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Antibodies to receptors are associated with biomarkers of inflammation and myocardial damage in heart failure

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ABSTRACT

Introduction: Naturally occurring antibodies are linked to inflammation, tissue injury and apoptosis, processes also linked to heart failure. Associations between antibodies, inflammation and myocardial damage, have not been elucidated in heart failure.
Objective: We investigated if 25 antibodies to receptors expressed in the cardiovascular system were associated with troponin-T, biomarkers of inflammation and clinical measures of disease severity, in patients with heart failure.
Methods: Antibodies in sera from patients (n = 191) with ischemic (n = 155) or non-ischemic (n = 36) heart failure were measured with full-receptor sandwich enzyme-linked immunosorbent assays. All patients underwent coronary angiography with determination of left ventricular ejection fraction (LVEF) and left ventricular end-diastolic pressure (LVEDP). Measured biomarkers included troponin-T, C-reactive protein, erythrocyte sedimentation rate, fibrinogen and neopterin.
Results: Stabilin-1-antibodies correlated with troponin-T (β 0.23 p = 0.008), soluble endoglin-antibodies with erythrocyte sedimentation rate (β 0.19, p = 0.007) and fibrinogen (β 0.28, p < 0.001). Platelet-derived growth factor subunit β-antibodies were associated with neopterin (β 0.17, p = 0.002). All antibodies were correlated (R 0.26 to 0.91) and formed 4 principal components (PCs). Patients with high CRP and high PC2 had higher NYHA class and patients with high troponin-T and high PC1 had lower LVEDP (interactions, all p < 0.05).
Conclusion: Antibodies to receptors are correlated and are associated with biomarkers of inflammation and myocardial damage, which further modifies their association with disease severity in heart failure. Their functional activity and immunological function, remain undecided.

1. Introduction

Antibodies (Abs) to key receptors in the cardiovascular system such as β_1-adrenoceptor (β_1AR-Abs) and angiotensin II type 1 receptor (AT1R-Abs) have been identified in idiopathic dilated cardiomyopathy (DCM) [1], ischemic heart failure [2] and hypertension [3].

Witebsky's criteria for defining an antibody as pathogenic postulate that there should be direct evidence from passive immune transfer or cellular action, while clinical associations are only suggestive [4]. β_1AR-Abs from patients with DCM show pathogenic effects on cells [5] and following passive transfer [6,7]. Levels of β_1AR-Abs are associated with mortality [2]. Thus, Witebsky's criteria support the potential presence of autoimmunity in DCM.

In contrast to antibodies with defined pathogenic effects, the majority of antibodies are naturally occurring antibodies (Nabs), also called physiological antibodies [8]. Most are of the immunoglobulin M class, but a significant proportion of Nabs are from the immunoglobulin G (IgG) class [9]. In contrast to high-affinity autoantibodies, Nabs are typically polyreactive and bind with low affinity [9]. Nabs, as part of the innate immune system, have homeostatic functions such as clearing apoptotic cells and oxidized proteins [10,11]. The anti-inflammatory effects of intravenous IgG demonstrate the immune-regulatory properties of Nabs [12] and their levels increase during inflammation [13].

Antibodies to receptors in heart failure are not limited to patients with DCM. Antibodies to both receptors (M_2R-Abs [14] and 5-HT_4R-Abs [15]),

Abbreviations: Abs, antibodies; Nabs, naturally occurring antibodies; IgG, immunoglobulin G; GPCR, G-protein-coupled receptor.

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calcium channels [16] and structural cardiac proteins (troponin-I-Abs and myosin-Abs) [17,18] are associated with heart failure, including ischemic heart failure. Increased inflammation and ongoing cellular injury in cardiac tissues could be associated with increased antibody production.

We measured IgG antibodies to a panel of 25 receptors expressed in the cardiovascular system in sera from patients with predominantly ischemic heart failure. We investigated the primary endpoints a) if there are associations with troponin-t, as a possible marker of cell death, b) associations with biomarkers of inflammation and c) to what extent the antibodies relate to each other, as an indirect measure of polyreactivity. Further, d) primary associations with clinical measures of disease severity and e) effect modification of the antibody-disease severity relationship by biomarkers of inflammation, troponin-t and medication use, were explored as secondary end-points.

