


Receptor autoantibodies: Associations with cardiac markers, histology, and function in human non-ischaemic heart failure

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Abstract

Aims A causal link between non-ischaemic heart failure (HF) and humoral autoimmunity against G-protein-coupled receptors (GPCR) remains unclear except for Chagas' cardiomyopathy. Uncertainty arises from ambiguous reports on incidences of GPCR autoantibodies, spurious correlations of autoantibody levels with disease activity, and lack of standardization and validation of measuring procedures for putatively cardio-pathogenic GPCR autoantibodies. Here, we use validated and certified immune assays presenting native receptors as binding targets. We compared candidate GPCR autoantibody species between HF patients and healthy controls and tested associations of serum autoantibody levels with serological, haemodynamic, metabolic, and functional parameters in HF.

Methods Ninety-five non-ischaemic HF patients undergoing transcatheter endomyocardial biopsy and 60 healthy controls were included. GPCR autoantibodies were determined in serum by IgG binding to native receptors or a cyclic peptide (for β_1 AR autoantibodies). In patients, cardiac function, volumes, and myocardial structural properties were assessed by cardiac magnetic resonance imaging; right heart catheterization served for determination of cardiac haemodynamics; endomyocardial biopsies were used for histological assessment of cardiomyopathy and determination of cardiac mitochondrial oxidative function by high-resolution respirometry.

Results Autoantibodies against β_1 adrenergic (β_1 AR), M5-muscarinic (M5AR), and angiotensin II type 2 receptors (AT2R) were increased in HF (all $P < 0.001$). Autoantibodies against α_1 -adrenergic (α_1 AR) and angiotensin II type 1 receptors (AT1R) were decreased in HF (all $P < 0.001$). Correlation of alterations of GPCR autoantibodies with markers of cardiac or systemic inflammation or cardiac damage, haemodynamics, myocardial histology, or left ventricular inflammation (judged by T2 mapping) were weak, even when corrected for total IgG. β_1 AR autoantibodies were related inversely to markers of left ventricular fibrosis indicated by T1 mapping ($r = -0.362$, $P < 0.05$) and global longitudinal strain ($r = -0.323$, $P < 0.05$). AT2R autoantibodies were associated with improved myocardial mitochondrial coupling as measured by high-resolution respirometry in myocardial biopsies ($r = -0.352$, $P < 0.05$). In insulin-resistant HF patients, AT2R autoantibodies were decreased ($r = -0.240$, $P < 0.05$), and AT1R autoantibodies were increased ($r = 0.212$, $P < 0.05$).

Conclusions GPCR autoantibodies are markedly altered in HF. However, they are correlated poorly or even inversely to haemodynamic, metabolic, and functional markers of disease severity, myocardial histology, and myocardial mitochondrial efficiency. These observations do not hint towards a specific cardio-pathogenic role of GPCR autoantibodies and suggest that further investigations are required before specific therapies directed at GPCR autoantibodies can be clinically tested in non-ischaemic HF.

Keywords Heart failure; Pathophysiology; Autoimmunity; Chronic non-ischaemic heart failure

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Introduction

Chronic heart failure (CHF) is a main cause for hospitalization and death in Western countries. Roughly one-third of the cases is due to non-ischaemic myocardial damage caused by genetic predisposition, metabolic stress, and, most notably, myocardial inflammation.¹ Inflammatory CHF is associated with abnormal or misled immune responses and with humoral autoimmunity against various antigens involved in energy metabolism and cardiovascular regulation.^{2–4} Heart-reactive autoantibodies potentially contribute to cardio-pathogenesis because CHF-like symptoms can be transferred from patients to mice via B lymphocytes.⁵ Consequently, B cells and autoantibodies are emerging targets in CHF therapy.^{6–10}

Among various heart-reactive autoantibodies discussed in the above context, autoantibodies against cardiac G-protein-coupled receptors (GPCRs) are candidates that have been studied earlier.^{2,11,12} CHF-associated GPCR autoantibodies are intriguing candidates because they impact receptor-mediated autonomous heart regulation and thereby damage the heart.¹³ Evidences of GPCR-directed/GPCR-mediated humoral autoimmune cardio-pathogenesis have been obtained *ex vivo* and by experimental immunization. These include aberrant activation of signalling pathways,^{14,15} receptor de-/hyper-sensitization,¹⁶ induction of cardiomyocyte apoptosis,¹⁷ and pro-fibrotic stimulation of cardiac fibroblasts.^{13,18} Clinical studies of non-ischaemic CHF patients have shown that activating GPCR autoantibodies (targeting most notably β_1 AR) are associated with a poorer cardiac function,¹⁹ a poorer disease outcome,²⁰ a higher incidence of atrial fibrillation,^{21,22} and a higher incidence of sudden heart death.²³ In one case, appearance of such autoantibodies even preceded clinical symptoms by several years.²² Moreover, removal^{24,25} or neutralization of IgG²⁶ can improve haemodynamics in CHF patients.

