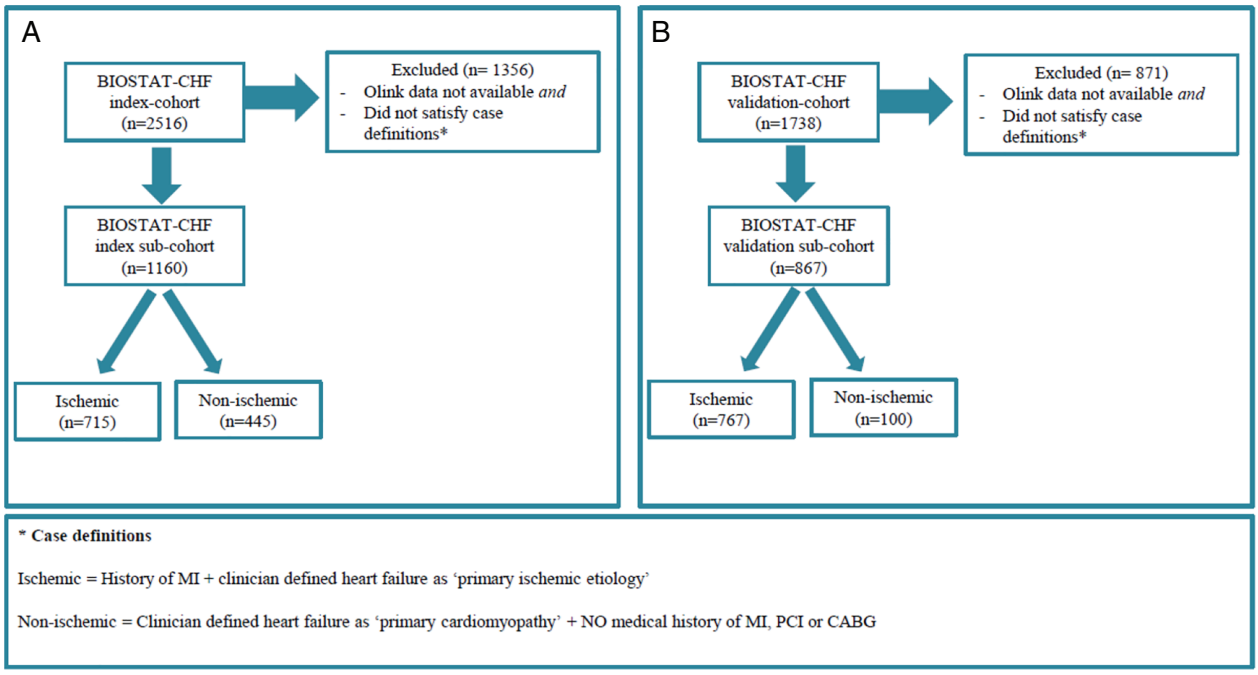

Figure 1 Consort diagram. Selection of ischaemic and non-ischaemic heart failure patients from BIOSTAT-CHF index (*A*) and validation (*B*) cohorts. CABG, coronary artery bypass graft; MI, myocardial infarction; PCI, percutaneous coronary intervention.

annotation option as reference set and reported only biological processes with a corrected *P*-value ≤0.05 to be significant.

Identification of key network proteins

Using the physically cohesive 'Olink PPI network' as template, we constructed a subnetwork by propagating [up to one neighbourhood away (N1-propagation)] interactions between proteins up-regulated in ischaemic relative to non-ischaemic patients to yield an enriched network. An N1-propagation minimizes loss of context (concept in online supplementary *Figure S1*) and would capture any of the 92 proteins having a previously established biological physical protein–protein interactions in the network knowledgebase. To enhance our understanding of the identified pathophysiological pathways, we identified the network proteins therein that were associated with mortality.

Statistical analysis

Statistical analyses were done in R.[15] In group comparisons, categorical variables were depicted as numbers with percentages. Normally distributed variables were depicted as means ± standard deviation, non-normally distributed variables as median with the first and third quartile (Q1–Q3). The means for continuous variables were compared by one-way analysis of variance (ANOVA) or the Kruskal–Wallis test, while categorical variables were compared by the Chi-squared test. Kaplan–Meier survival curves were compared using the log-rank statistic. Cox regression models were used to adjust for the effect of covariates and to calculate hazard ratios (HR). Cox proportional hazards assumptions were assessed (using the R-based Survival and Survminer packages) by visual inspection of Schoenfeld residuals against time plots. For additional pathway-level analyses, time-dependent area under receiver operating curve (AUCt) were obtained in a univariate fashion (per biomarker) and in aggregates (pathway biomarkers). AUCt is a measure of the discriminative ability of a marker at each time point under consideration. An AUCt <0.5 indicates decreasing while AUCt >0.5 indicates increasing mortality rate (in this study). The R-package, survivalROC was used for AUCt calculations. Baseline tables and univariate analyses were done using the R-based CompareGroups package. In general, a two-tailed *P*-value of <0.05 was considered statistically significant.

Results

Baseline characteristics

From the 2516 HF patients of the BIOSTAT-CHF index cohort we identified 715 patients with Olink-CVDIII data who met the stringent criteria for ischaemic HF and 445 patients with Olink-CVDIII data who met the stringent criteria for non-ischaemic HF. Patients in both groups were mainly men (82% in the ischaemic group and 73% in the non-ischaemic group; $P < 0.001$). Baseline characteristics of ischaemic vs. non-ischaemic HF patients are shown in *Table 1*. Ischaemic patients were older, had significantly lower heart rates and a more frequent history of HF hospitalization and smoking. In both study groups, most patients had either HF with reduced or mid-range LVEF and there was no significant difference in the prevalence of HF with preserved LVEF between the groups with ischaemic and non-ischaemic HF. Baseline characteristics of the patients in the validation cohort showed similar trends (online supplementary *Table S2*).

