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ORIGINAL ARTICLE

Circulating immune checkpoints predict heart failure outcomes

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Abstract

Aims There are limited data examining the role of immune checkpoint (IC) ligands in the pathophysiology of heart failure (HF). Therefore, we explore this in three HF animal models and in three different human cohorts (healthy, stable, and worsening HF).

Methods and results Transcriptomic analyses of cardiac tissue of three different HF mouse models revealed differentially expressed IC receptors and their ligands compared with control mice. Based on this observation, serum levels of three well-known IC ligands (i.e. sPD-L1, sPD-L2 and galectin-9) were measured in stable HF patients from the Vitamin D Chronic Heart Failure (VitD-CHF) study (n = 101), as well as healthy individuals from the Prevention of Renal and Vascular End-stage Disease (PREVEND) study (n = 58). sPD-L1, sPD-L2, and galectin-9 were all associated with New York Heart Association classification. In multivariate linear regression analyses, all three IC ligands were associated with galectin-3 (β = 0.230, β = 0.283, and β = 0.304, respectively). sPD-L1 and galectin-9 were also associated with hs-troponin-T (β = 0.386 and β = 0.314). Regarding prognosis, higher serum levels of sPD-L1 and galectin-9 were significantly associated with increased risk for HF hospitalization and all-cause mortality [hazard ratio 1.69 (1.09–2.59) and hazard ratio 1.50 (1.06–2.12)]. Furthermore, the importance of IC ligands was tested in another stage of HF, namely worsening HF patients. In the worsening HF cohort (The BIOlogy Study to Tailored Treatment in Chronic Heart Failure) (n = 2032), sPD-L2 and galectin-9 were associated with New York Heart Association classification and significantly predicted outcome with an increased relative risk of 15% and 20%, after multivariable adjustment, respectively.

Conclusions IC ligands are expressed in cardiac disease models, and serum levels of IC ligands are elevated in HF patients, are associated with disease severity, and significantly predict prognosis. These data indicate a potential role for IC ligands in HF pathogenesis.

Keywords Immune checkpoints; PD-L1; PD-L2; Galectin-9; Heart failure

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All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

Introduction

Immune checkpoints (IC) serve as 'brakes' on the immune system and include programmed cell death protein-1 (PD-1) and T-cell immunoglobulin and mucin-domain containing molecule-3 (TIM-3), expressed on immune cells.[1] ICs are targeted by immune checkpoint inhibitors (ICI) for effective cancer therapy but can lead to inflammatory heart disease underscoring a critical role for IC receptor/IC ligand signalling in cardiovascular homeostasis.[2,3]

Over the past decades, it has been recognized that the pathophysiology of heart failure (HF) is complex with different comorbidities[4–6] and involves a significant inflammatory component, irrespective of the cause of disease.[7,8] This is

evident from the fact that modulation of the immune system decreases the rate of cardiovascular events effectively.[9] More importantly, recent studies suggest that IC ligands might also contribute actively in immune modulatory processes in HF.[10] However, their exact role has not been fully elucidated.

Circulating forms of IC ligands are known to be actively involved in immune regulation and provide a non-invasive tool in gaining a broader understanding of IC ligands in HF. Therefore, we determined circulating levels of IC ligands (specifically soluble PD-L1/2 and galectin-9), as a function of clinical correlates and prognosis using sera from previously well-characterized HF cohorts and healthy controls.

Methods

Mouse studies

Transcriptomic analyses of IC receptors and their ligands were performed on left ventricle (LV) tissue samples from three different HF mouse models and their respective controls, namely, genetic cardiomyopathy (PLN-R14del), left ventricular pressure overload (transverse aortic constriction, TAC), and myocardial infarction (MI). In each group, three mice were evaluated and compared with three respective control mice.

PLN-R14del

Homozygous phospholamban-R14del (PLN-R14del) mice were generated as described previously.[11] In short, PLN-R14del mice have a heterozygous deletion of arginine 14 of the PLN protein. This results in dilated cardiomyopathy with severe LV dysfunction, decreased electrocardiogram (ECG) potentials and PLN protein aggregation, mimicking human disease. Mice were sacrificed within 4–8 weeks, depending on onset of disease.

