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RESEARCH ARTICLE

Pathophysiological pathways related to high plasma growth differentiation factor 15 concentrations in patients with heart failure

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Aims Elevated concentrations of growth differentiation factor 15 (GDF-15) in patients with heart failure (HF) have been consistently associated with worse clinical outcomes, but what disease mechanisms high GDF-15 concentrations represent remains unclear. Here, we aim to identify activated pathophysiological pathways related to elevated GDF-15 expression in patients with HF.

Methods and results In 2279 patients with HF, we measured circulating levels of 363 biomarkers. Then, we performed a pathway over-representation analysis to identify key biological pathways between patients in the highest and lowest GDF-15 concentration quartiles. Data were validated in an independent cohort of 1705 patients with HF. In both cohorts, the strongest up-regulated biomarkers in those with high GDF-15 were fibroblast growth factor 23 (FGF-23), death receptor 5 (TRAIL-R2), WNT1-inducible signalling pathway protein 1 (WISP-1), tumour necrosis factor receptor superfamily member 11a (TNFRSF11A), leucocyte immunoglobulin-like receptor subfamily B member 4 (LILRB4), and trefoil factor 3 (TFF3). Pathway over-representation analysis revealed that high GDF-15 patients had increased activity of pathways related to inflammatory processes, notably positive regulation of chemokine production; response to interleukin-6; tumour necrosis factor and death receptor activity; and positive regulation of T-cell differentiation and inflammatory response. Furthermore, we found pathways involved in regulation of insulin-like growth factor (IGF) receptor signalling and regulatory pathways of tissue, bones, and branching structures. GDF-15 quartiles significantly predicted all-cause mortality and HF hospitalization.

Conclusion Patients with HF and high plasma concentrations of GDF-15 are characterized by increased activation of inflammatory pathways and pathways related to IGF-1 regulation and bone/tissue remodelling.

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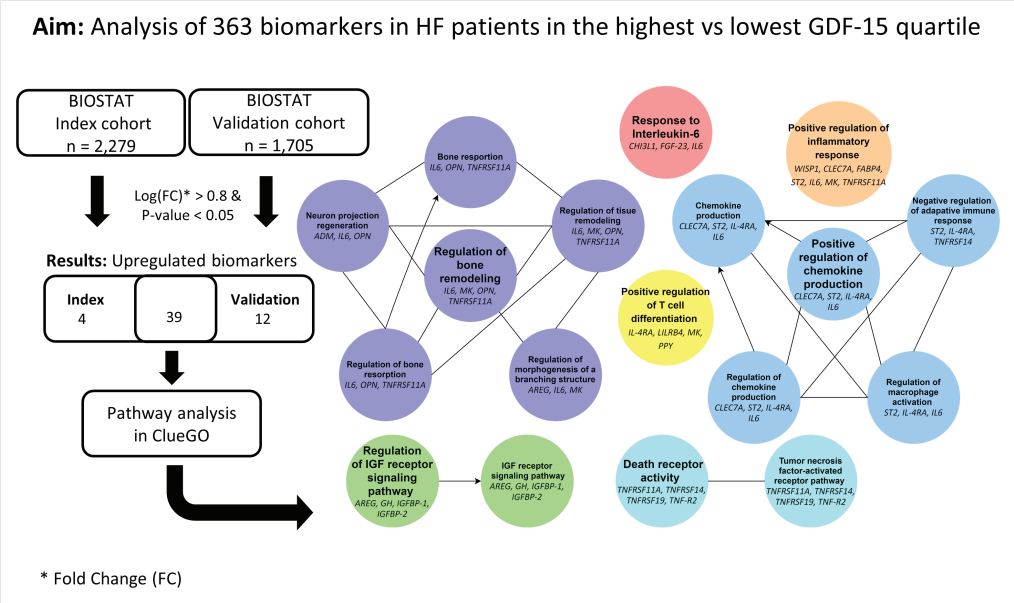
Graphical Abstract



We identified activated pathophysiological pathways related to elevated plasma growth differentiation factor 15 (GDF-15) levels in patients with heart failure (HF) using pathway over-representation analysis of 363 circulating biomarkers. Patients with HF with high plasma concentrations of GDF-15 were characterized by increased activation of inflammatory pathways and pathways related to insulin-like growth factor (IGF)-1 regulation and bone/tissue remodelling.

Keywords Heart failure • Pathway analysis • Growth differentiation factor 15 • Biomarkers

Introduction

The benchmark prognostic biomarker for heart failure (HF) is N-terminal pro-B-type natriuretic peptide (NT-proBNP), a hormone that is secreted by cardiomyocytes after stretching of the ventricle.[1] This measure of mechanical stretching and pressure is valuable in HF. However, NT-proBNP does not cover the full pathophysiology of HF and other biomarkers might reflect other disease mechanisms.[1–4]

Elevated concentrations of growth differentiation factor 15 (GDF-15) have been consistently associated with a higher risk of mortality and HF hospitalization in patients with both HF with preserved ejection fraction (HFpEF) and HF with reduced ejection fraction,[2,5–9] even on top of NT-pro-BNP and established clinical predictors.[10,11] GDF-15 is part of the transforming growth factor-β cytokine family and is a pleiotropic protein involved in various biological processes including inflammation, apoptosis, fibrosis, hypertrophy, and endothelial dysfunction in both the acute and chronic phase.[6] GDF-15 is non-cardiac specific, and is lowly expressed by many tissues in physiological states, but is elevated in renal, pulmonary, and cardiac disease among others.[6] GDF-15 could thus serve as a predictor for various systemic processes in HF.[2] However, due to its complex function, it remains unclear what specific disease mechanisms elevated GDF-15 concentrations represent in HF. This study utilizes differential expression and pathways analysis of a large number of biomarkers from different disease domains to identify the pathophysiological mechanisms related to elevated GDF-15 expression in HF.

Methods

Study patients

Overall, 2279 patients with new onset or worsening signs of HF from the multicentre, multinational, prospective, observational index trail of BIOSTAT-CHF were included between 2010 and 2012.[12] Data were validated in the BIOSTAT-CHF validation cohort consisting of 1705 hospitalized or outpatients with HF from six centres in Scotland that were enrolled between 2010 and 2014. Inclusion criteria for both cohorts are in the online supplementary material. Patients were grouped into quartiles based on their GDF-15 levels, after which the highest versus the lowest quartiles were compared.