However, in clinical medicine, a pathogenic role of humoral autoimmunity against β_1 AR and other GPCR in non-ischaemic, inflammatory CHF is not unequivocally accepted for several reasons: (i) GPCR autoantibodies are also associated with a variety of human diseases not involving CHF or cardiac injury^{27,28}; (ii) in certain autoimmune diseases, GPCR autoantibodies are thought to play a physiological or even protective role²⁹; (iii) incidence and circulating levels of CHF-associated GPCR autoantibodies do not stringently correlate with biomarkers of cardiac injury³⁰ and (iv) it remains unclear which autoantibody species is targeted by the beneficial effects of IgG removal or neutralization in CHF.^{31–33}

Hesitations regarding the assumption of a specific pathogenetic role of GPCR autoantibodies for the heart is in part due to an analytical issue: Only GPCR autoantibodies that stimulate the receptors or otherwise modulate their function^{13,16} are believed to exert the pathogenic effects.^{2,34} However, assessment of such functional effects is impractical in the setting of clinical studies because it requires functional tests or immune assays employing native receptors or other immunological targets faithfully mimicking the pathogenic conformational receptor autoepitope.^{35–37} Consequently, many clinical studies published so far on GPCR autoantibodies in CHF and other diseases have instead assessed GPCR autoantibody via IgG-binding to linear immobilized peptides, a procedure demonstrated to be unsuitable to detect functional GPCR autoantibodies.³⁵ Thus, it is quite possible that many published clinical studies available to date may have altogether overlooked the pathogenetically relevant species of GPCR autoantibodies.^{36,37}

Meanwhile, assays appropriate for the determination of antibodies targeting presumably cardio-pathogenic conformational epitopes have been certified for clinical use.³⁸ Our study is one of the first making use of these assays on a broad basis. It encompasses all species of GPCR autoantibodies that are currently suspected to play a role in CHF and cardiovascular dysfunction.^{2,27} Furthermore, to better understand a putative pathophysiological role of GPCR autoantibodies, we have investigated their association with histological, haemodynamic, and metabolic parameters of disease activity obtained by deep phenotyping of the patients with non-ischaemic CHF. We compare circulating levels of presumably disease-relevant GPCR autoantibodies with a plethora of patient data on inflammation, cardiac injury, cardiac fibrosis, haemodynamics, metabolism, and myocardial mitochondrial function, which comprehensively delineate disease activity, progression, and prognosis of non-ischaemic HF.

Methods

Study protocol

These registered clinical trials' protocols were approved by the local ethics board of Heinrich-Heine University Düsseldorf (study number 5263R and 3786) and registered at ClinicalTrials.gov (NCT03386864). The investigation conforms with the principles outlined in the World's Medical Association *Declaration of Helsinki*. Before inclusion, all participants have given written informed consent.

Patient enrolment

We included 95 patients with recently diagnosed heart failure (HF) of unknown origin at the University Hospital Düsseldorf. Patients scheduled for transcatheter endomyocardial biopsy were invited to participate in the study. Patient surveillance in the course of routine clinical healthcare included transthoracic echocardiography, cardiac magnetic resonance imaging (whenever feasible), coronary angiography, and right heart catheterization with endomyocardial biopsy.

A cohort of 60 healthy humans served as controls in this study. Exclusion criteria included systemic antibiotic treatment within 6 months prior to study enrolment, history of endocarditis, bleeding disorders, organ transplantation, dialysis, pregnancy, and lactation. Absence of HF at the time of blood drawing was in addition confirmed by determination of serum levels of N-terminal pro-brain natriuretic peptide (pBNP) at <125 ng/L and troponin T (TpT) at <4 ng/L. Absence of chronic systemic inflammation was ascertained by determination of serum levels of C-reactive protein (CRP) at <5 mg/L and interleukin 6 (IL-6) at <7 ng/L.

None of the study participants (patients or controls) had a history of Chagas' disease and/or had lived in areas where Chagas' disease was endemic within the past 20 years.

Cardiac magnetic resonance imaging

In 39 participants, cardiac magnetic resonance (CMR) was conducted on a 1.5-T scanner (Achieva, Philips, Best, Netherlands). In addition to standard protocols for assessment of ventricular volumes and function, including global longitudinal strain (GLS) and diastolic strain rate, left ventricular T2 relaxation time reflecting myocardial oedema and inflammation was quantified using the Gradient and SpinEcho (GraSE) sequence as described previously.³⁹ T1 relaxation time reflecting diffuse myocardial fibrosis was quantified according to current recommendations.⁴⁰

Catheterization procedures and endomyocardial biopsies

Right heart catheterization was performed to measure right atrial pressure (RAP), pulmonary artery pressure (PAP), and pulmonary capillary wedge pressure (PCWP). Afterwards, endomyocardial biopsies were taken from the interventricular septum. One additional biopsy (1–2 mg) was taken for respirometry.

High-resolution respirometry

Respirometry was carried out using the Oxygraph-2k (OROBOROS Instruments, Innsbruck, Austria) as recently established and described in detail.⁴¹ Oxidative phosphorylation capacity (OXPHOS) was measured for octanoyl-carnitine, glutamate, and succinate. The leak control ratio was calculated as the ratio of the LEAK state induced by addition of oligomycin divided by electron transfer system capacity.^{42,43} Integrity of the outer mitochondrial membrane was confirmed by assessing respiration increase after cytochrome C addition by less than 10%.