Transverse aortic constriction

Eight-week old C57Bl/6J mice (Charles River, France) underwent TAC surgery by placement of an 0.56 mm Nitrile O-ring (Apple Rubber, Lancaster, NY, USA) around the aorta. Mice were intubated and mechanically ventilated under 2% isoflurane. An incision was made in the third intercostal space. Sham-operated mice underwent the same procedure, without placement of the O-ring. Postoperatively, all mice received 5.0 mg/kg carprofen for analgesic purposes. Mice were sacrificed six weeks post-TAC.

Myocardial infarction

MI was induced in eight-week old C57Bl/6J mice (Charles River, France) by permanent left anterior descending coronary artery ligation. Mice were intubated and mechanically ventilated under 2% isoflurane. After incision via the fourth intercostal space, ligation was performed using a 6-0 prolene suture. In sham-operated animals, the suture was placed under the artery and removed without ligation. Mice received adequate painkilling with carprofen (5.0 mg/kg) post-MI. Six weeks post-MI, mice were sacrificed.

Transcriptomic analyses

Total RNA was isolated from LV using the SPLIT RNA Extraction Kit (Lexogen). RNA quality was determined using the DNF-471 RNA Kit (15 nt) (Agilent, CA, USA) on a Fragment Analyser System and libraries were prepared using high-quality RNA with the QuantSeq 3′ mRNA-Seq FWD Library Prep Kit (Lexogen). Sequencing was performed using NextSeq 500 instrument with SR75 High Output Kit (Illumina). Reads were aligned to Mus musculus reference genome using the splice-aware aligner (STAR version 2.6.1a). Gene expression analysis was performed using DESeq2 (version 1.18.1).

Human studies

Stable heart failure (VitD-CHF)

Circulating levels of sPD-L1 (Abcam, ab214565), sPD-L2 (Invitrogen, BMS2215), and galectin-9 (R&D Systems, DGAL90) were measured according to the manufacturer's protocol in sera of stable outpatient HF patients from The Vitamin D Chronic Heart Failure (VitD-CHF) study (n = 101). The VitD-CHF study was a single-centre, blinded endpoint trial, designed to study the effects of vitamin D supplementation on plasma renin activity in stable chronic HF patients with reduced ejection fraction. In total, 101 patients were enrolled. Details of this study have been described in detail elsewhere.[12]

Healthy controls (PREVEND)

To compare serum levels of IC ligands with healthy controls, a suitable control group was composed from the Prevention of Renal and Vascular End-Stage Disease (PREVEND) study. This cross-sectional study was designed to determine the natural course of microalbuminuria in non-diabetic patients and its relation to new-onset renal and cardiovascular disease, as described in detail before.[13] In total, serum samples of 8592 subjects were available and were stored at −80°C until further analysis. For the present study, subjects were excluded if they met one of the following criteria: History of cardiovascular disease (CV) or new-onset CV disease during follow-up, history of cancer or new-onset cancer during follow-up, and a history of renal disease requiring dialysis or estimated glomerular filtration rate (eGFR) $<$60 mL/min/1.73 m^2 during follow-up. Out of the remaining 3636 subjects, 58 age- and sex-matched individuals were randomly selected.

Worsening heart failure (BIOSTAT-CHF)

To study the prognostic value of IC ligands in different stages of HF, data of worsening HF patients from The BIOlogy Study to Tailored Treatment in Chronic Heart Failure (BIOSTAT-CHF) were used. Design and primary results of BIOSTAT-CHF have been described in detail elsewhere.[14,15] In brief,

BIOSTAT-CHF was a multicentre, prospective, observational study in which 2516 patients with new-onset or worsening HF were included from 11 European countries, considered to be on suboptimal HF medication. In this cohort, plasma levels of sPD-L2 and galectin-9 were available and measured using an immuno-assay based on proximity extension assay technology (Olink Bioscience analysis service, Cardiovascular panel II, Uppsala, Sweden). Data generated are expressed as relative quantification on the log2 scale of normalized protein expression (NPX) values. For the present study, patients were excluded from further analyses if plasma concentrations of IC ligands were not measured. Final analyses included 2032 patients.