Biomarkers

Four Olink Proseek Multiplex panels each measuring 92 biomarkers were used to measure circulating biomarker concentrations: cardiovascular (CVD) II, CVD III, immuno-oncology, and inflammation

[https://www.olink.com/products/]. The kits use a proximity extension assay (PEA) technology, which binds oligonucleotide-labelled antibody probe pairs to the target biomarker.[13] Real-time polymerase chain reaction was performed for further quantification. For overlapping biomarkers present in multiple panels (B-type natriuretic peptide and NT-proBNP; amphiregulin; stem cell factor; and interleukin-6 [IL-6]), the mean values of duplicates were calculated leading to a final number of 363 biomarkers. Details of assay reliability for the six most strongly associated with GDF-15 biomarkers are in the online supplementary *Table S1*.[14] Further, sample storage conditions can be found in the online supplementary material. GDF-15 concentrations were measured using electrochemiluminescence by a Cobas e 411 analyser at baseline using a standard Roche Diagnostics GmbH method in the lab of the Experimental Cardiology department in the UMCG, Groningen. GDF-15 assays had a level of detection of ≤400 pg/ml and intra-assay coefficients of variation of <1.5% regardless of GDF-15 concentration.[15]

Statistical analysis

We performed Kaplan–Meier analysis to evaluate differences in survival between patients in GDF-15 quartiles for two outcomes: all-cause mortality and HF hospitalization at 2 years. Further, we performed a Cox proportional hazard analysis to investigate the predictive strength of GDF-15 quartiles on 2-year all-cause mortality and hospitalization (with competing risk for mortality) when adjusted for age, sex, estimated glomerular filtration rate (eGFR), diabetes, NT-proBNP and New York Heart Association (NYHA) class. To establish the strongest associated variables of high GDF-15 concentrations, we carried out a multivariable logistic regression model in the index cohort. Variables that were significantly different between the highest and lowest GDF-15 quartile in both cohorts (*Tables 1* and *2*) and had missing data in <20% of patients were included in a multivariable model. Backward selection was performed with a *p*-value cut-off of 0.05 using the *fastbw* function from the rms package in R.[16] The differential expression analysis was performed using the Limma package in R.[17] In the differential expression analysis, the patients with the highest GDF-15 quartile were compared to the patients with the lowest GDF-15 quartile (GDF-15 high vs. low) in both cohorts. Biomarkers with false discovery rate (FDR, Benjamini–Hochberg) <5% and log fold change value >0.8 were considered to be significantly upregulated. Only biomarkers that were found upregulated in both the index and validation cohorts were used in the pathway analysis. Further, we performed a differential expression analysis for the highest versus the lowest quartiles for each of the six most strongly associated with GDF-15 biomarkers separately in the index cohort. Then, we created a heatmap, also including GDF-15 expression results, including all biomarkers that were significant in at least one analysis. As a sensitivity analysis, using full data (without exclusion of the second and third quartiles), we performed linear regression analyses for each biomarker as a dependent variable of log-transformed GDF-15 levels. Pathway analysis was performed using a Cystoscope plug-in, ClueGO, utilizing Gene Ontology Biological Process (08-05-2020); more details are in the online supplementary material.[18] *P*-values of each pathway term were Bonferroni corrected.

Results

Clinical characteristics

Tables 1 and *2* show the baseline characteristics for the index and validation cohorts, respectively. Patients in the highest GDF-15 quartile had significantly higher serum creatinine and urea, lower eGFR and lower haemoglobin compared to patients in the lowest GDF-15 quartile. In both the index and validation cohorts, patients with higher GDF-15 had significantly more often a history of a diabetes mellitus, percutaneous coronary intervention, stroke, atrial fibrillation (AF), myocardial infarction (MI), a higher NYHA class, hepatomegaly, higher NT-proBNP concentrations and lower weight. Multivariable logistic regression analysis showed that the strongest associated variables for higher GDF-15 were diabetes, higher NT-proBNP, lower eGFR, lower haemoglobin and smaller left ventricular end-diastolic diameter as shown in online supplementary *Table S2*. Baseline characteristics for all four quartiles are presented in online supplementary *Tables S3* and *S4*. Higher GDF-15 quartiles had significantly higher probability of all-cause mortality and HF hospitalization at 2 years (*Figure 1*). Further, after adjusting for potential confounders including age, sex, eGFR, diabetes, NT-proBNP and NYHA class, the association between GDF-15 quartiles and all-cause mortality remained significant and was gradually increased per quarter (compared to the first quarter): hazard ratio (HR) of quartile 2 = 1.46 and 2.82, HR of quartile 3 = 1.78 and 3.24, and HR of quartile 4 = 2.26 and 5.26 in the index and validation cohort, respectively. The associations were not significant for 2-year hospitalization (online supplementary *Table S5*).

Differential expression analysis

In both the index and validation cohorts, higher GDF-15 was associated with upregulation of several other biomarkers (*Figure 2*). A total of 43 and 51 proteins were upregulated in the index and validation cohort, respectively, of which 39 upregulated proteins were replicated in both cohorts (*Figure 3*). In both cohorts, the strongest upregulated biomarkers in patients with high GDF-15 concentrations were fibroblast growth factor 23 (FGF-23), death receptor 5 (TRAIL-R2), WNT1-inducible signalling pathway protein 1 (WISP-1), tumour necrosis factor (TNF) receptor superfamily member 11a (TNFRSF11A), leucocyte immunoglobulin-like receptor subfamily B member 4 (LILRB4) and trefoil factor 3 (TFF3) (*Figure 2* and online supplementary *Table S6*). Furthermore, 7 out of 39 biomarkers belonged to the TNF receptor superfamily. Heatmap analysis (online supplementary *Figure S1*) showed GDF-15 had a distinct expression profile compared to FGF-23, TRAIL-R2, WISP-1, TNFRSF11A, LILRB4 and TFF3. Results from the linear regression analyses (sensitivity analysis) after correction for FDR of 5% are presented in online supplementary *Table S7*.

Pathway analysis

Pathway over-representation analysis revealed that patients in the highest GDF-15 quartile had increased activity of pathways related to inflammatory processes, notably regulation of chemokine production, macrophages, and adaptive immune response; response to IL-6; TNF and death receptor activity; and positive regulation of T-cell differentiation and inflammatory response (*Figure 4*), as compared to patients in the lowest GDF-15 quartile. Furthermore, pathways involved in regulation of insulin-like growth factor (IGF) receptor signalling were found, in addition to regulatory pathways

Table 1 Baseline characteristics of the highest versus lowest growth differentiation factor 15 quartile in the index cohort