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Table 1 **Baseline characteristics of the index cohort stratified by ischaemic and non-ischaemic heart failure**

Demographics and medical history	Ischaemic (*n* = 715)	Non-ischaemic (*n* = 445)	*P*-value
Male sex	585 (81.8)	324 (72.8)	<0.001
Age (years)	71.0 [63.0–78.0]	64.0 [54.0–73.0]	<0.001
Ischaemic heart disease			<0.001
Primary	715 (100)	0 (0.00)	
Not present	0 (0.00)	382 (85.8)	
Unknown	0 (0.00)	63 (14.2)	
Hypertension			<0.001
Primary	16 (2.24)	0 (0.00)	
Contributory	401 (56.1)	153 (34.4)	
Not present	284 (39.7)	277 (62.2)	
Unknown	14 (1.96)	15 (3.37)	
Cardiomyopathy			<0.001
Primary	5 (0.70)	445 (100)	
Contributory	135 (18.9)	0 (0.00)	
Not present	543 (75.9)	0 (0.00)	
Unknown	32 (4.48)	0 (0.00)	
Valvular disease			0.150
Primary	5 (0.70)	0 (0.00)	
Contributory	220 (30.8)	155 (34.8)	
Not present	471 (65.9)	276 (62.0)	
Unknown	19 (2.66)	14 (3.15)	
Previous HF hospitalization(s) in last year	272 (38.0)	136 (30.6)	0.011
Myocardial infarction	715 (100)	0 (0.00)	<0.001
CABG	263 (36.8)	0 (0.00)	<0.001
Valvular surgery	44 (6.15)	15 (3.37)	0.050
PCI	339 (47.4)	0 (0.00)	<0.001
Atrial fibrillation	276 (38.6)	189 (42.5)	0.213
Stroke	92 (12.9)	24 (5.39)	<0.001
Peripheral arterial disease	116 (16.2)	20 (4.49)	<0.001
Hypertension	495 (69.2)	188 (42.2)	<0.001
Smoking			<0.001
None	180 (25.2)	196 (44.0)	
Past	437 (61.2)	183 (41.1)	
Current	97 (13.6)	66 (14.8)	
Current alcohol use	188 (26.3)	139 (31.2)	0.082
Diabetes	290 (40.6)	99 (22.2)	<0.001
Diet	192 (66.2)	68 (68.7)	0.742
Insulin	130 (44.8)	37 (37.4)	0.240
Oral anti-diabetic drugs	169 (58.3)	61 (61.6)	0.642
COPD	137 (19.2)	61 (13.7)	0.020
Renal disease	258 (36.1)	84 (18.9)	<0.001
Current malignancy	23 (3.22)	13 (2.92)	0.914
Physical examinations			
Height (cm)	172 [165–177]	172 [167–179]	0.026
Weight (kg)	80.0 [70.0–90.0]	81.0 [70.0–93.0]	0.194
Heart rate (bpm)	72.0 [64.0–81.0]	76.0 [68.0–88.5]	<0.001
Systolic blood pressure (mmHg)	120 [110–135]	120 [109–130]	0.375
Diastolic blood pressure (mmHg)	70.0 [65.0–80.0]	73.0 [68.0–80.0]	0.014
Pulmonary congestion/oedema with rales/crackles			<0.001
No	307 (44.6)	247 (57.0)	
Single base	84 (12.2)	54 (12.5)	
Bi-basilar	297 (43.2)	132 (30.5)	

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Table 1 (Continued)

Demographics and medical history	Ischaemic (*n* = 715)	Non-ischaemic (*n* = 445)	*P*-value
Elevated JVP			0.342
No	329 (63.8)	216 (67.1)	
Yes	167 (32.4)	90 (28.0)	
Uncertain	20 (3.88)	16 (4.97)	
Signs and symptoms of HF			
NYHA class			0.086
I	11 (1.58)	11 (2.52)	
II	248 (35.6)	181 (41.5)	
III	339 (48.6)	196 (45.0)	
IV	99 (14.2)	48 (11.0)	
Dyspnoea VAS score	50.0 [30.0–65.0]	60.0 [40.0–70.0]	0.031
LVEF (%)	30.0 [25.0–35.0]	26.5 [21.2–31.8]	<0.001
HFrEF (LVEF <40%)	545 (86.1)	387 (93.5)	<0.001
HFmrEF (LVEF 40–<50%)	75 (11.8)	21 (5.07)	<0.001
HFpEF (LVEF ≥ 50%)	13 (2.05)	6 (1.45)	0.631
Orthopnoea present	245 (34.4)	141 (31.8)	0.395
Medications			
ACEi/ARB	497 (69.5)	354 (79.6)	<0.001
Beta-blocker	634 (88.7)	380 (85.4)	0.122
Aldosterone antagonist	396 (55.4)	266 (59.8)	0.159
Diuretics	715 (100)	444 (99.8)	0.384
Statin	545 (76.2)	159 (35.7)	<0.001
Laboratory data			
Serum creatinine (μmol/L)	110 [89.2–142]	95.5 [79.6–119]	<0.001
LDL (mmol/L)	2.30 [1.64–2.96]	2.90 [2.16–3.48]	<0.001
NT-proBNP (pg/mL)	2831 [1246–5868]	2130 [906–4734]	0.002
Mortality			
All-cause death	201 (28.1)	76 (17.1)	<0.001
Cardiovascular death	142 (19.9)	47 (10.6)	<0.001

Values are given as *n* (%), or median [interquartile range].
ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CABG, coronary artery bypass graft; COPD, chronic obstructive pulmonary disease; HF, heart failure; HFmrEF, heart failure with mid-range ejection fraction; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; JVP, jugular venous pressure; LDL, low-density lipoprotein; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro-B-type natriuretic peptide; NYHA, New York Heart Association; PCI, percutaneous coronary intervention; VAS, visual analogue scale.