Ethics

Mouse studies were approved by the animal ethical committee of the University of Groningen. The VitD-CHF, PREVEND, and BIOSTAT-CHF studies all conform to the principles drafted in the *declaration of Helsinki*. All study participants provided written informed consent.

Statistical analyses

Associations of IC ligands with baseline characteristics were assessed with linear regression analyses using data from the stable outpatient HF cohort (VitD-CHF). IC ligands and biomarker levels were log-transformed prior to analysis to obtain approximately normal distributions. For multivariable regression analysis, all variables with $P < 0.05$ in univariable analysis were included and subjected to the backward elimination method. Biomarker performance was assessed using cox regression analyses, with the composite of HF rehospitalization and all-cause mortality as primary outcome. Analyses in the worsening HF cohort were adjusted for the database-specific risk model,[15] consisting of age, HF hospitalization in last year, presence of peripheral oedema, systolic blood pressure, NT-proBNP, haemoglobin, HDL, sodium and beta-blocker use at baseline (referred to as 'Model 1'), and a second model that consisted of the database-specific risk model, including device therapy and estimated glomerular filtration rate (eGFR) (referred to as 'Model 2'). All analyses were conducted using Stata 14.2 and GraphPad Prism 9.1.0, with 2-tailed significance set at $P < 0.05$.

Results

Transcriptomic analyses

To determine if IC receptors and their ligands are expressed specifically in cardiac tissue, transcriptomic analyses of left ventricular tissue samples from three different HF mouse models (i.e. PLN-R14del, TAC, and MI) was performed. Data revealed differentially expressed IC receptors and their ligands compared with control mice (*Supporting information, Figure S1*).

Patient characteristics

Baseline characteristics of the 101 stable and 2032 worsening HF patients are presented in *Table 1*. In the stable HF cohort, mean age was 64 ± 10 years and 7 (7%) patients were female. Sixty-seven patients (66%) showed reduced ejection fraction [left ventricular ejection fraction (LVEF) <40] and 1 (1%) preserved ejection fraction (LVEF ≥50). Eighty-nine patients (88%) were New York Heart Association (NYHA) Class II and 12 (12%) NYHA Class III. Median NT-proBNP level was 376 (203–782) ng/L. Mean follow-up time was 4.3 (*SD* 1.4) years. At 4 year follow-up, 11 (11%) patients were rehospitalized for HF and 17 (17%) patients died of any cause.

In the worsening HF cohort, mean age was 69 ± 12 years, and 538 (27%) patients were female. There were 1464 patients (81%) that showed reduced EF and 121 (7%) preserved EF. The majority of patients could be classified as NYHA Class II and III and median NT-proBNP level was 2677 (1200–5620) ng/L. Mean follow-up was 1.7 (*SD* 0.8) years. At 2 year follow-up, 493 (24%) patients were rehospitalized for HF and 480 (24%) patients died of any cause.

Serum immune checkpoint ligand levels in patients with heart failure

In patients with stable HF, serum levels of sPD-L1, sPD-L2 and galectin-9 were higher compared to age- and sex-matched healthy individuals (*Figure 1A*). All three IC ligands were associated with disease severity, as reflected by New York Heart Association (NYHA) classification (*P*-value for trend 0.003, 0.043, and 0.003 for sPD-L1, sPD-L2, and galectin-9, respectively) (*Figure 1A*). In multivariate linear regression analyses, all three IC ligands were significantly associated with galectin-3, a marker of cardiac remodelling (β = 0.230, β = 0.283, and β = 0.304; P = 0.012, P = 0.004 and P = 0.001, respectively) (*Table 2*). sPD-L1 and galectin-9 were furthermore associated with high-sensitivity cardiac troponin-T (hs-troponin-T), a marker of cardiomyocyte damage (β = 0.386, $P < 0.0001$ and β = 0.314, P = 0.001). Also in patients with worsening HF, serum sPD-L2 and galectin-9 levels were associated with NYHA classification (*P* value for trend <0.001 for both, data not shown).