Index cohort characteristics	Lowest GDF-15 quartile (n = 570)	Highest GDF-15 quartile (n = 569)	p-value
Female sex	156 (27.4)	139 (24.4)	0.287
Age	63.7 [54.5–71.8]	73.9 [65.1–80.2]	<0.001
Race			0.696
Caucasian	563 (98.8)	565 (99.3)	
Asian	2 (0.35)	2 (0.35)	
Black	2 (0.35)	0 (0.00)	
Other	3 (0.53)	2 (0.35)	
BMI (kg/m^2)	27.5 [24.6–30.8]	26.9 [23.9–30.4]	0.054
Weight (kg)	82.0 [71.0–93.2]	79.0 [69.0–90.0]	0.002
Height (cm)	172 [166–178]	170 [165–176]	0.008
NYHA class			<0.001
I	23 (4.11)	7 (1.27)	
II	291 (52.0)	125 (22.6)	
III	207 (37.0)	321 (58.2)	
IV	39 (6.96)	99 (17.9)	
LVEF (%)	30.0 [25.0–35.0]	30.0 [21.0–37.0]	0.867
Pulmonary congestion/oedema with rales/crackles			<0.001
No	326 (59.7)	196 (35.1)	
Single base	67 (12.3)	85 (15.2)	
Bi-basilar	153 (28.0)	278 (49.7)	
Orthopnoea present	130 (22.8)	258 (45.7)	<0.001
Peripheral oedema	455 (79.8)	492 (86.6)	0.003
Pulmonary congestion/oedema >1/3 up lung fields	27 (12.2)	77 (21.3)	0.008
Elevated JVP			<0.001
No	311 (78.1)	186 (46.4)	
Yes	67 (16.8)	185 (46.1)	
Uncertain	20 (5.03)	30 (7.48)	
Hepatomegaly	51 (8.95)	119 (21.1)	<0.001
SBP (mmHg)	125 [110–140]	120 [106–130]	<0.001
DBP (mmHg)	79.0 [70.0–85.0]	70.0 [62.0–80.0]	<0.001
Heart rate (bpm)	75.0 [66.0–85.0]	77.0 [67.5–90.0]	0.039
Admitted to ICU/CCU	20 (32.8)	22 (20.0)	0.094
Years since first diagnosis	0.12 [0.01–1.80]	0.52 [0.01–4.44]	0.333
Ischaemia	233 (41.7)	325 (57.8)	<0.001
Previous HF hospitalizzation(s) in last year	146 (25.6)	215 (37.8)	<0.001
Hypertension	325 (57.0)	364 (64.0)	0.019
Atrial fibrillation	194 (34.0)	311 (54.7)	<0.001
Myocardial infarction	175 (30.7)	252 (44.3)	<0.001
PCI	99 (17.4)	136 (23.9)	0.008
CABG	62 (10.9)	143 (25.1)	<0.001
Diabetes mellitus	105 (18.4)	262 (46.0)	<0.001
COPD	63 (11.1)	105 (18.5)	0.001
Peripheral arterial disease	33 (5.79)	81 (14.2)	<0.001
Stroke	36 (6.32)	74 (13.0)	<0.001
Haemoglobin (g/dl)	13.9 [12.8–14.9]	12.4 [11.1–13.7]	<0.001
Serum creatinine (μmol/L)	88.4 [75.0–106]	126 [99.9–166]	<0.001
Urinary creatinine (mmol/L)	7.10 [3.48–12.0]	3.85 [2.20–6.53]	<0.001
Urea (mmol/L)	8.60 [6.30–13.3]	15.9 [9.84–25.3]	<0.001
Sodium (mmol/L)	140 [138–142]	139 [136–141]	<0.001
Potassium (mmol/L)	4.30 [4.00–4.60]	4.20 [3.90–4.60]	0.069
BNP (pg/ml)	417 [185–738]	1101 [678–1989]	<0.001
NT-proBNP (pg/ml)	1108 [476–2233]	5962 [2800–12 855]	<0.001
eGFR (ml/min/1.73 m^2)	75.1 [60.4–90.5]	46.0 [32.3–60.1]	<0.001
LVEDD (mm)	62.0 [57.0–68.0]	60.0 [53.0–66.0]	<0.001
LVESD (mm)	50.0 [45.0–57.0]	49.0 [42.0–56.8]	0.033
Left atrial diameter (mm)	46.0 [42.0–51.0]	49.0 [43.5–55.0]	<0.001
GDF-15 (pg/ml)	1224 [943–1472]	7071 [5598–10 510]	<0.001

Data are presented as n (%), or median [quartiles].
BMI, body mass index; BNP, B-type natriuretic peptide; CABG, coronary artery bypass graft; COPD, chronic obstructive pulmonary disease; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; GDF-15, growth differentiation factor 15; HF, heart failure; ICU, intensive care unit; JVP, jugular venous pressure; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic diameter; NT-proBNP, N-terminal pro-B-type natriuretic peptide; NYHA, New York Heart Association; PCI, percutaneous coronary intervention; SBP, systolic blood pressure.

Table 2 Baseline characteristics of the highest versus lowest growth differentiation factor quartile in the validation cohort

Validation cohort characteristics	Lowest GDF-15 quartile (n = 427)	Highest GDF-15 quartile (n = 426)	p-value
Female sex	155 (36.3)	128 (30.0)	0.062
Age	68.2 [60.9–75.4]	78.9 [71.7–84.4]	<0.001
Race			0.624
Caucasian	425 (99.5)	423 (99.3)	
Black	1 (0.23)	0 (0.00)	
Asian	0 (0.00)	2 (0.47)	
Hispanic	1 (0.23)	1 (0.23)	
BMI (kg/m^2)	28.7 [25.6–33.2]	27.1 [23.9–32.0]	<0.001
Weight (kg)	83.5 [71.0–98.0]	78.0 [65.0–89.0]	<0.001
Height (cm)	170 [162–178]	168 [160–175]	0.003
NYHA class			<0.001
I	11 (2.58)	2 (0.47)	
II	251 (58.9)	105 (24.6)	
III	143 (33.6)	219 (51.4)	
IV	21 (4.93)	100 (23.5)	
LVEF (%)	39.5 [35.0–48.0]	40.0 [35.0–50.0]	0.131
Pulmonary congestion/oedema with rales/crackles			<0.001
No	302 (75.7)	152 (36.9)	
Single base	16 (4.01)	33 (8.01)	
Bi-basilar	81 (20.3)	227 (55.1)	
Peripheral oedema	363 (85.0)	400 (94.1)	<0.001
Pulmonary congestion/oedema >1/3 up lung fields	5 (4.07)	15 (9.04)	0.158
Elevated JVP			<0.001
No	288 (80.9)	226 (59.6)	
Yes	67 (18.8)	152 (40.1)	
Uncertain	1 (0.28)	1 (0.26)	
Hepatomegaly	4 (1.08)	30 (7.52)	<0.001
SBP (mmHg)	124 [112–140]	121 [107–137]	0.044
DBP (mmHg)	72.0 [64.2–80.0]	66.0 [59.0–75.0]	<0.001
Heart rate (bpm)	70.0 [60.0–80.0]	74.0 [64.0–87.0]	<0.001
Years since first diagnosis	0.99 [0.13–3.66]	1.59 [0.05–5.46]	0.134
Ischemia	263 (94.9)	282 (92.2)	0.232
Previous HF hospitalization(s) in last year	100 (23.7)	136 (32.6)	0.005
Hypertension	212 (49.6)	268 (63.1)	<0.001
Atrial fibrillation	155 (36.7)	214 (50.6)	<0.001
Myocardial infarction	193 (45.2)	213 (50.1)	0.171
PCI	97 (22.9)	69 (16.3)	0.020
CABG	66 (15.5)	83 (19.5)	0.140
Diabetes mellitus	80 (18.8)	208 (49.1)	<0.001
COPD, n (%)	64 (15.1)	79 (18.8)	0.178
Peripheral arterial disease	64 (15.4)	100 (24.3)	0.002
Stroke	58 (13.7)	88 (20.8)	0.009
Haemoglobin (g/dl)	14.0 [13.1–15.2]	12.1 [10.9–13.3]	<0.001
Serum creatinine (μmol/L)	83.0 [69.0–96.0]	127 [100–168]	<0.001
Urinary creatinine (mmol/L)	4.10 [2.00–7.90]	3.80 [2.20–6.50]	0.286
Urea (mmol/L)	6.80 [5.50–8.22]	12.5 [9.00–17.1]	<0.001
Sodium (mmol/L)	140 [138–141]	139 [136–141]	0.001
Potassium (mmol/L)	4.30 [4.10–4.50]	4.30 [3.90–4.60]	0.268
NT-proBNP (pg/ml)	548 [229–1357]	3994 [1459–8712]	<0.001
eGFR (ml/min/1.73 m^2)	70.3 [58.8–84.6]	38.0 [27.0–52.2]	<0.001
LVEDD (mm)	56.0 [50.0–62.0]	54.0 [47.0–60.0]	0.001
LVESD (mm)	44.6 (13.1)	42.6 (12.0)	0.226
Left atrial diameter (mm)	44.0 [40.0–49.0]	46.0 [42.0–50.2]	0.002
GDF-15 (pg/ml)	1343 [1084–1600]	6955 [5436–9583]	<0.001

Data are presented as n (%), median [quartiles], or mean (standard deviation).
BMI, body mass index; BNP, B-type natriuretic peptide; CABG, coronary artery bypass graft; COPD, chronic obstructive pulmonary disease; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; GDF-15, growth differentiation factor 15; HF, heart failure; ICU, intensive care unit; JVP, jugular venous pressure; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic diameter; NT-proBNP, N-terminal pro-B-type natriuretic peptide; NYHA, New York Heart Association; PCI, percutaneous coronary intervention; SBP, systolic blood pressure.