Results of differential protein expression analyses

Differential protein expression in ischaemic vs. non-ischaemic HF patients yielded 23/92 differentially expressed proteins amongst which 21 were up-regulated and 2 down-regulated (*Figure 2*). The two most prominently up-regulated proteins in patients with ischaemic HF were galectin 4 (LGALS4) and growth differentiation factor 15 (GDF15), while paraoxonase 3 (PON3) was the most prominently down-regulated protein in patients with ischaemic HF. These differences remained statistically significant after correction for age, sex and the use of statins at baseline (online supplementary *Figure S3*). The differentially expressed proteins in the validation cohort largely overlapped with those in the index cohort (*Figure 2B*).

In addition, the differential expression results were consistent between both cohorts as no protein was found to be up-regulated in one cohort but down-regulated in the other, or vice versa. For brevity, detail results for the index cohort are presented in online supplementary *Table S3*.

Results of enriched physical protein–protein interaction network

The 21/92 up-regulated proteins in ischaemic vs. non-ischaemic HF patients yielded an enriched network consisting of 18 proteins (online supplementary *Figure S4*). In non-ischaemic HF patients, only two proteins were up-regulated, which was insufficient to build a network. Therefore, we focused on networks specific for ischaemic HF.

Results of pathway over-representation analyses

Pathway over-representation analyses of the 21 up-regulated proteins in ischaemic HF patients led to the identification of only two

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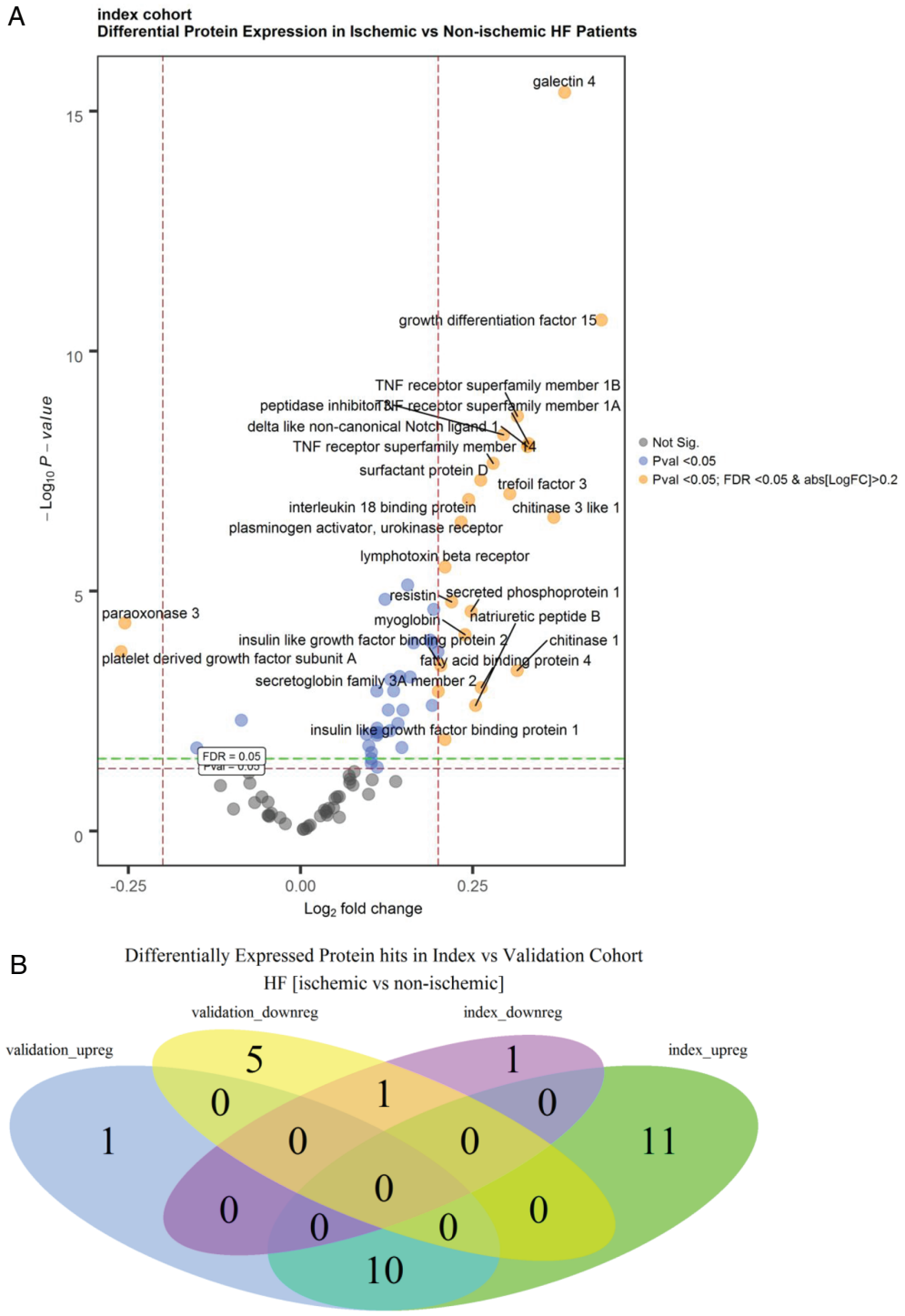

Figure 2 Differential protein expression in ischaemic relative to non-ischaemic heart failure (HF) patients. (*A*) Volcano plot of differential protein expression (y-axis significance, x-axis effect size (positive = up-regulated, negative = down-regulated; labelled = significant differentially expressed proteins). (*B*) Venn diagram of number of significantly differentially expressed proteins in index cohort (main) and validation cohort. Consistently, no proteins were up-regulated in one cohort but down-regulated in the other, nor vice versa. FC, fold change; FDR, false discovery rate; TNF, tumour necrosis factor.