Table 1 Baseline characteristics preceding incident clinical outcomes in the stable and worsening heart failure cohort.

Characteristics	Stable HF cohort $n = 101$	Worsening HF cohort $n = 2032$
Age (years), mean (*SD*)	64 (10)	69 (12)
Female sex, *n* (%)	7 (7)	538 (27)
BMI (kg/m^2), mean (*SD*)	28 (4)	28 (6)
SBP	118 (18)	125 (22)
DBP	72 (13)	75 (13)
LVEF (%), mean (*SD*)	35 (8)	31 (11)
HF subtype, *n* (%)		
HFpEF	1 (1)	121 (7)
HFmrEF	33 (33)	229 (13)
HFrEF	67 (66)	1464 (81)
NYHA class, *n* (%)		
I		174 (9)
II	89 (88)	937 (46)
III	12 (12)	572 (28)
IV		67 (3)
Biomarker levels		
NT-proBNP (ng/L), median [IQR]	376 [203–782]	2677 [1200–5620]
Galectin-3 (μg/L), median [IQR]	17 [15–19]	21 [15–29]
Creatinine (μmol/L), mean (SD)	90 (18)	115 (55)

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; HFmrEF, heart failure with mildly-reduced ejection fraction; HFrEF, heart failure with reduced ejection fraction; IQR, inter-quartile range; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro-B-type natriuretic peptide; NYHA, New York Heart Association; SBP, systolic blood pressure.

Prognostic value of immune checkpoint ligands in patients with heart failure

In the stable HF cohort, serum sPD-L1 and galectin-9 levels significantly predicted HF hospitalization and all-cause mortality [unadjusted HR 1.68; CI (1.09, 2.59) and HR 1.50; CI (1.06, 2.12), *Figure 1B*, upper panel]. This also applied to patients with worsening HF: Both sPD-L2 and galectin-9 showed to be independent predictors for adverse outcome with an increased relative risk of 15% and 20% for HF hospitalization and all-cause mortality combined, respectively, after adjustment for the database-specific risk model (Model 1) (*Figure 1B*, lower panel).

Discussion

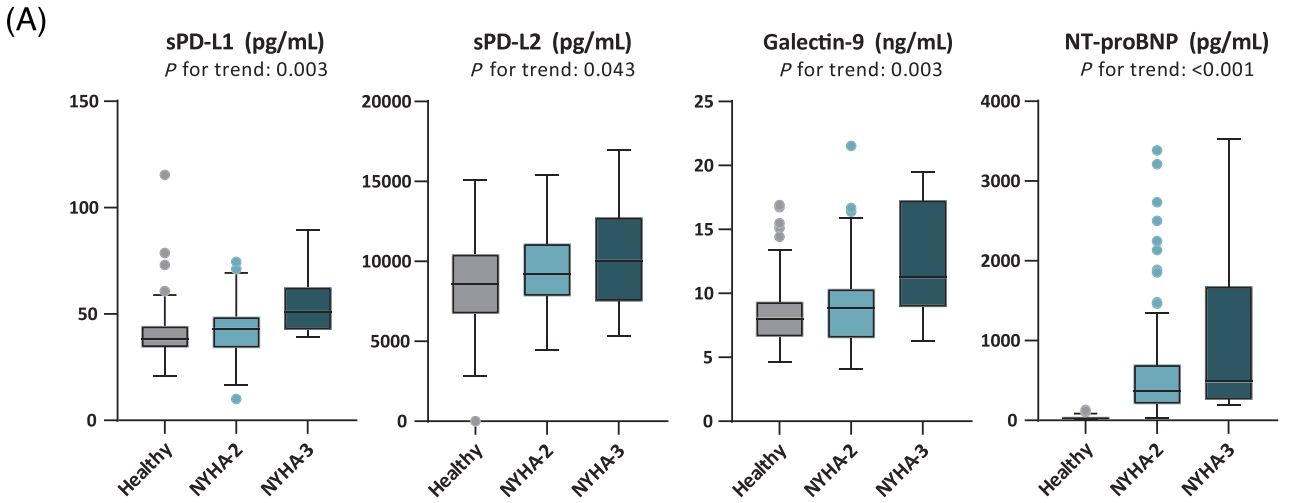
In this study, we report for the first time that serum levels of circulating IC ligands are elevated in HF and are associated with disease severity. sPD-L1, sPD-L2, and galectin-9 all strongly correlate with galectin-3 in multivariate analyses, indicating a clear association with myocardial fibrosis and inflammation,[16,17] underpinning hallmark processes of myocardial remodelling in HF. sPD-L1, and galectin-9 were also associated with hs-troponin-T, a reflection of ongoing myocardial injury. Additionally, higher levels of IC ligands are independently associated with a higher risk of death and HF hospitalization in patients in different stages of HF, namely, stable and worsening HF. Finally, transcriptomic analyses revealed differentially expressed IC ligands in cardiac tissue specifically, supporting our hypothesis that IC ligands potentially play a role in cardiac biology and HF pathology.