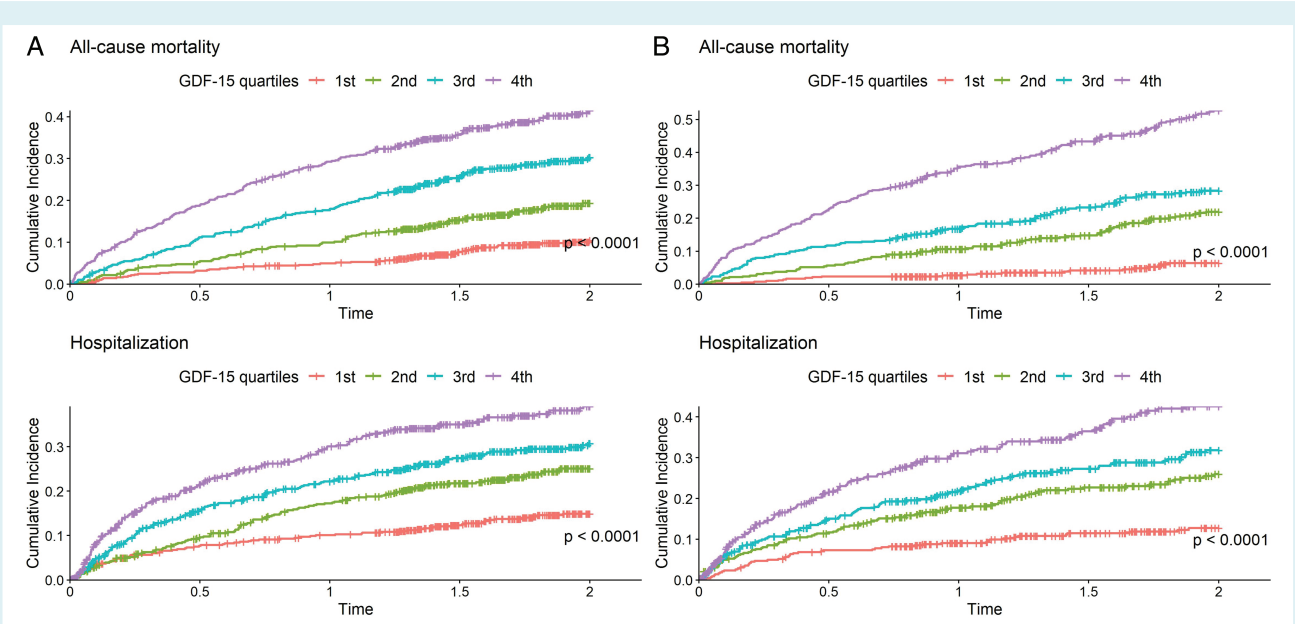


Figure 1 Kaplan–Meier survival curves and *p*-value of log-rank test for all-cause mortality and hospitalization at 2 years in the index cohort (*A*) and the validation cohort (*B*) stratified according to growth differentiation factor 15 (GDF-15) quartiles.

of bones, tissues, neurons and branching structures. Biomarkers related to each pathway are presented in *Figure 4A*.

Discussion

In two independent cohorts of patients with HF we consistently found that high GDF-15 concentrations identified patients with increased activity of pathways related to inflammation and those involved in regulation of IGF receptor signalling and bone/tissue remodelling (*Graphical Abstract*).

Normal physiology of GDF-15

GDF-15 is expressed in the kidney, liver, lung, pancreas, gastrointestinal tract and heart, with physiological serums levels between 0.1–1.2 ng/ml.[19,20] Increased GDF-15 levels are linked to older age, excessive exercise, pregnancy, oxidized low-density lipoprotein (oxLDL) and smoking.[2,19] Furthermore, GDF-15 elevations are seen in many pathological conditions, including metabolic disease, cancer, inflammation, and cardiovascular disease.[2,19,20] It is believed that GDF-15 plays a protective, anti-inflammatory role in the liver, kidney, heart and lungs after injury and inflammation.[2,19,21] Further, GDF-15 has a bone regulatory function, as it attenuates osteocyte differentiation in response to hypoxia and in cancer and spondylarthritis.[22–25] Furthermore, GDF-15 blocks polymorphonuclear leucocytes recruitment which reduces cardiac rupture after MI and inhibits atherosclerosis progression,[2,20] in addition to inhibiting nuclear factor kappa B (NF-κB).[19] Moreover, GDF-15 protects against ischaemia–reperfusion injury by inhibiting neutrophil infiltration and apoptosis, decreases thrombus formation by inhibiting platelet integrin activation, and GDF-15 treatment or cardiac overexpression decreases cardiac hypertrophy.[20] The exact protein–receptor interactions through which GDF-15 exerts these effects, are still unclear.[19,20] GDF-15 also inhibits appetite, thereby reducing obesity and potentially increasing insulin sensitivity.[20] Moreover, GDF-15 produced by cardiomyocytes inhibits growth hormone (GH) signalling in the liver by reducing plasma IGF-1 and IGF binding protein 3 (IGFBP-3), without affecting GH concentrations, potentially inducing the GH-tolerance seen in HF.[26]

Inflammatory pathways

We found an association between elevated GDF-15 and biomarkers related to macrophage and adaptive immune response. These findings are in line with earlier studies, where GDF-15 directly decreased myeloid cell adhesion and transmigration after myocardial infarction, protecting the heart in the chronic phase.[27] Macrophages and monocytes are the major source for TNF in the human body, what may explain the link between GDF-15 and TNF. As shown in a previous study, TNF has a direct negative inotropic and hypertrophic effect on myocardial cells; uncouples adenylyl cyclase from beta-adrenergic receptors via its effect on G inhibitory protein; leads to release of cytokines; is directly cytotoxic to endothelial cells and activates metalloproteinases which leads to extracellular matrix remodelling.[28] TNF is expressed in cardiac cells in HF, but not in healthy cells, and is known to be associated with HF severity.[28] Moreover, TNF is increased earlier in the disease process than NT-proBNP.[29] Furthermore, similar to GDF-15, TNF is linked to cachexia, anorexia and muscle wasting.[28,30–33] TNF-α exerts its cachexic effect through activation of death receptors TNF receptor 1 and 2 (TNFR1 and 2), which