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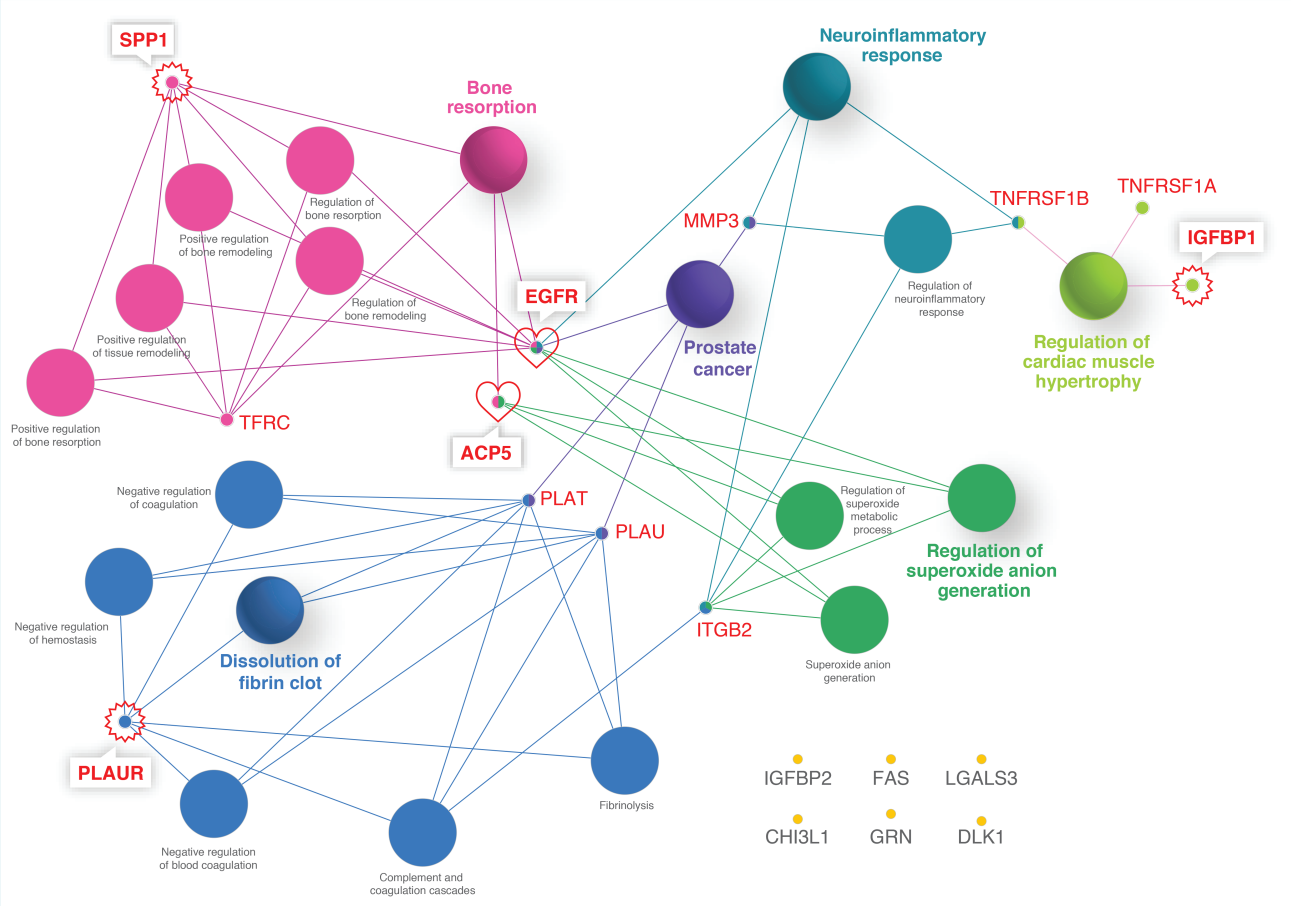

Figure 3 Pathophysiological pathways related to ischaemic heart failure. Proteins (small nodes) linking the pathways (large nodes) are depicted. The six-pathway modules are labelled by the most significant group term. The five-protein nodes highlighted were significantly associated with mortality. ACP5, acid phosphatase 5; CHI3L1, chitinase-3-like protein 1; DLK1, delta like non-canonical notch ligand 1; EGFR, epidermal growth factor; GRN, progranulin; IGFBP1, insulin-like growth factor binding protein 1; IGFBP2, insulin-like growth factor binding protein 2; ITGB2, integrin subunit beta 2; LGALS3, galectin 3; MMP3, matrix metalloproteinase 3; PLAT, plasminogen activator, tissue type; PLAU, plasminogen activator, urokinase; PLAUR, plasminogen activator, urokinase receptor; SPP1, secreted phosphoprotein 1; TNFRSF1A, tumour necrosis factor receptor superfamily member 1A; TNFRSF1B, tumour necrosis factor receptor superfamily member 1B; TRFC, transferrin receptor.

pathways (regulation of cardiac muscle hypertrophy, and tumour necrosis factors (TNFs) bind their physiological receptors), with the majority of the proteins not successfully captured in the analysis (online supplementary *Figure S5*). We therefore proceeded to perform pathway over-representation analysis of the enriched network.