Measurement of GPCR antibodies in serum samples

Antibodies against α_1 - and β_1 -adrenergic receptors, the muscarinic acetylcholine receptor 5, the receptor for complement C5a, angiotensin II receptor types 1 and 2, endothelin receptors A and B, and ACE were measured with commercially available immunoassays (CellTrend GmbH, Luckenwalde, Germany) according to the instructions of the manufacturer. These assays provide native receptors presented in their physiological membrane environment as immunogenic targets for autoantibodies and were calibrated with polyclonal standard sera, yielding quantitative values of autoantibody levels expressed in arbitrary units/mL. Antibodies against β_1 -adrenergic receptors (β_1 AR-Aabs, the most prominent candidate) were determined by IgG binding to a cyclic peptide providing a valid representation of the conformational epitope within the second extracellular loop of the receptor associated with the active receptor conformation. It has been demonstrated that pre-absorption with this peptide neutralizes the cardio-pathogenic potency of stimulatory receptor antibodies in immunized mice.³⁸ The cyclic peptide was coated onto microtitre plates by established procedures, and these plates were processed in a similar manner as the above commercial immunoassays. It was calibrated with a humanized mouse monoclonal antibody against the autoepitope (kindly provided by Drs Holthoff and Ungerer, AdvanceCor, Munich, Germany), allowing expression of autoantibody levels in ng IgG/mL. The two assays for β_1 AR-Aabs exhibited a reasonable correlation (Figure S1) with a few outliers, which are explained by the presence of β_1 AR-Aabs not directed against the second extracellular loop of the receptor. It should be noted, however, that some correlations with corroborative clinical data such as left ventricular T1-relaxation times and myocardial mitochondrial leak control ratio were only seen with levels of β_1 AR autoantibodies determined by the cyclopeptide assay (Table S1).

Statistical analyses

Quantitative measurements of receptor autoantibodies were optionally normalized to total IgG levels of the respective patients' serum by a simple division and subsequent ($\log + 1$) transformation. The influence of confounders was tested for each antibody species in a separate general linear model. All statistical analyses were carried out using GraphPad Prism version 9.1.2 (GraphPad Software, San Diego, CA, USA) and SPSS Statistics 25.0.0.2 (International Business Machines Corporation, Armonk, NY, USA). The statistical significance threshold was $\alpha = 0.05$. The homeostasis model assessment was used to estimate the participants' insulin resistance (HOMA-IR) based on fasting insulin and glucose concentrations.⁴³

Results

Basal characteristics

Characteristics of the two groups are summarized in *Table 1*. A total of 155 participants were recruited for the study. Ninety-five of these were HF patients (HF group) undergoing

endomyocardial biopsy. Advanced left-sided HF within the HF group was established by cardiac index (2.17 ± 0.62 L/min/m²), left ventricular ejection fraction ($28.95 \pm 10.26\%$), and elevated pulmonary artery pressures (18.71 ± 8.55 mmHg). The remaining 60 were healthy humans, who tended to be slightly older and comprised more females. In the HF group, supra-normal serum levels of CRP and IL-6 indicated evidence for mostly subclinical or low-grade systemic inflammation, whereas in the control group, total serum IgG was higher. Of note, the groups also differed in biomarkers of kidney function and cardiac injury.

HF-associated differences in circulating levels of GPCR autoantibodies

Out of all GPCR autoantibodies here examined, significant differences between the HF group and the control group were observed for autoantibodies against β_1 AR, M5R, α_1 AR, AT1R, and AT2R, but not for antibodies against component 5a receptor, angiotensin converting enzyme II receptor, Endothelin A receptor or Endothelin B receptor. As illustrated in *Figure 1*, levels of β_1 AR, M5R, and AT2R autoantibodies were higher in the HF group than in the control group, whereas levels of α_1 AR and AT1R autoantibodies were lower