2. Material and methods

2.1. Subjects

Patients were recruited from the Bergen Coronary Angiography Cohort (BECAC) and Western Norway B Vitamin Intervention study (WENBIT) [19]. These cohorts included 4241 patients who underwent elective coronary angiography at Haukeland University Hospital, Bergen, or Stavanger University Hospital, Stavanger, between 1999 and 2004. One hundred and ninety one patients met the criteria for heart failure. Inclusion criteria were left ventricular ejection fraction (LVEF) ≤40% or LVEF <50 and New York Heart Association (NYHA) class >1. Exclusion criteria were severe pulmonary disease, primary valvular heart disease, known cancer at baseline, dialysis-demanding or stadium IV renal failure, later acceptance for heart transplant, known toxic cause for heart failure (e.g. cytostatics or alcohol) and other terminal illness. Patient characteristics were obtained by clinical examination performed by a physician at inclusion, self-administered questionnaire and from hospital records. LVEF and left ventricular end-diastolic pressure (LVEDP) were obtained by cine ventriculography during cardiac catheterization performed by trained cardiologists. Definition of hypertension and diabetes was based on clinical diagnoses. Smoking habits were self-reported by the patients. Routine laboratory analyses were performed at hospital laboratories at the two hospitals. Estimated glomerular filtration rate (eGFR) was obtained using the Chronic Kidney Disease Epidemiology Collaboration CKD-EPI) formula. Specialized metabolic profiles were determined at the laboratory Bevital (http://www.bevital.no).

2.2. Ethics

Ethical approval was granted from the Regional Ethics Committee (REK approval no.: 2013/2022). Patients provided written consent.

2.3. Antibody measurements

Sera were collected at baseline and stored at minus 80 °C until thawed and analyzed by staff blinded to the clinical status of patients. Mean storage time was 14.3 ± 1.1(SD) years. Recombinant receptors used as antigens were expressed in Chinese hamster ovary (CHO) cells, purified from membranes and used in enzyme-linked immunosorbent assays (ELISA) [20], CellTrend GmbH. A list of all the antibodies measured can be found in Appendix Table A.1.

2.4. Antigen selection

Receptor-antigens were selected based on their expression in the cardiovascular system, or had a potential link to the pathophysiology of heart failure. Antibodies to cardiovascular receptors of the adrenergic, dopaminergic, serotonergic and cholinergic muscarinic receptor systems were measured. Stabilin-1(Stab1) is an immune receptor expressed on tissue macrophages that is involved in scavenging [21]. Activation of the calcium sensing receptor (CaSR) induces myocardial apoptosis in rats and mice [22]. Soluble endoglin (sENG), platelet derived growth factor subunit β (PDGFB) and the two thrombin receptors protease activated receptor 1 (PAR1) and 2 (PAR2) are linked to cardiac remodeling [23–26]. Vascular endothelial growth factor receptor 1 (VEGFR1) and 2 (VEGFR2) regulate angiogenesis [27]. Binding of endothelin to endothelin receptor type A (ETAR) and type B (ETBR) causes vasoconstriction and vasodilatation, respectively [28]. A total of 25 different antibodies were measured.

2.5. Troponin-T and biomarkers of inflammation

Troponin-T (TnT) was measured in serum by a high-sensitivity TnT assay on Modular E170 (Roche Diagnostics). The lower detection limit was 3 ng/L.

Serum C-reactive protein (CRP) was measured using an ultrasensitive immunoassay, with a detection limit of 0.17 mg/L, applying the Behring nephelometer II system (CV 8.1–11.4%; N Latex CRP mono, Behring Diagnostics, Marburg, Germany). Neopterin was analyzed by liquid chromatography–tandem mass spectrometry [29]. Analyses of erythrocyte sedimentation rate (ESR) and fibrinogen were performed at hospital laboratories at Haukeland University Hospital, Bergen, or Stavanger University Hospital.