Using the 18 proteins of the enriched network (online supplementary *Figure S4*) yielded six significant pathways ($P < 0.001$, details in online supplementary *Table S4*) interconnected with each other. This pathway network nexus is the main result of this study and is presented in *Figure 3*. The six identified pathways/biological processes are officially named 'dissolution of fibrin clot'; 'bone resorption'; 'regulation of superoxide anion generation'; 'prostate cancer'; 'neuroinflammatory response' and 'regulation of cardiac muscle hypertrophy'.

Results of identification of key network proteins associated with mortality

Out of the 18 proteins in the enriched network, five showed significant associations with mortality, correcting for case study group (i.e. ischaemic vs. non-ischaemic) and the other network proteins. The Cox proportional hazard assumption was supported by a non-significant relationship between residuals and time (global $P = 0.15$). In addition, the independent variables did not indicate multicollinearity (variance inflation factor < 10). Amongst these five key network proteins, increased levels of acid phosphatase 5 (ACP5) (HR 0.76, $P = 0.045$) and epidermal growth factor receptor (EGFR) (HR 0.46, $p = 0.005$) were associated with significantly lower risks of death, while increased levels of insulin-like growth factor binding protein 1 (IGFBP1) (HR 1.19, $P = 0.005$), plasminogen activator urokinase receptor (PLAUR) (HR 1.95, $P = 0.009$),

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Table 2 Excerpts of biomarker association with all-cause mortality from multivariate Cox proportional hazards regression analyses of enriched-network models

Protein biomarker	HR (95% CI) *P*-value		
	Model 1	Model 2	Model 3
Index cohort			
ACP5	0.76 (0.58–0.99) 0.045	0.76 (0.58–1) 0.048	0.77 (0.59–1.01) 0.057
EGFR	0.46 (0.27–0.8) 0.005	0.48 (0.28–0.84) 0.010	0.47 (0.27–0.82) 0.008
IGFBP1	1.19 (1.06–1.35) 0.005	1.2 (1.06–1.35) 0.004	1.2 (1.07–1.36) 0.003
PLAUR	1.95 (1.18–3.21) 0.009	2.01 (1.22–3.32) 0.006	1.98 (1.2–3.27) 0.007
SPP1	1.47 (1.2–1.8) <0.001	1.47 (1.2–1.8) <0.001	1.46 (1.19–1.78) <0.001
Validation cohort			
ACP5	0.7 (0.53–0.94) 0.016	0.72 (0.54–0.95) 0.022	0.7 (0.52–0.94) 0.016
EGFR	0.44 (0.23–0.83) 0.011	0.5 (0.26–1) 0.048	0.53 (0.27–1.06) 0.073
IGFBP1	1.23 (1.07–1.4) 0.003	1.22 (1.07–1.4) 0.004	1.23 (1.07–1.41) 0.003
PLAUR	2.3 (1.38–3.81) 0.001	2.47 (1.47–4.15) <0.001	2.44 (1.45–4.12) <0.001
SPP1	1.57 (1.2–2.07) 0.001	1.56 (1.18–2.05) 0.002	1.55 (1.18–2.05) 0.002

N/B: only significant results are shown.
ACP5, acid phosphatase 5, tartrate resistant; CI, confidence interval; EGFR, epidermal growth factor receptor; HR, hazard ratio; IGFBP1, insulin-like growth factor binding protein 1; PLAUR, plasminogen activator, urokinase receptor; SPP1, secreted phosphoprotein 1.
Model 1: adjusted for enriched network nodes and study group.
Model 2: adjusted for Model 1 covariates, plus age and sex.
Model 3: adjusted for Model 2 covariates, plus angiotensin-converting enzyme inhibitor/angiotensin receptor blocker and beta-blocker use at baseline.

and secreted phosphoprotein 1 (SPP1) (HR 1.47, $P < 0.001$) were associated with significantly higher risks of death (*Table 2* and online supplementary *Figure S7*). Therefore, these five proteins were identified as the five key pathway proteins.

Results of time-dependent area under the curve analysis per pathway

To provide an overall prognostic impression of the protein biomarkers together as a group per identified pathway, we performed AUC(t) analyses. We observed an overall mortality drop over time from baseline to about 15 months. This was mainly driven by four/six pathways (bone resorption, dissolution of fibrin clots, neuroinflammatory response and regulation of cardiac muscle hypertrophy). For two/six pathways (i.e. prostate cancer and regulation of superoxide anion generation) prognosis was better and earlier. For these two pathways, although generally low in both cohorts, mortality dropped from the first trimester in the index cohort, but worsened (albeit at medium levels) over time in the validation cohort (*Figure 4*).

Discussion

Using a physical protein–protein interaction network analysis to identify pathophysiological pathways distinguishing ischaemic from non-ischaemic HF, we showed that ischaemic HF pathways were related to inflammation, endothelial dysfunction, superoxide production, coagulation, and atherosclerosis. The network was connected by 18 circulating proteins, of which five had a statistically significant association with all-cause death. These five identified proteins represent potential novel treatment targets specifically for patients with ischaemic HF.

Differentially expressed proteins

Most of the proteins (75%) showed similar expression levels in both disease populations, which suggests that there is a substantial pathophysiological overlap between ischaemic and non-ischaemic HF (at least as informed by the set of 92 proteins). The majority of the differentially expressed proteins [21/23 (91%)] were up-regulated in ischaemic vs. non-ischaemic HF patients, prompting us to focus on the ischaemic phenotype in this manuscript.