The IC receptor/IC ligand interactions play a critical role in immune regulation. In cardiac biology, previous clinical and pre-clinical studies have shown that IC ligands, such as PD-L1/2 and galectin-9, are significantly up-regulated in inflammatory heart disease[3] and in hearts of patients after heart transplant rejection.[18] Intriguingly, our study results indicate a potential role for IC ligands across the overall spectrum of HF—irrespective of evident auto-immunity or infection—further implicating the importance of IC ligands in the pathophysiology of heart disease in general.

While normally IC ligands are expressed on 'host' cells including cardiomyocytes or endothelial cells,[19] the circulating forms (such as sPD-L1, sPD-L2, and galectin-9) are acknowledged as functional parts of membrane-bound IC ligands that regulate immune activity in a similar fashion. In oncology, changes in plasma levels of circulating IC ligands have been shown to affect development, prognosis, and treatment of several types of cancer.[20] Our study extends these observations to HF.

Despite these results, it remains unclear whether increased levels of circulating IC ligands represent a pathological or compensatory process. Nor does this study define the cell types or organs responsible for this up-regulation—although our results indicate that the heart might be a possible source. Nevertheless, our data implicate a potential role for IC ligands as biomarkers in HF. Further research is needed to elucidate the exact mechanism of IC ligands in the pathophysiology of HF and their potential as target for therapy.

Figure 1 Association of circulating immune checkpoint (IC) ligands with disease severity and prognosis. (*A*) Levels of circulating ICR ligands in healthy subjects (*n* = 58) and patients with stable heart failure (HF) (*n* = 101), categorized by NYHA II and III. NT-proBNP levels are depicted as a positive control. Levels are displayed as Tukey boxplot [median (inter-quartile range)]. (*B*) Forest plot showing the unadjusted hazard ratio (95% CI) associated with circulating IC ligands per 1 log-*SD* increase to the primary combined endpoint (HF hospitalization and all-cause mortality) in stable HF (upper panel) and worsening HF (lower panel) cohort. Data from the latter cohort are adjusted for Model 1 and Model 2. Model 1: The database-specific risk model, consisting of age, HF hospitalization in last year, presence of peripheral oedema, systolic blood pressure, NT-proBNP, haemoglobin, HDL, sodium, and beta-blocker use at baseline. Model 2: The database-specific risk model + device therapy + estimated glomerular filtration rate. Abbreviations: BIOSTAT-CHF, The BIOlogy Study to Tailored Treatment in Chronic Heart Failure; CI, confidence interval; HF, heart failure; HR, hazard ratio; NYHA, New York Heart Association; NT-proBNP, N-terminal pro-B-type natriuretic peptide; sPD-L1, soluble programmed death-ligand 1; sPD-L2, soluble programmed death-ligand 2; Vit-D-CHF, Vitamin D Chronic Heart Failure study.