2.6. Statistics

P-values <0.05 were considered statistically significant for secondary end-points and below a false discovery rate (FDR) of 0.05, for primary end-points (Benjamini-Hochberg). Both antibodies and biomarkers followed right-skewed distributions. To approximate normality, antibodies were transformed by the Rankit method and the biomarkers were transformed by their log or square root. The Spearman correlations between the antibodies were used to generate a correlation network (spring electric network visualization). FDR-adjusted, univariate Pearson correlations were used as a variable selection method to identify the strongest correlations and avoid multicollinearity. The strongest associations identified in univariate analyses were adjusted for potential confounders in multivariate analyses. We included age, gender, eGFR and LVEF as potential confounders. Regressions were standardized to generate comparable effect sizes. TnT followed a censored-normal distribution after transformation and Tobit regression were employed. A generalized linear model was used to analyze fibrinogen and ESR (normal distribution), with neopterin being analyzed by robust regression by MM-estimation due to the presence of outliers (Cook's distance > 8/sample size, leverage > 2 * number of predictors/sample size). Prior to assessing associations with clinical outcomes (secondary end-points), the antibodies were reduced to their principal components by optimal scaling (rank discretization, varimax rotation), retaining components with an eigenvalue > 1. The association between antibodies and clinical parameters of disease severity, as well as any modification of this relationship by biomarkers of inflammation, cell death or medications, was then assessed in multivariate analysis. We applied a proportional odds model (NYHA), robust regression by MM-estimation (LVEF and LVEDP) and logistic regression (medications). Compared to M estimation, MM estimation is also robust to bad leverage [30]. Clinical measures were analyzed with age, gender and eGFR, PCs and their interactions with TnT, biomarkers of inflammation and medications as potential modifiers of the association between antibodies, as their PCs, and measures of disease severity. Medications were analyzed by logistic regression for association with antibodies. We assessed the use of β-blockers and use of either angiotensin converting enzyme inhibitors (ACE-I) or angiotensin receptor blockers (ARB), as a combined variable. NYHA classes 3 and 4 were collapsed to one, in order to fulfill the proportional odds- and adequate cell count assumptions of ordinal regression.

Analyses were performed using SPSS Statistics version 22.0 (SPSS Inc., Chicago, IL, USA), STATA 14 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP) and Cytoscape 3.4.0 (Cyni toolbox and AllegroLayout).

3. Results

3.1. Study participants

Among a total of 191 patients, 155 (81%) had ischemic heart failure. Twenty percent of patients had normal coronary arteries. Baseline characteristics are shown in detail in Table 1.

3.2. Antibody measurements: coefficients of variation

Eleven out of 25 antibodies measured, had both inter- and intra-variability below 10% (good), 11 were below 20% (acceptable) and three were over 20% (5-hydroxytryptamine 7 receptor-Abs, muscarinic acetylcholine receptor M_2-Abs and endothelin B receptor-Abs). See Appendix Fig. B.1 for details.

3.3. Antibodies, troponin-T and inflammation: univariate analyses

Median levels and interquartile ranges of antibodies in U/mL can be found in Appendix Table A.1. There were 42 significant correlations between biomarkers and antibodies where 14 were discoveries after correcting for multiple testing (Appendix Fig. C.1).

The highest correlation coefficients were between TnT and Stab1-Abs (R 0.30, $p \leq 0.001$), neopterin and PDGFB-Abs (R 0.24, $p = 0.001$), sENG-Abs and both fibrinogen (R 0.31, $p < 0.0001$) and ESR (R 0.24, $p = 0.001$). There were no significant correlations between the antibodies and CRP. The overall direction of all determined effect sizes (R) between antibodies and biomarkers was skewed towards positive associations.

3.4. Multivariate adjustment

The antibodies most strongly correlated with TnT and each of the inflammatory biomarkers was selected for multivariate analysis with age, gender, LVEF and eGFR as covariates (Table 2). The significant

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Table 1
Heart failure patient's characteristics.