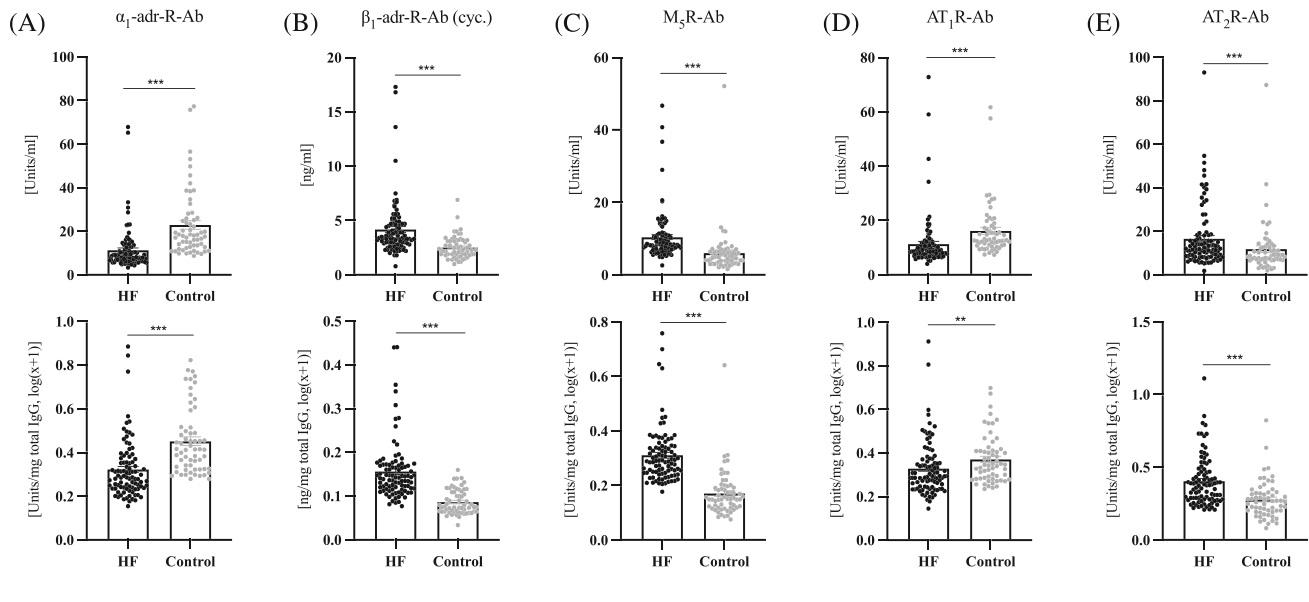
Table 1 Patient characteristics

	Heart failure	Control	Total	P
Overall, n	95	60	155	
Anthropometry				
Male, %	74	47	63	0.001
Age, y	56.7 \pm 12.1	59.1 \pm 13.6	57.6 \pm 12.7	0.069
Cardiac measurements				
Cardiac index, L/min/m ²	2.17 \pm 0.62	-	-	-
EF, %	28.95 \pm 10.26	-	-	-
RA (M), mmHg	7.87 \pm 5.64	-	-	-
PA (M), mmHg	27.64 \pm 10.68	-	-	-
PA (sys), mmHg	42.10 \pm 14.63	-	-	-
PA (dia), mmHg	17.93 \pm 8.50	-	-	-
PCWP, mmHg	18.71 \pm 8.55	-	-	-
Cardiac MRI				
T1 relaxation time, msec	1058 \pm 74.16	-	-	-
T2 relaxation time, msec	58.80 \pm 10.90	-	-	-
Laboratory results				
Creatinine (serum), mg/dL	1.22 \pm 1.03	0.78 \pm 0.15	1.04 \pm 0.84	<0.001
Creatine kinase, U/L	75.74 \pm 58.17	84.85 \pm 74.93	79.31 \pm 65.17	0.0330
Total protein, g/dL	14.27 \pm 75.87	7.39 \pm 0.63	11.58 \pm 59.12	<0.001
Total IgG, mg/dL	955.0 \pm 306.3	1139 \pm 230.2	1028 \pm 292.0	<0.001
Troponin, ng/L	64.38 \pm 59.14	3.64 \pm 4.03	40.87 \pm 54.97	<0.001
nt-proBNP, pg/mL	8075 \pm 36 169	44.54 \pm 34.01	4966 \pm 28 529	< 0.001
IL-6, pg/mL	15.97 \pm 44.89	1.71 \pm 0.75	10.45 \pm 35.76	< 0.001
Cystatin C, mg/L	1.40 \pm 0.81	0.80 \pm 0.12	1.16 \pm 0.70	< 0.001
CRP, mg/dL	1.96 \pm 5.94	0.19 \pm 0.40	1.27 \pm 4.72	< 0.001
Medication				
Beta-blockers, %	94	-	-	-
AT ₁ -receptor antagonists, %	12	-	-	-
Angiotensin receptor-neprilysin inhibitor, %	24	-	-	-

Patient characteristics of the heart failure and the control group. Data given as mean \pm SD, P calculated using unpaired t test or Mann-Whitney test.

CRP, C-reactive protein; EF, ejection fraction; IgG, immunoglobulin G; IL-6, interleukin 6; nt-proBNP, n-terminal pro-brain natriuretic peptide; PA, pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; RA, right atrial pressure.

Figure 1 Levels of GPCR-Abs in the heart failure group and the control group. Top row: Native data. Bottom row: Data normalized to total IgG and log (x + 1) transformed. HF, heart failure; IgG, immunoglobulin G. (A) α_1 -Adrenergic receptor antibodies. (B) β_1 -Adrenergic receptor antibodies, measured via cyclic peptide assay. (C) Muscarinic receptor M₅ antibodies. (D) Angiotensin II receptor type 1 antibodies. (E) Angiotensin II receptor type 2 antibodies. ** $P \leq 0.01$, *** $P \leq 0.001$ vs. control group. P calculated using Mann–Whitney test. $n = 95$ – 91 vs. $n = 60$.



in the HF group than in the control group. All these differences were highly significant at $P < 0.0001$. The above findings could be replicated in each of the following settings: (i) in non-transformed raw data; (ii) in IgG-normalized data; (iii) in IgG-normalized and log-transformed data; and (iv) in IgG-normalized and log-transformed data corrected for age and gender in linear regression models.

Subsequently, we divided the HF cohort according to histopathology into subgroups of myocarditis ($n = 30$), dilated cardiomyopathy ($n = 29$), other cardiomyopathy ($n = 25$), and no evidence of pathology ($n = 11$). Neither the increases in β_1 AR, M5R, and AT2R autoantibodies nor the decreases of α_1 AR and AT1R autoantibodies were found clustered to any of these histopathological categories (Table S2).