Amongst the most prominent differentially expressed proteins, LGALS4 and GDF15 were up-regulated while PON3 was down-regulated in ischaemic vs. non-ischaemic HF patients. These proteins remained significantly different even after correction for age, sex and statin use. LGALS4 is involved in the regulation of inflammation and promotion of angiogenesis.[16] In animal studies, GDF15 protected the heart through inhibition of hypertrophic, inflammatory and apoptotic processes.[17] PON3 is an inhibitor of both oxidative modification of low-density lipoproteins and monocyte activation, both of which are important stages in atherosclerotic plaque formation.[18] Lower levels of PON3 in the ischaemic group might be associated with increased oxidation of low-density lipoproteins leading to atherosclerosis.

The six interconnecting pathways related to ischaemic heart failure

We identified the following six pathways in relation to ischaemic HF.

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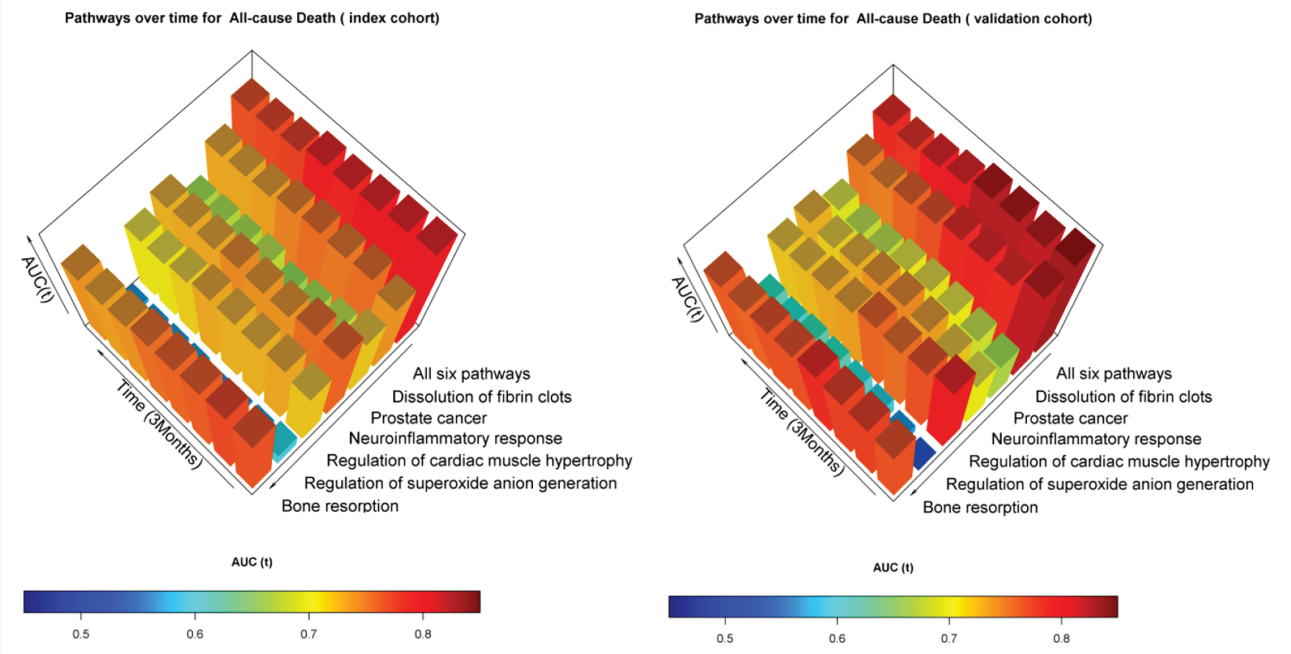

Figure 4 Temporal mortality prognostic trend of ischaemic nexus pathways. The time-dependent area under receiver operating curve [AUC(t)] bins per 3 months (i.e. trimesters) for all six pathways are shown, in addition to those per pathway (left, index cohort; right, validation cohort). Overall AUC(t) dropped over time. Although generally low in both cohorts, mortality prognostics based on two pathways ('prostate cancer' and 'regulation of superoxide anion generation') dropped from the first trimester in the index cohort, but worsened (albeit at medium levels) over time in the validation cohort.

Dissolution of fibrin clots

'Dissolution of fibrin clots' was the largest pathway identified and involves the proteins plasminogen activator urokinase tissue type (PLAT), plasminogen activator urokinase (PLAU), PLAUR and integrin subunit beta 2 (ITGB2). ITGB2 is involved in the activation of plasminogen on the surface of neutrophils.[19] In addition, conversion of plasminogen to plasmin, and subsequent promotion of fibrinolysis is mediated by both PLAT and receptor bound PLAU.[20] A study by Minami *et al.*[21] reported that while acute up-regulation of PLAU prevented rupture of the infarcted zone, chronic and persistent activation of plasminogen through PLAUR–PLAU binding may lead to fibrotic changes in uninjured myocardium. In addition, several studies have reported macrophage-associated overexpression of PLAUR and PLAU in atherosclerotic lesions, leading to the conclusion by Svensson *et al.*[22] that the PLAU–PLAUR system may play a vital role in plaque instability. In our study, higher levels of PLAUR were also associated with increased risk of death.