Clinical parameters and risk factors	Total N = 191
Age, mean ± SD	63.5 ± 9.5
Body mass index, mean ± SD	26.3 ± 4.2
Male, N (%)	151 (79.1)
Smoking, N (%)	56 (41.5)
Diabetes, N (%)	33 (17.3)
Hypertension, N (%)	95 (49.7)
LDL, mean ± SD	3.0 ± 0.9
Creatinine (μmol/L), mean ± SD	98.7 ± 23.1
eGFR, mean ± SD	80.5 ± 18.6
Disease severity	
Coronary artery stenosis, N (%)	
0	38 (19.9)
1	30 (15.7)
2	31 (16.2)
3	92 (48.2)
NYHA, N (%)	
1	54 (28.3)
2	93 (48.7)
3	34 (17.8)
4	4 (2.1)
Ischemic heart failure, N (%)	155 (81.2)
Left ventricular ejection fraction[a], mean ± SD	34.9 ± 8.6
Left ventricular end-diastolic pressure, mean ± SD	22.1 ± 8.5
Systolic blood pressure, mean ± SD	132.8 ± 20.6
Diastolic blood pressure, mean ± SD	79.0 ± 11.4
Heart rate, mean ± SD	69.7 ± 13.8
Biomarkers	
Troponin T (ng/L), mean ± SD	20.4 ± 23.8
C-reactive protein (mg/L), mean ± SD	5.2 ± 8.9
ESR (mm), mean ± SD	15.5 ± 13.1
Fibrinogen (g/L), mean ± SD	3.9 ± 0.7
Neopterin (nmol/L), mean ± SD	10.6 ± 5.0
Medications, N (%)	
β-blocker	154 (80.6)
ACE-I	131 (68.6)
ARB	31 (16.2)
Either ACE-I/ARB	162 (84,8)
Aldosterone antagonist	22 (11.5)
Loop diuretic	113 (59.2)
Thiazide diuretic	17 (8.9)
ASA	127 (62.8)
$P2Y_{12}$ receptor antagonist	24 (12.6)
Warfarin	46 (24.5)
Statin	146 (76.4)
Other lipid lowering medication	23 (12.0)
Calcium channel blocker	34 (17.8)
Long acting nitrates	74 (38.7)
Digitalis	32 (16.8)

Abbreviations: N = number of patients, SD = standard deviation, LDL = low density lipoprotein, NYHA = New York Heart Association Functional Classification, ESR = erythrocyte sedimentation rate, ACE-I = angiotensin converting enzyme inhibitor, ARB = angiotensin receptor blocker, ASA = Acetylsalicylic acid.
[a] Left ventricular ejection fraction measured by cine ventriculography.

antibody associations with TnT, neopterin, fibrinogen and ESR remained in adjusted analyses (table 2).

3.5. Correlation network between antibodies

The antibodies correlated with each other (*R* 0.26 to 0.91, Spearman Rho's) and formed a dense correlation network (Fig. 2). The network analysis shows that groups of correlating antibodies are related to the different biomarkers indicated by the labeled results in fig. 1. Antibodies previously found in patients with heart failure (particularly β_1AR-Abs and AT1R-Abs) were also highly correlated with other antibodies.

3.6. Principle component analysis of antibodies

Four components were extracted from 25 Abs in categorical principal component analysis. The total variance explained was 72.9%.

Table 2
Antibodies, biomarkers and clinical parameters[a].

Biomarkers					
Variables	Troponin T[b]		Variables	Neopterin[c]	
	β	p		β	p
Age	0.002	0.985	Age	0.006	0.932
Male	0.273	0.199	Male	−0.124	0.370
LVEF	−0.206	0.026	LVEF	0.012	0.835
eGFR	−0.360	0.001	eGFR	−0.562	<0.001
Stabilin-1	0.233	0.007	PDGFB	0.176	0.003
	Fibrinogen[d]			ESR[d]	
	β	p		β	p
Age	0.052	0.559	Age	0.065	0.452
Male	0.009	0.957	Male	−0.527	0.003
LVEF	0.018	0.808	LVEF	−0.033	0.653
eGFR	−0.178	0.047	eGFR	−0.148	0.097
sENG	0.287	<0.001	sENG	0.200	0.006
Clinical parameters and interactions					
	NYHA[e]			LVEDP[c]	
	OR	p		β	p
Age	1.295	0.135	Age	0.035	0.768
Male	0.586	0.138	Male	0.141	0.547
eGFR	0.956	0.795	eGFR	−0.013	0.926
CRP	1.534	0.134	TnT	0.267	0.005
PC2	0.679	0.080	PC1	−0.189	0.009
PC2 * CRP	1.846	0.037	PC1 * TnT	−0.217	0.003

Abbreviations: LVEF = left ventricular ejection fraction, PDGFB = platelet derived growth factor subunit β, sENG = soluble endoglin, ESR = erythrocyte sedimentation rate, LVEDP = left ventricular end-diastolic pressure.
[a] R^2 of models: Troponin $T = 0.24$, Neopterin = 0.42, Fibrinogen = 0.17, ESR = 0.16, NYHA = 0.035, LVEDP = 0.24.
[b] Standardized Tobit regression.
[c] Standardized robust regression by MM estimation.
[d] Standardized general linear model with a normally distributed dependent variable.
[e] Standardized ordered logistic regression.