Association of GPCR autoantibodies with biomarkers of cardiac injury and inflammation

GPCR autoantibody levels were quantitatively compared with serum markers of inflammation (IL-6 and CRP) or cardiac injury (pBNP and TpT). β_1 AR, M5R, and AT2R autoantibodies exhibited a positive correlation with pBNP, α_1 AR and AT1R autoantibodies exhibiting an inverse correlation with pBNP when we analysed the complete cohort consisting of HF and healthy patients (Figure 2). However, within the HF group, the above associations of GPCR autoantibody levels with pBNP or high-sensitive TpT were not detectable. Moreover, within the HF group, an apparent association of β_1 AR

autoantibody levels with IL-6 and CRP disappeared upon normalization to total IgG content (Table 2).

Association of GPCR autoantibodies with parameters of impaired haemodynamics

Within the HF group, we observed an inverse correlation between pulmonary vascular resistance and α_1 AR autoantibodies, which, however, disappeared upon normalization of the autoantibody levels to total IgG (Table S3). Apart from that, we failed to find any significant association of GPCR autoantibodies (unprocessed or IgG-normalized serum levels) with haemodynamic parameters of the patients including ejection fraction, cardiac index, PCWP, PAP, or RAP of the HF patients studied.

Associations of GPCR autoantibodies with parameters of cardiac structure and contractility

We compared serum levels of GPCR autoantibodies in the HF patient group with myocardial T2 mapping reflecting myocardial oedema and inflammation³⁹ and myocardial T1 mapping considered a marker of tissue fibrosis as well as lipid and iron accumulation.⁴⁴ These results are summarized in Table S4. Global left ventricular T1-relaxation times were significantly correlated to serum levels of M5R and β_1 AR autoantibodies. The inverse correlation of T1-mapping data with β_1 AR auto-

Figure 2 Association of GPCR-Abs and nt-proBNP; GPCR-Abs were normalized to total IgG and logarithmized. Red: Heart failure group. Blue: Control group. C, control; HF, heart failure; IgG, immunoglobulin G. Data given as Spearman rho. (A) α_1 -adrenergic antibodies. (B) β_1 -Adrenergic receptor antibodies, measured via cyclic peptide assay. (C) Muscarinic receptor M_3 antibodies. (D) Angiotensin II receptor type 1 antibodies. (E) Angiotensin II receptor type 2 antibodies. $n = 91$ vs. $n = 60$.