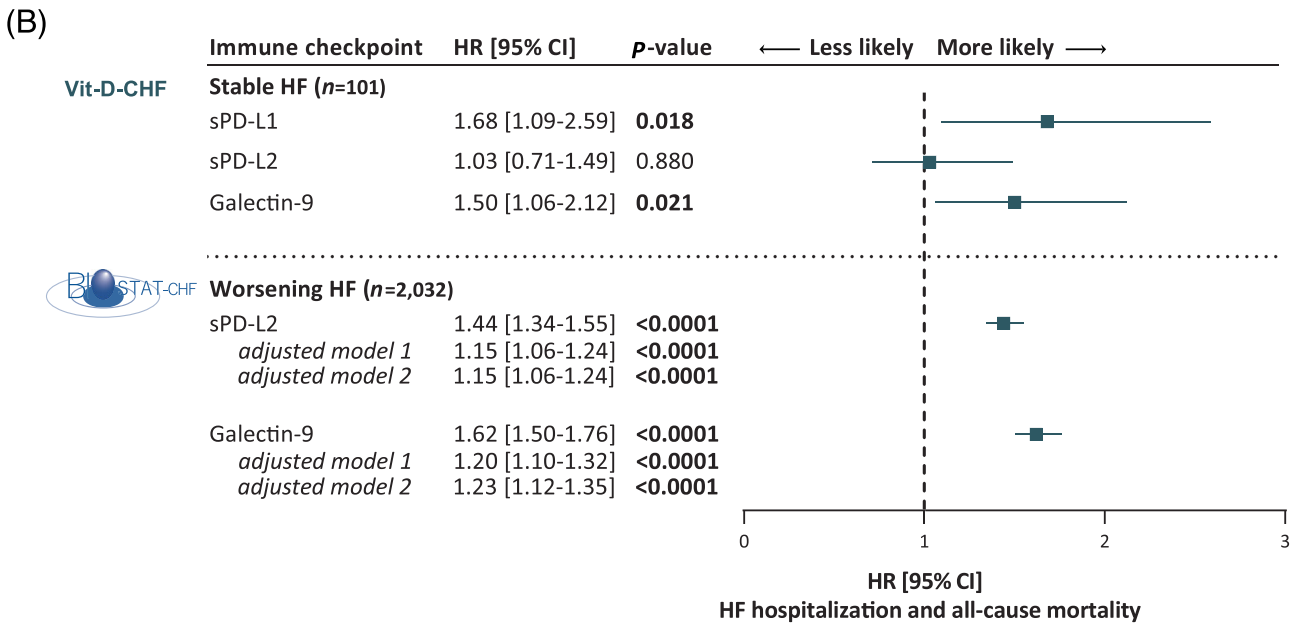


Table 2 Univariable and multivariable linear regression analysis of baseline characteristics with serum levels of circulating immune checkpoint ligands in heart failure patients.

	sPD-L1				sPD-L2				Galectin-9			
	Univariable Sβ	*P* value	Multivariable Sβ[a]	*P* value	Univariable Sβ	*P* value	Multivariable Sβ[a]	*P* value	Univariable Sβ	*P* value	Multivariable Sβ[a]	*P* value
Characteristics												
Age	0.250	**0.012**			0.020	0.839			0.324	**0.001**		
Female sex	−0.040	0.666			−0.064	0.526			−0.023	0.818		
BMI	0.163	0.103			0.049	0.630			0.309	**0.002**	0.179	**0.044**
LVEF	−0.005	0.961			0.077	0.447			−0.112	0.263		
Medical history												
Ischaemic aetiology	−0.040	0.694			0.139	0.167			0.044	0.666		
Diabetes mellitus	0.105	0.294			0.193	0.053			0.225	**0.024**		
Hypertension	0.245	**0.013**			0.035	0.726			0.279	**0.005**		
Hypercholesterolemia	−0.205	**0.040**			−0.206	**0.039**			−0.178	0.074		
Obesity	0.228	**0.022**			0.062	0.535			0.238	**0.017**		
Laboratory measurements												
NT-proBNP	0.326	**0.001**			0.210	**0.036**			0.235	**0.019**		
hs-troponin-T	0.437	<**0.0001**	0.386	<**0.0001**	0.145	0.149			0.422	<**0.0001**	0.314	**0.001**
Galectin-3	0.316	**0.001**	0.230	**0.012**	0.283	**0.004**	0.283	**0.004**	0.408	<**0.0001**	0.304	**0.001**
Creatinine	0.273	**0.006**			0.216	**0.030**			0.232	**0.020**		
Medication												
Diuretics	0.155	0.122			0.091	0.363			0.328	**0.001**		
Betablocker	−0.003	0.978			0.171	0.087			−0.026	0.794		
ACEi/ARB	N/A[b]				N/A[b]				N/A[b]			
MRA	0.090	0.370			0.148	0.140			0.238	**0.017**		