Principal component loadings and percentage of explained variance of each principal component can be found in Appendix Table D.1.

3.7. Clinical measures: multivariate analyses

There were no primary associations between antibodies and measures of disease severity. However, PC2 was increasingly associated with NYHA class in patients with CRP levels above the median (interaction of PC2 and CRP, Table 2 and Fig. 2), while PC1 was associated with lower LVEDP in patients with higher TnT (interaction of PC1 and TnT, table 2 and fig. 2). There were no associations between antibodies and the current use of β-blockers and either ACE-I or ARB, nor did we identify interaction between antibodies and use of these medications, with disease severity measures as outcomes (data not shown).

4. Discussion

In this study on antibodies in patients with heart failure, several antibodies to receptors were related to biomarkers of inflammation and TnT. These antibodies formed a highly correlated, dense network. Analyses of the principal components of the antibodies and interactions with biomarkers were associated with worse clinical measures of disease severity in patients with high CRP. Conversely a protective association was identified when TnT was high. Previous evidence supports a possible pathogenic role for autoantibodies in patients with idiopathic dilated cardiomyopathy [7,31]. Our study indicates that antibodies have a close relationship to inflammation and elevated levels of TnT, which suggests that there is a broad spectrum of physiological antibodies in heart failure, directed at receptors. Further, associations between antibodies and measures of disease severity are determined

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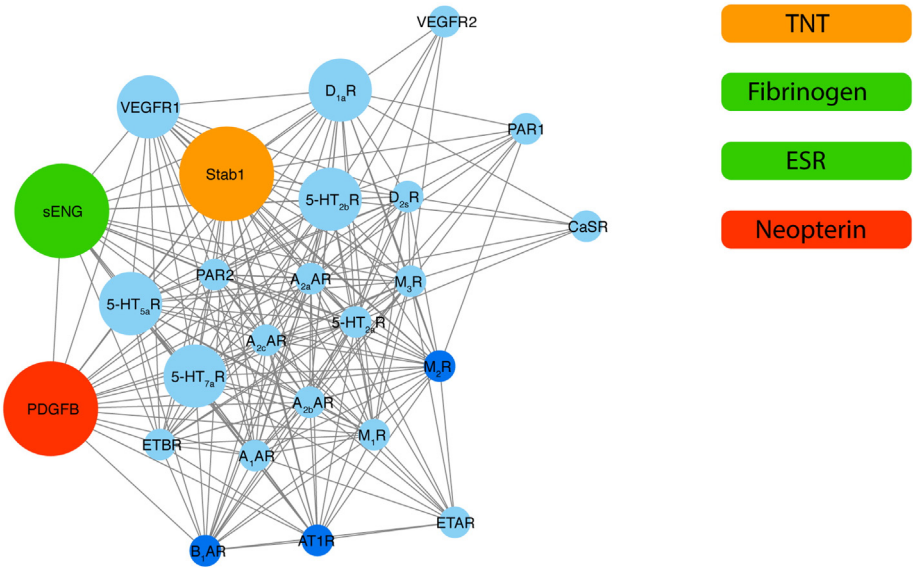

Fig. 1. Spearman correlation network of all antibodies and summary of findings. Spearman correlation network with node size representing: Small size: no association after FDR correction. Medium sized: significant after FDR in univariate analyses. Big size: Significant after multivariate analyses, with color labels representing their corresponding correlation to biomarkers. Univariate correlations with biomarkers after FDR not indicated by labels: Stab1 with neopterin, fibrinogen and ESR, sENG with TnT, ESR and neopterin. VEGFR1 with TnT, 5-HT_{5A}R with TnT, 5-HT_{2B}R with TnT, 5-HT_7R with TnT and D_{1a}R with ESR. Dark blue color representing antibodies in previous studies associated with heart failure. Abbreviations: PDGFB = platelet derived growth factor subunit beta, sENG = soluble endoglin, ESR = erythrocyte sedimentation rate, TnT = troponin-T, VEGFR1 = Vascular endothelial growth factor receptor 1, 5-HT_{5A}R = 5-hydroxytryptamine receptor 5A, 5-HT_{2B}R = 5-hydroxytryptamine receptor 2B, 5-HT_7R = 5-ydroxytryptamine receptor 7, D_{1a}R = Dopamine receptor D1a.

by the level of biomarkers of cell death and inflammation. While Nabs may have both local pro-inflammatory actions and may also suppress inflammation, Nabs are likely beneficial when cell death is increased. We cannot confirm functional activation from these antibodies on cardiac receptors, the clinical relevance of the observed interactions or if there is an association with transition to pathogenic autoimmunity. However, this can neither be excluded.