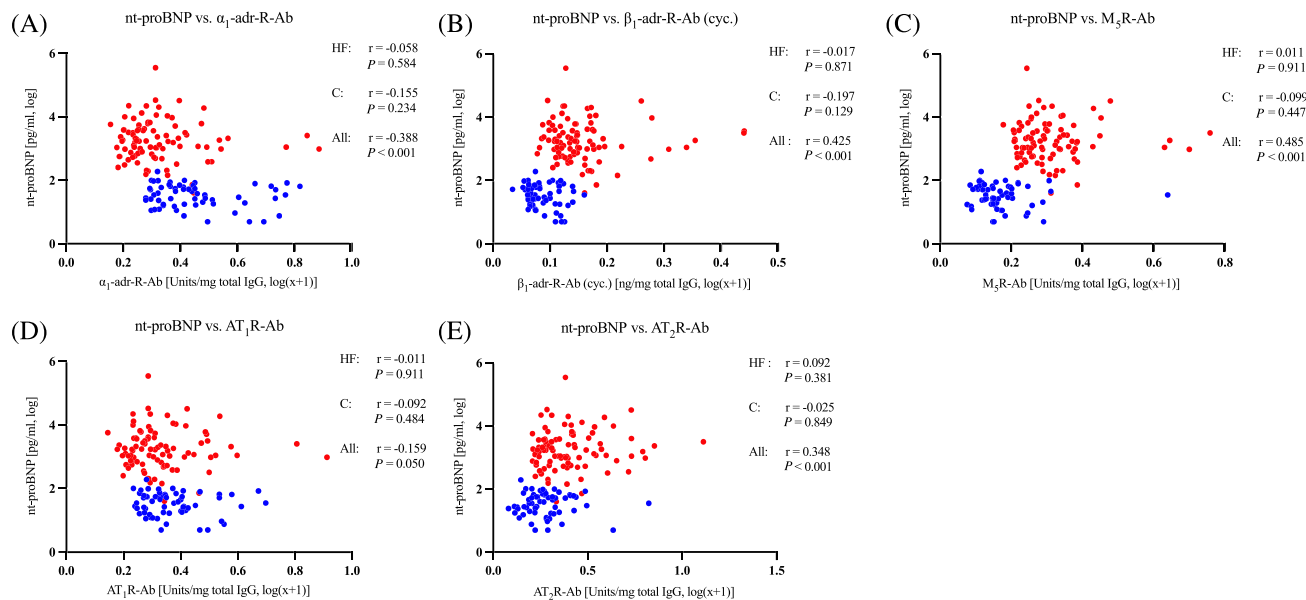


Table 2 GPCR antibodies and cardiac and inflammatory markers

	IL-6	CRP	Troponin	nt-proBNP
Panel A				
α_1 -adr-R-Ab	0.194	0.059	0.095	-0.026
β_1 -adr-R-Ab (cyc.)	0.327*	0.226*	0.084	-0.052
M_3 R-Ab	0.171	0.017	0.004	0.017
AT ₁ R-Ab	0.174	-0.000	0.043	0.013
AT ₂ R-Ab	0.139	-0.127	-0.029	0.105
Panel B				
α_1 -adr-R-Ab	0.053	-0.034	0.070	-0.021
β_1 -adr-R-Ab (cyc.)	0.184	0.116	0.185	-0.094
M_3 R-Ab	-0.009	-0.164	0.076	-0.003
AT ₁ R-Ab	0.020	-0.125	0.030	-0.008
AT ₂ R-Ab	0.054	-0.198	-0.014	0.095

Association of GPCR antibodies with IL-6, CRP, troponin, and BNP within the heart failure group. Panel A: Native data. Panel B: Antibodies normalized to total IgG and logarithmized. Data given as Spearman rho with * $P \leq 0.05$. $n = 95-90$. CRP, C-reactive protein; IL-6, interleukin 6; nt-proBNP, n-terminal pro-brain natriuretic peptide.

antibodies was retained after normalization to total IgG, but the correlation with M5R autoantibody levels disappeared upon normalization to total IgG. We failed to observe any correlations between the various GPCR autoantibody levels and T2-mapping data of the patients.

CMR-based left ventricular strain analyses indicated a weak association between elevated β_1 AR autoantibodies and impaired systolic GLS, as a marker of myocardial deformation (Table S4). Diastolic strain rate and left ventricular ejection fraction were not related to circulating autoantibodies.

Association of GPCR autoantibodies with serum markers of metabolic alterations

Within the HF group, 26 participants were normoglycaemic, 47 participants had prediabetes, and 22 participants had type 2 diabetes mellitus. Among the HF-associated GPCR autoantibodies, AT1R-AT2R autoantibodies exhibited associations with certain markers of metabolic alterations: AT1R autoantibody levels were correlated to circulating non-esterified fatty acids, and AT2R autoantibody levels were inversely related to insulin resistance, as assessed according to the homeostasis model (HOMA-IR). Furthermore, higher serum cholesterol and triacylglycerol related inversely to serum AT2R autoantibody levels. These associations were robust in as much as they were retained following normalization to total IgG (Table S5).

GPCR autoantibodies and myocardial mitochondrial function

Impaired mitochondrial function has been shown to play an important role in the development of HF.^{41,45} To follow up on possible interrelations between myocardial mitochondrial function and GPCR autoimmunity, we compared serum levels of HF-associated GPCR autoantibodies to data from high-resolution respirometry performed in corresponding myocardial specimen. There was no direct association of

the receptor autoantibodies with myocardial mitochondrial oxidative phosphorylation activity and electron transport capacity (Table S6). However, the leak control ratio, which is a marker of mitochondrial uncoupling, was related inversely to the IgG-normalized circulating antibodies of AT2R, M5R, and β_1 AR. Without IgG-normalization, the correlation of leak control ratio with M5R and β_1 AR autoantibody levels disappeared, whereas the correlation with AT2R autoantibody remained significant.

Discussion

The salient findings of this study are:

- 1 GPCR autoantibodies for α_1 AR, β_1 AR, M5R, AT1R, and AT2R differ between patients with non-ischaemic HF and healthy controls independent of total IgG when assessed with state-of-the-art assays.
- 2 In patients with non-ischaemic HF, increases in these autoantibodies do neither relate to histological diagnosis nor to cardiac haemodynamics, adverse prognostic parameters, or cardiac structural properties.
- 3 In patients with non-ischaemic HF, AT2R autoantibodies relate inversely to systemic insulin resistance (HOMA-IR) and myocardial mitochondrial uncoupling. M5R autoantibodies also relate to myocardial mitochondrial uncoupling but not to insulin resistance.

Key questions

Autoantibodies against adrenergic and muscarinic receptors are discussed as pathogenetic principle in chronic HF since more than four decades.^{46–50} The basic pathogenetic mechanism has been extensively demonstrated by *ex vivo* studies and animal models,^{13,15} and we know of a human disease (Chagas' cardiomyopathy) based thereon.⁵¹ However, published evidence regarding a putative role of humoral GPCR autoimmunity in human chronic HF other than Chagas' cardiomyopathy is inconsistent, and the measurements employed in most available clinical studies to determine potentially cardio-pathogenic GPCR autoantibodies are either impractical, not certified, and/or poorly reproducible^{52,53} or invalid.^{35–37}

Here, we aimed at a comprehensive re-assessment of the above issue in a cohort of thoroughly characterized, manifest HF patients of established aetiology (excluding Chagas' cardiomyopathy) using validated/certified and reproducible high-throughput assays³⁸ for a panel of GPCR autoantibodies currently suggested to play a role in HF.² Results thus obtained allow us to address three key questions: (i) Are any of the tested GPCR autoantibodies associated with HF? (ii)

Can serological patterns of such HF-associated GPCR autoantibodies distinguish non-ischaemic HF or even sub-entities thereof from the healthy condition? (iii) Are serum levels of HF-associated GPCR autoantibodies quantitatively correlated with established serological, haemodynamic, histological, radiological, and biochemical parameters of disease activity?