Prostate cancer

The second pathway is officially labelled as 'prostate cancer', involving PLAU, PLAT, EGFR and matrix metalloproteinase 3 (MMP3). Although we do not see a direct connection with prostate cancer, these proteins have potential associations with ischaemia, atherosclerosis and neovascularization. The role of PLAT and PLAU has been described under 'dissolution of fibrin clots'. EGFR is not only implicated in the promotion of tumour growth by promoting angiogenesis and revascularization,[23] but EGFR signalling pathways are also related to up-regulation of PLAU and PLAUR.[24] Plasmin is also capable of activating several MMPs, including MMP3, which may lead to extracellular matrix degradation.[23] Our network outcome model indicated that EGFR was significantly associated with lower mortality rates. This pathway is in close crosstalk with that of the 'dissolution of fibrin clots' and warrants further investigation in future studies.

Neuroinflammatory response

A third pathway is officially labelled as 'neuroinflammatory response', and involves the proteins MMP3, EGFR, ITGB2 and TNF receptor superfamily member 1B (TNFRSF1B). All of these proteins are involved in the regulation of inflammatory pathways. TNFRSF1B is implicated in T-cell-mediated immune responses.[25] MMP3 is an important downstream activator of MMPs, including MMP9 which is implicated in the cleavage of ITGB2 leading to exodus of macrophages from the area of inflammation, which is vital in limiting the local innate immune response.[26] Collectively, this implies a role for inflammatory pathways and their regulators specifically in the pathophysiology of ischaemic HF.

Regulation of cardiac muscle hypertrophy

IGFBP1, TNFRSF1A and TNFRSF1B are implicated in the pathway officially labelled as 'regulation of cardiac muscle hypertrophy' and are in crosstalk with the 'neuroinflammation' pathway. TNF

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receptors (TNFRSF1A1 and TNFRSF1B) mediate the downstream cellular effects of TNF-α, a pro-inflammatory cytokine important for repair following tissue injury. However, persistent up-regulation of TNF-α leads to chronic inflammation and is implicated in cardiac dysfunction and HF.[27]

IGFBP1 is part of a protein family implicated in the regulation of insulin-like growth factor I (IGF-I) bioavailability, transportation and localization. The role of IGF-1 in HF is not completely understood. However, there is growing evidence to suggest a pro-hypertrophic effect of IGF-1 since IGFBP1 attenuated pro-hypertrophic effects of IGF-1.[28] We found that higher levels of IGFBP1 were significantly associated with increased mortality.

Regulation of superoxide anion generation

The fifth pathway identified is officially labelled as 'regulation of superoxide anion generation', and involves ACP5, EGFR and ITGB2. These proteins are in crosstalk with other pathways. Previous research has indicated that endothelial dysfunction present in HF is due to superoxide-mediated inactivation of nitric oxide.[29] ACP5-deficient mice reportedly exhibited increased superoxide production, indicating its potential protective role in HF.[30] On the other hand, ITGB2 and EGFR mediate generation of superoxide anions through downstream signalling. Higher levels of ACP5 and EGFR were associated with lower risk of death.

Bone resorption

The final pathway identified is officially labelled as 'bone resorption', involving ACP5, EGFR, SPP1 and transferrin receptor. All four proteins in this pathway have been identified to be up-regulated in atherosclerosis.[31–34] SPP1, involved in the attachment of osteoclasts to the mineralized bone matrix, is implicated in a variety of cardiovascular diseases. Expression of SPP1 in the heart under physiological conditions is low. However, its expression increases dramatically after an acute myocardial infarction.[31] Research in both mice and humans has reported that increased SPP1 levels are associated with fibrosis and systolic dysfunction.[31,35] In our study, increased levels of SPP1 were significantly associated with increased mortality.

Therapies targeting the five key proteins

Amongst the five key proteins found, increased levels of ACP5 and EGFR were associated with improved clinical outcome while IGFBP1, PLAUR and SPP1 were associated with worse clinical outcome. In the Therapeutic Target Database[36] (available at: db.idrblab.net/ttd/), EGFR has been targeted (by e.g. cetuximab, an antibody agent) for the treatment of inflammatory breast cancer and colorectal cancer. The other four remain to be tested. Knock-out models or other perturbation of levels for these five targets would potentially enhance our understanding of their involvement in ischaemic heart disease.

ACP5 [also known as tartrate-resistant acid phosphatase (TRAP)] is involved in bone remodelling, a process that is gaining increasing interest in arterial calcification. Arterial plaque calcification is highly prevalent, closely parallels atherosclerotic burden and is related to risk of myocardial infarction and sudden cardiac death.[37] Using immunohistochemical analysis (TRAP enzyme staining), ACP5-positive multinucleated giant cells have been observed in suture granulomas, necrotic plaque cores, fibrotic/calcified plaque, and surrounded by foamy macrophages.[38] The suggestion that these ACP5-positive cells might degrade mineral deposits, prevent formation of calcification or both and therefore counterbalance the activity of the osteoblast-like cells in atherosclerosis[38] corroborate our pro-life findings for ACP5 as an interesting therapeutic target in ischaemic heart disease patients.

SPP1 also mediates cardiac fibrosis through the modulation of cellular adhesion and proliferation and is increased in cardiac hypertrophy. Studies on Spp1-knockout mice indicate that the lack of SPP1 attenuates fibrosis.[39]

Regarding circulating PLAUR (which is also associated with fibrosis and atherosclerosis), studies in humans indicate that higher protein levels correlate with poorer cardiovascular outcomes and are predictive of the presence of peripheral arterial disease.[40] Likewise, higher levels of IGFBP1 were associated with higher risks of mortality and incident HF.[41,42]

Amongst the five key proteins, PLAUR and SPP1 remained differentially expressed after correcting for patients' age, sex and statin use (online supplementary *Figure S3*) or diabetic status (online supplementary *Figure S8*). In a subgroup analysis wherein all ischaemic patients had received angiotensin-converting enzyme inhibitors (ACEi)/angiotensin receptor blockers (ARB) and statins, none of the five key proteins were significant (also after adjustment for patients' age, sex, diabetes status and atrial fibrillation status) (online supplementary *Figures S9–S11*). This suggests that their expression was not due to drug use but rather related to underlying ischaemic disease.