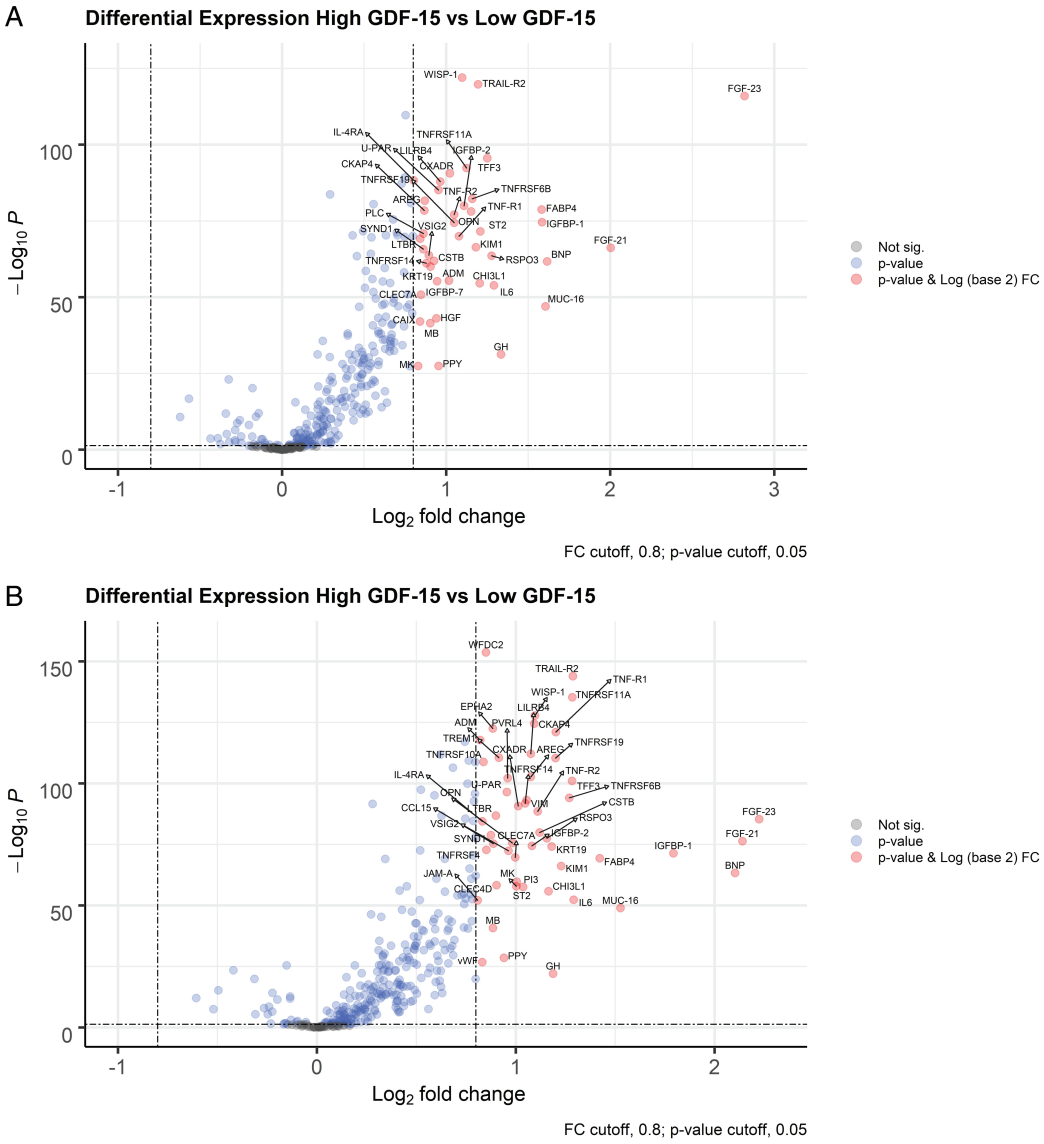


Figure 2 Volcano plots presenting differentially expressed biomarkers in heart failure patients with high growth differentiation factor 15 (GDF-15) concentrations compared to heart failure patients with low GDF-15 concentrations — (*A*) the index cohort, (*B*) the validation cohort. ADM, adrenomedullin; AREG, amphiregulin; BNP, B-type natriuretic peptide; CAIX, carbonic anhydrase 9; CCL15, C-C motif chemokine ligand 15; CHI3L1, chitinase 3 like 1; CKAP4, cytoskeleton associated protein 4; CLEC4D, C-type lectin domain family 4 member D; CLEC7A, C-type lectin domain containing 7A; CSTB, cystatin B; CXADR, CXADR Ig-like cell adhesion molecule; EPHA2, EPH receptor A2; FABP4, fatty acid binding protein 4; FGF-21, fibroblast growth factor 21; FGF-23, fibroblast growth factor 23; GH, growth hormone 1; HGF, hepatocyte growth factor; IGFBP-1, insulin-like growth factor binding protein 1; IGFBP-2, insulin-like growth factor binding protein 2; IGFBP-7, insulin-like growth factor binding protein 7; JAM-A, F11 receptor; KIM1, hepatitis A virus cellular receptor 1; KRT19, keratin 19; IL-4RA, interleukin-4 receptor; IL6, interleukin-6; LILRB4, leucocyte immunoglobulin like receptor B4; LTBR, lymphotoxin beta receptor; MB, myoglobin; MK, midkine; MUC-16, mucin 16, cell surface associated; PI3, peptidase inhibitor 3; PLC, heparan sulfate proteoglycan 2; PPY, pancreatic polypeptide; PVRL4, nectin cell adhesion molecule 4; RSPO3, R-spondin 3; OPN, secreted posphoporetin 1; ST2, interleukin 1 receptor like 1; SYND1, syndecan 1; TFF3, trefoil factor 3; TNF-R1, TNF receptor superfamily member 1A; TNF-R2, TNF receptor superfamily member 1B; TNFRSF4, TNF receptor superfamily member 4; TNFRSF6B, TNF receptor superfamily member 6b; TNFRSF10A, TNF receptor superfamily member 10a; TNFRSF11A, TNF receptor superfamily member 11a; TNFRSF14, TNF receptor superfamily member 14; TNFRSF19, TNF receptor superfamily member 19; TRAIL-R2, TNF receptor superfamily member 10b; TREM1, triggering receptor expressed on myeloid cells 1; U-PAR, urokinase plasminogen activator receptor; VIM, vimentin; VSIG2, V-set and immunoglobulin domain containing 2; vWF, von Willebrand factor; WFDC2, WAP four-disulfide core domain 2; WISP-1, WNT1-inducible signalling pathway protein 1.