Association of imbalances of GPCR autoantibodies with non-ischaemic HF

We observed that circulating levels of autoantibodies against α_1 AR, β_1 AR, M5R, AT1R, and AT2R differed significantly between HF patients and healthy controls. These differences remained significant upon correction for total IgG. Among a broad panel of GPCR autoantibodies analysed simultaneously by comparable assays, only these five GPCR autoantibody species were altered in the patients. The above alterations were equally distributed between histological sub-entities of non-ischaemic HF classified as *myocarditis*, *dilated cardiomyopathy*, *other cardiomyopathy*, and *no histological correlate*. Thus, our data support the notion that alterations of the above five GPCR autoantibody species are associated with non-Chagas' and non-ischaemic HF in a significant and possibly meaningful manner, but a distinction of histological sub-entities does not show a distinct GPCR autoantibody immunity profile. Interestingly, not all the observed HF-associated alterations of GPCR autoantibody levels pointed in the same direction: β_1 AR, M5R, and AT2R autoantibodies were increased, whereas α_1 AR and AT1R autoantibodies were decreased in the HF patients, and component 5a receptor, angiotensin-converting enzyme II receptor, and Endothelin A receptor or Endothelin B receptor autoantibodies were not significantly altered.

HF-associated increases in GPCR autoantibodies: The increase in β_1 AR autoantibodies conforms to a plethora of published data suggesting a specific association of that autoantibody species with various cardiovascular diseases, most notably with idiopathic dilated cardiomyopathy.² The increase in M5R autoantibodies observed here is reminiscent of a similar phenomenon recently observed in elderly persons subjected to invasive periodontal therapy, which, however, was correlated with the intensity and time course of periodontal surgery but not with cardiac markers.³⁰ The increase in AT2R autoantibodies finally, which we observed in HF patients, remains to be evaluated with respect to its possible relevance for non-ischaemic HF. To our knowledge, a similar humoral alteration has not been observed before in any human disease or syndrome. However, the role of the AT2R itself has been thoroughly investigated in HF. Recent research suggests that AT2R activation plays a protective role in HF.⁵⁴ The demonstrated HF-associated increase in circulating AT2R antibodies might therefore indicate a novel compensatory

mechanism after HF onset, provided the measured antibodies exert a stimulating effect on the AT2R, which still needs to be demonstrated.

HF-associated decreases of GPCR autoantibodies: α_1 AR, AT1R, ET_{A/B}R, and ACEII autoantibodies were included in this study, because increased levels thereof play an established role in vascular allograft rejection and blood pressure dysregulation.²⁷ However, levels of ET_{A/B}R and ACEII autoantibodies did not significantly differ between patients and controls, whereas α_1 AR and AT1R autoantibody levels were lower in HF patients. Decreases in GPCR autoantibodies could be just as relevant as increases given current pathogenetic concepts proposing a physiological regulatory role of GPCR antibodies¹⁵ that becomes imbalanced in autoimmune diseases.²⁹ α_1 AR and AT1R autoantibodies are known to upregulate blood pressure in various human pathologies,²⁷ and AT2R autoantibodies can counteract these effects in immunized rabbits.⁵⁵ The observed HF-associated alterations of AT1R, α_1 AR, and AT2R autoantibodies mirror the known effects of corresponding receptor activations in cardiovascular regulation^{54,56} and, thus, could be relevant for HF due to an impact on blood pressure regulation.

Association of GPCR autoantibodies with disease activity and myocardial damage

We have performed an extensive screen for associations or quantitative correlations between HF-associated alterations of GPCR autoantibodies and clinical and experimental parameters of disease activity, prognosis parameters, or myocardial damage. However, we have not detected any association with serum markers of myocardial damage or haemodynamic parameters of impaired systolic function. Neither have we observed any association with myocardial oedema and inflammation as judged by T2 mapping³⁹ or biopsy histology or inflammation serum markers.

These negative findings stand in contrast to some previous studies, which may be attributable to several factors. Firstly, most other studies have been based on quantitative determinations of circulating GPCR antibodies by IgG binding to linear peptides. However, such measurements do not necessarily reflect the impact of the antibodies on receptor function.^{35–37,53} Other previous studies have been exclusively based on functional readouts, which are only loosely related to circulating levels of the antibodies.^{52,57} Here, we employed a type of assay that to the best of our knowledge combines both approaches as it measures IgG binding to the native receptor or a circular peptide faithfully reflecting the conformational autoepitope related to the active receptor conformation.^{38,58} Another source of divergence may be a different representation of receptor

polymorphisms in the study population. At least for the β_1 AR, it has been demonstrated that certain frequent polymorphisms have a significant influence on binding as well as functional effects of GPCR autoantibodies.⁵⁹ Moreover, to our knowledge, in none of the previous studies, measurements of circulating levels of GPCR autoantibodies have been normalized to total IgG. Consequently, these studies cannot exclude confounding effects of global inflammatory phenomena on the circulating levels of certain GPCR autoantibodies. Such confounding effects have previously demonstrated in periodontitis patients³⁰ and were here observed in HF patients. Finally, all patients in the HF group of this study exhibited an advanced HF phenotype justifying endomyocardial biopsy. Thus, in contrast to most previous studies, not all existing phenotypes and earlier stages of non-ischaemic HF were included in our study population.