High levels of Stab1-Abs were associated with increased TnT. TnT is associated with cardiovascular death and heart failure in stable coronary artery disease [32] and a prognostic marker in patients with heart failure [33]. The mechanism behind increased levels of TnT not due to acute coronary syndrome is not clear. Several mechanisms have been proposed, including microvascular disease, ischemic strain and necrosis, apoptosis and inflammatory cytokines causing cardiomyocyte damage [33–35]. In addition to being expressed on monocytes and macrophages Stab1 is expressed within the myocardium [36] and deficiency of Stab1 is linked to increased levels of proinflammatory cytokines [37] and increased hepatic fibrosis [38].

sENG is a circulating antiangiogenic protein elevated in patients with increased LVEDP [39]. sENG-Abs were associated with increased

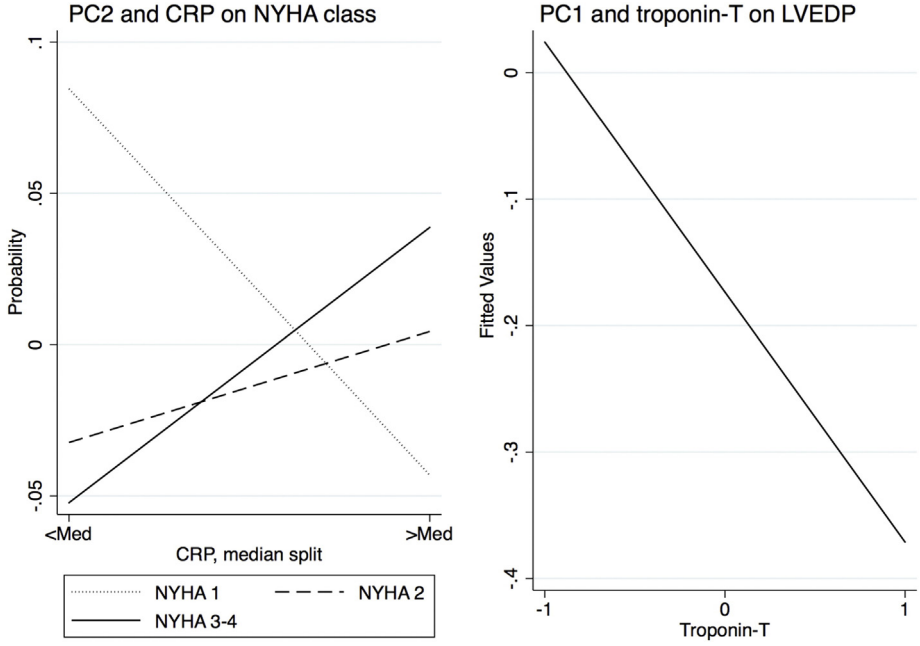

Fig. 2. Clinical measures, principal components and interactions with biomarkers. Graphs representing margins plots of interactions in ordered logistic regression and robust regression analyses. Left: Interaction of PC2 and CRP on probability for NYHA class. Right: Interaction of PC1 and troponin-T on LVEDP. Abbreviations: PC = principal component, CRP = C-reactive protein, NYHA = New York Heart Association, LVEDP = left ventricular end-diastolic pressure.

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fibrinogen, a marker of cardiovascular risk [40]. PDGFB has moderate protein expression in the myocardium. PDGFB-Abs were associated with neopterin, a marker of IFN-γ mediated activation of macrophages, that is associated with cardiac remodeling [41].

These associations could occur as a response to cellular injury with subsequent rise in the levels of antibodies. However, the pathways leading to generation of IgG Nabs from B cells are not yet fully understood. Tissue injury and apoptosis are associated with increased levels of Nabs [10,42]. Heart failure is characterized by chronic inflammation and infiltration of macrophages and T-cells [43], increase in oxidative stress and myocardial cell death and apoptosis [44,45]. Upregulated expressions of major histocompatibility complex II, are increased in the myocardium in heart failure [46]. Thus, pathophysiological changes in heart failure could favor an environment for increased production of antibodies directed at proteins expressed in the myocardium, including receptors.