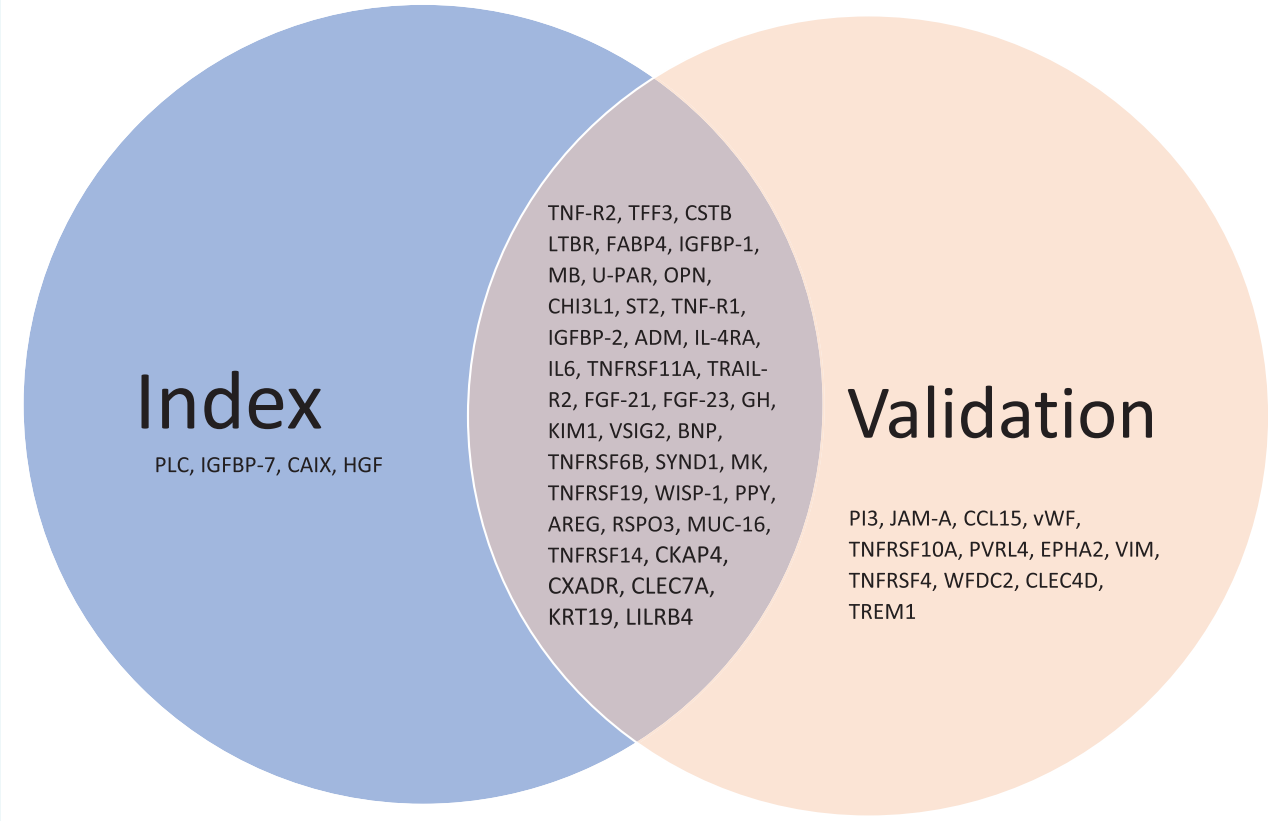


Figure 3 Upregulated biomarkers after differential expression analysis. For abbreviations see *Figure 2*.

in turn leads to long-term overexpression of NF-κB dependent gene products and results in increased apoptosis,[34,35] and through interfering with neuropeptide Y release in the hypothalamus.[34,35] The upregulation of TNF-related proteins could thus partly explain why weight was consistently decreased, and NYHA class increased in upper compared to the lower quartiles of GDF-15 patients in both cohorts.

Interleukin-6 is an important pleiotropic cytokine that has pro- and anti-inflammatory effects through its activation of transmembrane receptor subunit glycoprotein 130 (gp130). In the heart, IL-6 predominantly has its effect through the Janus kinase/signal transducer and activator of transcription 1/3 (JAK/STAT1/3) pathway.[36] Like TNF proteins, IL-6 has protective effects in the acute phase as a survival pathway, but leads to reduced contractility, diastolic disturbances, disturbed calcium handling and cardiac hypertrophy when chronically elevated or during unbalanced expression.[36,37] Further, JAK2/STAT3 activation by IL-6 increases hepcidin activation, which in turn reduces plasma iron levels through inhibition of ferroportin.[38] In large heterogeneous cohorts, elevated IL-6 can be found in over half of chronic HF patients, is significantly related to HFpEF, NT-proBNP, iron deficiency and AF.[39] Furthermore, IL-6 was found to be a significant predictor of mortality and hospitalization in HF.[37] The upregulation in the IL-6 pathway in patients with high GDF-15 levels could be representative of a chronic maladaptation and inflammatory state in patients with elevated concentrations of GDF-15 and contributes to the observed reduced haemoglobin levels.

GDF-15 was also related to positive chemokine production regulation through pathways including interleukin-4 receptor (IL4-RA), interleukin 1 receptor-like 1 (ST2) and IL-6, which are all increased in, or a predictor of, HF.[40] Other studies have found that GDF-15 inhibits chemokine production in HF, possibly highlighting its protective, anti-inflammatory function.[27] Further, we found an association between positive regulation of T lymphocytes and GDF-15. Global increases in T lymphocytes are an essential driving factor for both mortality and remodelling in ischaemic HF, with $CD4^+/CD28^{null}$ subsets playing a role in the development of AF, and an increase in $CD4^+/CD57^+$ being related to cardiovascular events and increased TNF-α expression.[41–43]

Insulin-like growth factor regulation

Similar to GDF-15, IGF-1 has long been linked to diabetes and HF.[44,45] IGFBP-1 and 2 and GH were found to be upregulated in patients with elevated GDF-15. IGFBP-2 is a significant predictor for mortality in HF.[46,47] This mechanism is likely through IGFBP-2 inhibition of IGF-1. IGF-1 has a cardioprotective effect through a downregulation of the renin–angiotensin system.[47] In addition, IGF-1 increases contractility, is indicated in physiological cardiac hypertrophy, delays myocyte apoptosis, and is found to