Abbreviations: ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; BMI, body mass index; LVEF, left ventricular ejection fraction; MRA, mineralocorticoid receptor antagonist; N/A, not applicable; NT-proBNP, N-terminal pro-B-type natriuretic peptide; sPD-L1, soluble programmed death-ligand 1; sPD-L2, soluble programmed death-ligand 2.

Note: All IC ligands and biomarkers were log-transformed prior to analysis, to obtain approximately normal distributions. Sβ, standardized beta coefficient, a reflection of the change in dependent variable for 1 log-*SD* change in the independent variable. Bold values denote statistical significance at the $P < 0.05$ level.

[a]For multivariable regression analysis, all variables depicted in the table with $P < 0.10$ in univariable analysis were included and subjected to the backward elimination method.

[b]Linear regression analysis between circulating IC ligands and ACE inhibitor or ARB use was not applicable, since all HF patients (100%) were treated with this medication.

Study limitations

Follow-up time of the worsening HF cohort was considerably shorter compared with the stable HF cohort. Considering that IC ligands concentrations—which might fluctuate over time—were only determined at one time point during the study, a follow-up duration of more than 1 year was considered sufficient to determine their prognostic value.

Conflict of interest

The UMCG, which employs/employed 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. Dr Moslehi received fees from Pfizer, Novartis, Bristol-Myers Squibb, Deciphera, Audentes Pharmaceuticals, Nektar, Takeda, Ipsen, Myokardia, AstraZeneca, GlaxoSmithKline, Intrexon, and Regeneron, and is supported by R01 HL141466. Dr Salem participated to BMS advisory boards. Dr Voors has received consultancy fees and/or research grants from Amgen, AstraZeneca, Bayer, Boehringer Ingelheim, Cytokinetics, Merck, Myokardia, Novartis, Novo Nordisk, and Roche Diagnostics. Dr de Boer received speaker fees from Abbott, AstraZeneca, Bayer, Novartis, and Roche. The remaining authors declare no competing interests.

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Supporting information

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

Figure S1. Heatmaps showing transcriptomic analyses of IC receptors and IC ligands in left ventricular tissue of three different HF mouse models, namely genetic cardiomyopathy (PLN-R14del), left ventricular pressure overload (TAC) and MI. Each coloured square represents one individual mouse. Abbreviations: *Cd40*, cluster of differentiation 40; *Cd80*, B7–1; *Cd86*, B7–2; *Gitr*, glucocorticoid-induced TNFR-related gene; *Gitrl*, glucocorticoid-induced TNF-related protein ligand; HF, heart failure; *Lgals3*, galectin-3; *Lgals9*, galectin-9; MI, myocardial infarction; *Nppa*, natriuretic peptide A; *Nppb*, natriuretic peptide B; *Pdcd1lg1*, programmed cell death 1 ligand 1; *Pdcd1lg2*, programmed cell death 1 ligand 2; TAC, transverse aortic constriction; *Tim3*, T-cell immunoglobulin and mucin-domain containing-3; WT, wild-type.

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