The only notable links between GPCR autoantibodies and indicators of HF disease activity found in this study were a correlation between circulating levels β_1 AR autoantibodies and global left ventricular T1-relaxation time as a marker of myocardial fibrosis as well as lipid and iron accumulation,⁴⁴ as well as left ventricular GLS as a marker of cardiac contractile function. These correlations were robustly conserved following normalization to total IgG. However, both correlations were inverse, which is inconsistent with data obtained by experimental immunization and transfer of immune components to animals suggesting that β_1 AR autoantibodies stimulate myocardial fibrosis and may impair cardiac contractile function.^{5,60–62} However, it is conceivable that humoral β_1 AR autoimmunity disappears again once myocardial fibrosis has established, which would be the case in the advanced state of disease of the HF patients included in our study. Recent research also hints towards a compensatory increase of β -adrenergic receptor autoantibodies in paediatric myocarditis.⁶³ As only cross-sectional non-causal correlations are provided in our study, future studies of the timing of events linking cardiac fibrosis and functional impairment to humoral β_1 AR autoimmunity are required to address a presumed biphasic time course of β_1 AR autoantibodies.

Association of GPCR autoantibodies with metabolic disease

Our data demonstrate an inverse correlation of AT2R autoantibodies with insulin resistance, whereas an elevation in circulating free fatty acids, as often found in the insulin resistant state,⁶⁴ was associated with higher AT1R autoantibody titres. As known from previous studies, AT2R stimulation exhibits protective effects in heart failure as well as diabetes-associated atherosclerosis.^{54,65} AT2R

activation through angiotensin II increases capillary blood flow and raises insulin-mediated glucose usage. In contrast, AT1R activation leads to reduced nitric oxide bioavailability, impaired insulin signalling, vasoconstriction, and insulin resistance.⁶⁶

Furthermore, we find that increased levels of AT2R autoantibodies are correlated to increased mitochondrial efficiency as estimated via leak control ratio.^{41,67} These findings could support the hypothesis of GPCR antibodies having a physiological regulatory role.²⁹ In this case, a role of AT2R autoantibodies in the adaptation of mitochondrial metabolism would further corroborate the concept of protective effects of AT2R on the myocardium in HF.

On the one hand, our data could indicate that low levels of AT2R autoantibodies might promote insulin resistance, which in turn is a key factor for a developing type 2 diabetes mellitus.^{68–70} On the other hand, an effect of insulin resistance on AT2R activity and their cardioprotective effects via circulating AT2R autoantibodies cannot be excluded.⁷¹ Therefore, it is tempting to speculate that the link between AT2R autoantibodies and insulin resistance here observed in HF patients may be mechanistically involved in diabetes-related heart disease or in the worse outcomes of HF patients with diabetes mellitus or both.^{71–75}

Limitations

This study comes with several limitations. Firstly, and most importantly, the observational and cross-sectional design of the study and the correlations shown in this study do not allow to infer causation or timing of events and should therefore be regarded as hypothesis-generating. Moreover, we were not able to fully phenotype the control group in the same fashion as the HF group. Finally, our groups differed in age and sex distribution, which we accounted for by adjusting for those parameters in linear regression analysis in the main group comparisons of GPCR autoantibodies in HF and controls.

Concluding remarks

Our data clearly show a link between non-ischaemic HF and alterations of GPCR autoantibody-mediated immunity. Although the associations shown in this hypothesis-generating study alone cannot imply or preclude causality, the consistency of these alterations among different causes of non-ischaemic HF does not hint towards a direct and global cardio-pathogenic effect of GPCR autoantibodies as demonstrated for other anti-heart autoantibodies.^{76,77} On the contrary, correlations of GPCR autoantibodies with cardiac metabolic or structural parameters lend support to the concepts of protective rather than pathogenic properties

of specific autoantibodies and reactive alterations of GPCR autoantibodies in HF. Therefore, it seems counterintuitive that removal or neutralization of HF-associated GPCR autoantibodies (as opposed to unselective removal total IgG or certain IgG subclasses) should improve human HF in the same manner as it does in certain animal models.⁷ Overall, our study stresses the importance to further differentiate the effects of GPCR autoantibodies within their class prior to establishing GPCR autoantibody-regulating therapies.

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Conflict of interest

None of the authors have a conflict of interest with respect to the content of this manuscript to declare.

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Supporting information

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

Table S1. Comparison of immune assays.

Table S2. Histopathological classification.

Table S3. GPCR-Antibodies and cardiac parameters.

Table S4. GPCR-Antibodies and cardiac MRI.

Table S5. GPCR-Antibodies and metabolic markers.

Table S6. GPCR-Antibodies and mitochondrial parameters.

Figure S1. Correlation between β_1 -adr-R-Antibodies measured via cyclopeptide or membrane assay within the heart failure group; GPCR-Antibodies were normalized to total IgG and logarithmized. *IgG* immunoglobulin G. Data given as Spearman rho. n = 91.

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