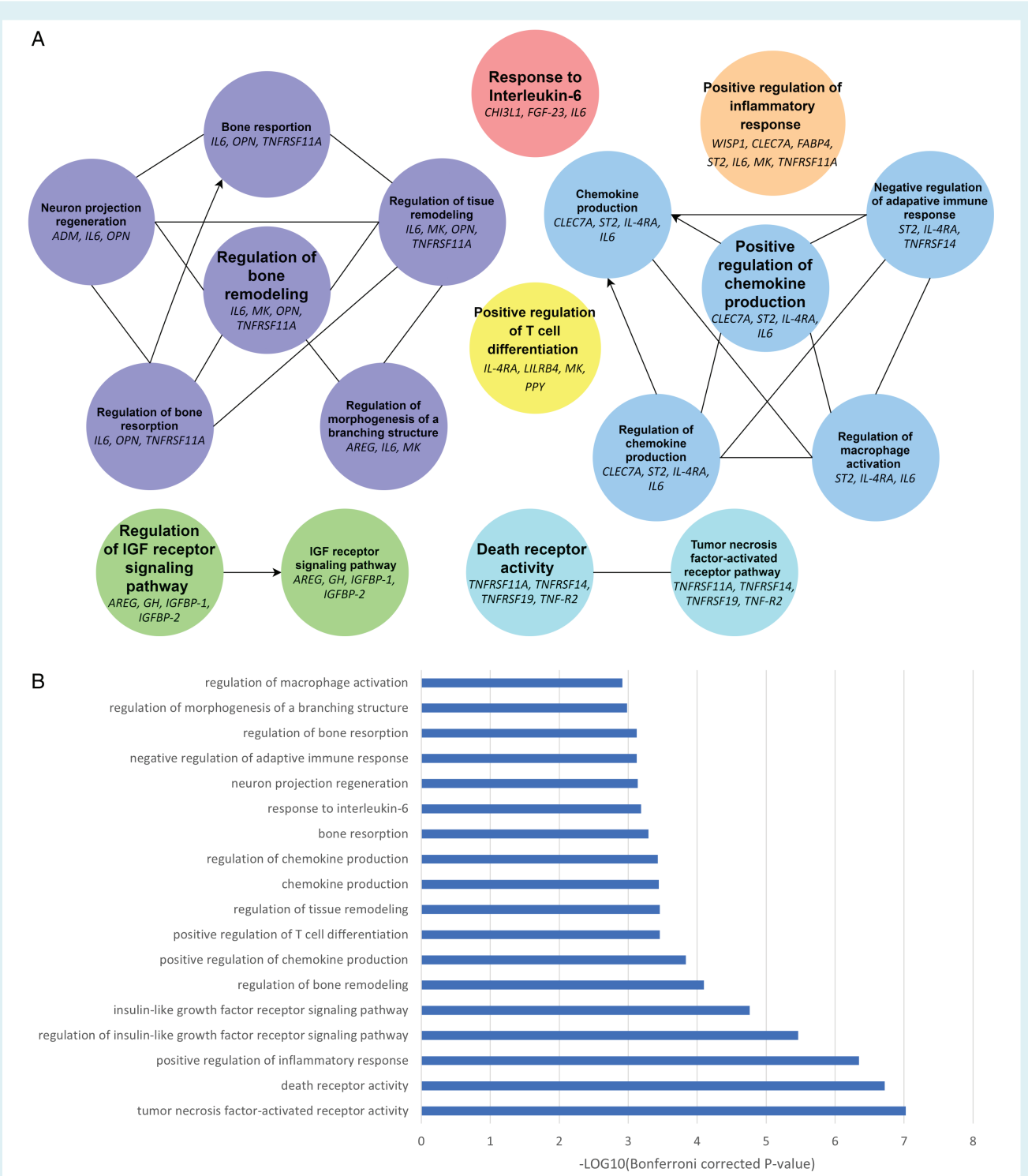


Figure 4 Pathway over-representation results for heart failure patients with high growth differentiation factor 15 (GDF-15) concentrations compared to heart failure patients with low GDF-15 concentrations. (*A*) Pathway networks, (*B*) Bonferroni corrected *p*-values per pathway term. AREG, amphiregulin; CHI3L1, chitinase 3 like 1; CLEC7A, C-type lectin domain family 7 member A; FABP4, fatty acid binding protein 4; FGF-23, fibroblast growth factor 23; GH, growth hormone; IGFBP-1, insulin like growth factor binding protein 1; IGFBP-2, insulin like growth factor binding protein 2; IL-4RA, interleukin-4 receptor; IL6, interleukin-6; LILRB4, leucocyte immunoglobulin like receptor B4; MK, midkine; OPN, secreted posphoporetin 1/osteopontin; ST2, interleukin 1 receptor-like 1; TNF-R2, TNF receptor superfamily member 1B; TNFRSF11A, TNF receptor superfamily member 11a; TNFRSF14, TNF receptor superfamily member 14; TNFRSF19, TNF receptor superfamily member 19; WISP-1, WNT1-inducible signalling pathway protein 1.

be reduced in HF, particularly with comorbid cachexia.[45,48] These findings suggest that IGF-1 might become a novel treatment target of cachectic HF patients. Paradoxically, patients with higher GDF-15 concentrations also had higher GH concentrations. GH increases IGF production and improves cardiac function.[49] However, in severe HF, especially in cachexic patients, GH resistance occurs, increasing circulating GH concentrations but reducing its cardio-protective effects.[48,49] GDF-15 seems thus to be related to an unbalanced IGF/GH axis.

Bone, tissue, and branched structure remodelling

Pathway analysis found upregulation in bone and tissue remodelling, which is in line with previous literature, as GDF-15 has been related to osteoclast differentiation, being attenuated by bone hypoxia.[23,50] Key players in these pathways were osteopontin, IL-6 and TNFRSF11A. Osteopontin inhibits myeloid cell recruitment and metalloproteinase expression, regulates osteoclast differentiation, and has a pleiotropic effect on vascular remodelling. Moreover, osteopontin has strong links to left ventricular dysfunction and remodelling, particularly in HFpEF.[51–53] GDF-15 has been found to suppress osteopontin secretions in fibrotic liver disease, likely acting as a liver protective protein, in line with our findings, as high GDF-15 was significantly linked to hepatomegaly.[54,55] The bone regulatory effects of GDF-15 have been linked to bone metastasis in cancer and spondylarthritis, however its exact role in HF remains unclear.[24,25,50]

Strongest GDF-15 associated biomarkers

WISP-1 is an extracellular matrix protein expressed by fibroblasts in the heart, lungs, kidneys, spleen, pancreas and brain among others.[56] Further, WISP-1 is highly expressed in visceral adipose tissue where it may reflect adipose tissue inflammation and insulin resistance, and is elevated in obesity and in reaction to oxLDL.[56] Moreover, it is upregulated in the left ventricle after MI where it might be a therapeutic target for revascularization as it stimulates cardiac-specific angiogenesis.[57] Although early results are promising, effects were measured 7 days post-MI. Future studies using knockout mice and/or recombinant WISP-1 should investigate both the long- and short-term effects of WISP-1 attenuation and aim identify the receptor for WISP-1 on endothelial cells.[57]

TFF3 belongs to the trefoil factor family, which are expressed in a variety of mucin-producing cells in the intestines and kidney.[58] It is increased in diabetes, after use of antihypertensive medication and in kidney injury.[58] In the latter it may reflect regenerative function through restitution, apoptosis inhibition and modulation of differentiation/immune function.[58] TFF3 is a Food and Drug Administration-approved biomarker of drug-induced kidney injury and is associated with chronic kidney disease.[58] Moreover, TFF3 is upregulated and cardioprotective after MI,[59] and predicts a composite endpoint including HF hospitalization and cardiac death in HF, even after adjustment for established risk predictors.[60] Why TFF3 is highly predictive of HF outcomes is unknown, future studies utilizing TFF3 knockout in HF-induced mice models could shed light onto the mechanisms of TFF3-related pathophysiology.

LILRB4 is part of the immunoglobulin superfamily and is expressed on the surface of monocytes, macrophages and dendritic cells.[61,62] LILRB4 may be a future therapeutic target as it protects against cardiac hypertrophy and fibrosis by inhibition of NF-κB.[62,63] Moreover, LILRB4 improves plaque stability due to decreasing NF-κB, IL-6 and TNF-α mediated inflammation.[61,62] The upregulation of LILRB4 in antigen presenting cells might thus reflect a cardioprotective function. Further, LILRB4 expression is reduced in pressure-overloaded hearts,[63] thus LILRB4 might improve established cardiac hypertrophy which warrants additional investigation, for example by LILRB4 induction in hypertrophic mice models.