Unlike PLAUR, SPP1 was the most stable differentially expressed protein after adjustment for significant baseline clinical confounders (age, sex, ACEi/ARB use, beta-blocker use, statin use, diabetes status, NT-proBNP, smoking status) (online supplementary *Figures S12* and *S13*). SPP1 remains an interesting target for further investigations.

Our finding on the loss of differential expression of PLAUR after correction for the use of ACEi/ARB and/or beta-blockers is supported by previous findings. Treatment with beta-blockers has been associated with lower levels of PLAUR even after correction for age, gender, hypertension, coronary artery disease, and statin usage.[43] Reduced levels of PLAUR in carotid plaques of patients on beta-blockers suggest their possible protective role in plaque inflammation and prevention of cardiovascular disease,[43] and could assuage eventual culmination to HF.

Looking at the prognostic patterns of the pathways in the ischaemic nexus, we observed that for two/six pathways (i.e. prostate cancer and regulation of superoxide anion generation) AUC(t)s were low (*Figure 4*). Because there is increasing evidence that the use of beta-blockers have anti-cancer properties (also against prostate cancer),[44–46] this is possibly due to comparatively more men and more baseline use of beta-blockers in the index than in the validation cohort.[3]

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Fine-tuning ACEi/ARB, beta-blocker or statin use to abrogate levels of PLAUR, SPP1 and IGFBP1 might assuage HF deaths, especially for the more vulnerable ischaemic cases.

Study limitations

Some potential limitations of this study should be noted. Due to the comparison between ischaemic and non-ischaemic HF, interpretations are limited to relative comparisons. However, the strength of this study is the presence of two distinct HF groups with clear definitions, enabling us to identify mechanisms that are quantitatively relevant to ischaemic heart disease. A second limitation of this study is the pre-selection bias of the 92 protein biomarkers. However, we have checked for this bias in our network enrichment analyses and found it to be a physically cohesive network, meaning that the proteins are functionally coherent. A third limitation is the limited number of proteins investigated. It is therefore likely that the pathways identified are incomplete, limiting our interpretation. However, the hypergeometric test for pathway over-representation analysis takes into account the number of input proteins, assuaging this limitation for the identified pathways. Further analyses using whole-scale transcriptomics and proteomics are expensive, but may unveil more pathways. In addition, further analyses using class prediction methods like prediction analysis of microarrays using nearest shrunken centroids[47] could add further insights.

A major strength of this study are the consistent outcome findings in an independent validation cohort, even though it had fewer non-ischaemic patients (a potentially heterogeneous population), limiting the phenotypic (ischaemic vs. non-ischaemic) contrast.

The propagation of the network, based on differentially expressed proteins, and further restriction to the circulating proteins has enabled identification of member proteins in putative functional modules of ischaemic heart disease and should be further investigated.

Our results will likely inform future studies aimed at designing clearer and more objective diagnostics and/or therapeutics for ischaemic HF.

Conclusions

We identified pathophysiological pathways distinguishing ischaemic from non-ischaemic heart disease. These pathways were related to inflammation, endothelial dysfunction, superoxide production, coagulation, and atherosclerosis. We propose five key pathway proteins as potential treatment targets specifically for ischaemic HF patients.

Supplementary Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Annotation of the 92 Olink biomarkers studied herein.

Table S2. Baseline characteristics – validation cohort stratified for ischaemic and non-ischaemic heart failure patient groups.

Table S3. Differential protein expression results in ischaemic vs. non-ischaemic heart failure.

Table S4. Pathway over-representation results of network proteins.

Figure S1. Concepts.

Figure S2. Construction of the general PPI ('HsapiensPPI') and derivation of a physically cohesive Olink PPI network.

Figure S3. Volcano plot of adjusted differential protein expression in the index cohort for ischaemic vs. non-ischaemic patients.

Figure S4. The enriched network (N1-propagated network) of proteins up-regulated in ischaemic heart failure relative to non-ischaemic patients.

Figure S5. Gene ontology/pathway analyses of the 21 differentially expressed proteins.

Figure S6. Summary of physical interaction methods from BioGrid.

Figure S7. Forest plot showing impact of enriched-network nodes and study group on clinical outcome of all-cause death.

Figure S8. Volcano plot of diabetes-adjusted differential protein expression in the index cohort for ischaemic vs. non-ischaemic patients.

Figure S9. Subgroup analysis volcano plot results of differential protein expression in the index cohort for ischaemic vs. non-ischaemic patients.

Figure S10. Subgroup analysis volcano plot results of differential protein expression in the index cohort for ischaemic vs. non-ischaemic patients (adjusted for patient's age, sex and diabetes status).

Figure S11. Subgroup analysis volcano plot results of differential protein expression in the index cohort for ischaemic vs. non-ischaemic patients (adjusted for patient's age, sex, diabetes status and atrial fibrillation status).

Figure S12. Volcano plot of differential protein expression in the index cohort for ischaemic vs. non-ischaemic patients (adjusted for age, sex, angiotensin-converting enzyme inhibitor/angiotensin receptor blocker use, beta-blocker use, statin use and diabetes status).

Figure S13. Volcano plot of differential protein expression in the index cohort for ischaemic vs. non-ischaemic patients (adjusted for age, sex, angiotensin-converting enzyme inhibitor/angiotensin receptor blocker use, beta-blocker use, statin use, diabetes status, N-terminal pro-B-type natriuretic peptide, smoking status).

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