FGF-23 is a glycoprotein mainly expressed by osteoblasts and is associated with cardiovascular diseases, chronic kidney disease, HF and mortality.[64] FGF-23 contributes to arterial stiffness/inflammation, left ventricular hypertrophy, renin–angiotensin system stimulation and endothelial dysfunction, although it might stabilize and improve contractility acutely post-MI.[64] Our finding of elevated FGF-23 is likely due to both increased skeletal and cardiac expression, because even though not expressed in the healthy heart, cardiac specific FGF-23 is increased post-MI, in cardiomyopathy and is augmented by left ventricular hypertrophy.[64] Currently, it is unclear whether FGF-23 secreted by the myocardium is protective or deleterious. Future studies could investigate optimal systemic and cardiac FGF-23 levels, explaining the conflicting results so far.[64]

TRAIL-R2 is a cell surface death receptor that is expressed on endothelium, vascular smooth muscle cells (VSMCs) and macrophages, is highly expressed in the heart, and can also be released in a soluble form (sTRAIL).[65–67] It predicts all-cause mortality after MI[68] and HF development.[67] In most cells TRAIL-R2 stimulates apoptosis, whereas in cardiomyocytes it promotes hypertrophy while inhibiting fibrosis after injury.[67,69] However, more research is needed if TRAIL-R2 therapy has a future in established HF, as TRAIL-R2 expression and decoy receptor mediated regulation may be altered in HF.[67]

TNFRSF11A, or receptor activator of NF-kB (RANK), binds to RANK ligand (RANKL) resulting in NF-kB signalling and osteoprotegerin (OPG) production.[70] The RANK–RANKL–OPG signalling axis is a main regulator of vessel wall calcification/inflammation, bone remodelling, and T-cell functioning.[70] Although widely expressed in the gastrointestinal tract, adipose tissue and arteries among others, cardiomyocyte RANK expression is increased response to systemic RANKL, but also in VSMCs and endothelial cells in the failing myocardium.[71] Future studies utilizing TNFRSF11A knockout in mice might elucidate TNFRSF11A exact role in HF pathophysiology.

Utility and diagnostics

This study found that patients in the upper GDF-15 quartiles had significantly lower weight. GDF-15 exerts its anorexic effect through binding to glial cell-derived neurotrophic factor (GDNF) family receptor alpha-like (GFRAL) in the nucleus of the solitary

tract and the area postrema, leading to a reduction in appetite.[32] Furthermore, acute induction of the GDF-15/GFRAL pathway has been shown to combat obesity in mice and non-human primates, whilst blockade of the pathway helps prevent anorexia and weight loss in cancer patients undergoing chemotherapy.[72,73] Moreover, the weight lowering effects of metformin may be mediated through its acute upregulation of GDF-15.[74] Chronic elevation of GDF-15 through genetic proxying, however, relates to higher body mass index.[75] This study thus strengthens the argument for additional research on GDF-15 therapy in HF to promote weight loss.

We found that patients in high GDF-15 quartiles were more likely to have AF. Previous studies have identified GDF-15 as a predictor for bleeding risk, but not stroke, in AF patients undergoing anticoagulation therapy.[76,77] Therefore, GDF-15 was included into the ABC (age, biomarkers, clinical history) bleeding score which improved discrimination and utility to pre-existing models. In line with literature, GDF-15 quartiles showed to significantly predict both all-cause mortality and HF hospitalization, highlighting the value of GDF-15 inclusion in future prognostic multi-marker models.[2,5–7] Furthermore, we found that GDF-15 is associated with various inflammatory and metabolic pathways, giving insight into why GDF-15 is such a powerful predictor for patients with HF.

Clinical implications

To our knowledge, this is the first study linking GDF-15 with WISP-1, TFF3, LILRB4, TRAIL-R2 and TNFRSF11A in HF. Besides improving prognosis, GDF-15 could be a valuable marker that indicates repair processes after myocardial and renal damage and can potentially guide therapy based on several repair mechanisms found in this study. Studies applying the Mendelian randomization analysis have found a causal effect of genetically predicted TNF levels on coronary artery disease and ischemic stroke.[78] Further, there was a causal relation established between IL-6 signalling and coronary artery disease and AF.[79] Thus, we cannot exclude the possibility that there are causal links between biomarkers tested in our study and HF, and this could be addressed in the future with the Mendelian randomization approach. Moreover, further studies could help understand whether WISP-1, TFF3, LILRB4, FGF-23 and TRAIL-R2 carry a clinical potential, and whether GDF-15 could predict treatment response for these agents. Furthermore, the link between GDF-15 and the IGF-1/GH signalling axis could be further investigated, as GDF-15 might be a therapeutic target to alleviate GH resistance in HF.

Strengths and limitations

The main strength in the design of our study is the validation of our findings in an independent cohort. Another strength is the high number of analysed biomarkers ($n = 363$), which cover a broad range of proteins such as cytokines, enzymes, apoptotic and growth factors. Further, biomarkers were analysed using PEA, which shows good performance in plasma samples due to its high sensitivity, specificity, scalability, and low sample consumption,[13,80] and is shown not to be affected by freeze–thaw cycles.[81] We acknowledge limitations of our study. Firstly, since the analysed biomarkers come from the following panels: cardiovascular II and III, oncological, and immunological, any upregulated pathway could be potentially biased into one of these four categories. Secondly, since gene ontology annotation data are continuously manually updated by curators, and more extensively studied proteins might be more comprehensively annotated, updates and choice of GO data source might slightly affect results of the pathway analysis. In addition, although circulating plasma protein levels are influenced by factors such as metabolism and clearance besides tissue expression, an increasing amount of evidence suggests that plasma proteins reliably detect myocardial tissue changes.[82] However, because tissue sample analytes were not measured, verification that the 363 circulating biomarkers truly reflect tissue-based events was limited. Furthermore, although the enrichment/depletion analysis (two-sided hypergeometric test) in ClueGO showed that the relation between the identified pathways and the upregulated biomarkers was highly significant (*Figure* 4B), whether the number of biomarkers was sufficient to comprehensively populate the pathways is still uncertain. Lastly, although our findings reflected previous literature, enrolled patients were largely of Caucasian descent (99%), limiting generalizability. Further studies in more diverse populations will have to be performed to replicate our findings.

Conclusion

Patients with HF and high plasma concentrations of GDF-15 are characterized by increased activation of inflammatory pathways and pathways related to IGF-1 regulation and bone/tissue remodelling.

Supplementary Information

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

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Conflict of interest: The UMCG, which employs several of the authors, has received research grants and/or fees from AstraZeneca, Abbott, Boehringer Ingelheim, Cardior Pharmaceuticals Gmbh, Ionis Pharmaceuticals, Inc., Novo Nordisk, and Roche. J.T. has received speaker and or consultancy fees from Roche diagnostics, Us2.ai and holds a patent (U.S. Patent No. 10,702,247) unrelated to the present work. R.A.d.B. received speaker fees from Abbott, AstraZeneca, Bayer, Novartis, and Roche. C.C.L. declares receiving consultancy fees and/or research grants from Amgen, Applied Therapeutics, AstraZeneca, Boehringer Ingelheim, MSD, Novartis, and Novo Nordisk. S.D.A. reports receiving fees from Abbott, Bayer, Boehringer Ingelheim, Cardiac Dimension, Cordio, Impulse Dynamics, Novartis, Occlutech, Servier, and Vifor

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