The highly correlated network formed by the antibodies as demonstrated in our study suggests that these antibodies may be polyreactive, due to overlapping primary amino-acid sequences on G-protein-coupled receptors (GPCRs) and conformational epitopes that could have molecular similarities. The polyreactivity typically seen for Nabs indicates lower affinity that would likely result in a more indiscriminate binding to similar epitopes on several receptor-antigens in the assays. Taken together with the previously mentioned correlations with markers of inflammation and cell death, our results suggest that these are Nabs. Due to the likely presence of pathogenic antibodies in DCM, the continuum between Nabs and true markers of autoimmune disease is important to study further. The search for biomarkers of true autoimmunity by measuring antibodies to receptors may be complicated by the presence of multiple physiological antibodies to receptors. In theory, these would respond with increased levels to many forms of cellular injuries, resulting in low specificity. Distinguishing which antibodies are detrimental and which are not, would likely require cell-based immune-assays that can assess functional consequences of antibody binding.

The associations between clinical measures and principal components interacting with biomarkers and medications found in multivariate analyses, although not as strong as the interactions with biomarkers, strengthen the association between antibodies and heart failure. High levels of PC2 (PAR1, Stab1, M_3R, $D_{1a}R$, $D_{2s}R$) predicted higher NYHA class in patients with elevated CRP (interaction of PC2 and CRP). High levels of PC1 (ETBR, α_1AR, α_{2a}AR, α_{2b}AR, α_{2c}AR, β_1AR, M_1R, M_2R, 5-$HT_{2A}R$, 5-HT_7R) were associated with lower LVEDP in patients with higher levels of TnT (interaction of PC1 and TnT). This suggest a protective effect of the antibodies and supports a possible role of antibodies in modifying the inflammatory response and clearing apoptotic or necrotic cells, in heart failure, well in line with known physiological functions of Nabs [9,10,12].

Carvedilol therapy in β_1AR-Ab positive patients with heart failure had a higher percentage increase in LVEF compared to the β_1AR-Ab negative group [47]. We could not identify such associations, but our study was not designed to primarily study drug-antibody interactions. The low frequency of patients not taking, or taking medications, made our study under-powered. Further, antibody levels prior to commencing treatment, was unknown. Still, an interesting question arising from our study, that identified moderate-to-high correlation between antibodies, is to what degree different antibodies have functional activity on one, or likely several targets, with potentially overlapping epitopes. If the antibodies have functional activity on multiple receptors, it could have a complex relationship to drug response. This needs to be addressed in future studies.

β_1AR-Abs and AT1R-Abs have been extensively studied due to their ability to activate receptors [31,48]. β_1AR-Abs and AT1R-Abs are found in several cardiovascular diseases and are thus not specific to DCM. Despite a lack of specificity, the number of publications addressing functional aspects of these antibodies leaves little doubt that some indeed have agonist function [49]. However, in this study, these were highly correlated with other antibodies. Of note, studies suggesting functional activity of these antibodies have been identified by the same immune-assays as used here [48]. Whether high-affinity autoantibodies can arise from low-affinity Nabs is a matter of debate [50].

Strengths of this study included a population with heart failure that was systematically assessed with invasive measurement of LVEF and LVEDP as well as an adequate power to detect small-to-medium effect sizes. We did not include a control group because none was available that was matched for time in cryopreservation, which may be a potential confounder. Weaknesses include lack of functional data on the effect of antibodies on their targets in our study and little previous characterization of many of these antibodies. Information about medication use was based on current drugs used at baseline, and not information about duration of treatment. Echocardiographic measures of left ventricular function, such as tissue Doppler and left ventricular longitudinal strain, could have added additional information about association between antibodies and clinical measures, but were not available at the time of inclusion. Further, other markers of immune-activation and cell death could have been measured, but several, such as cytokines, are vulnerable to long term cryopreservation.

4.1. Conclusion

In summary, we found that antibodies to sENG, Stab1 and PDGFB were correlated with known markers of inflammation and myocardial damage. Antibodies were associated with higher NYHA class, in patients with increased CRP, and with lower LVEDP, in patients with high TnT. We identified a highly correlated network of antibodies to multiple receptors and our results suggest that these are physiological antibodies that are related to biomarkers of homeostatic functions, where physiological antibodies are important. This relationship might be clinically relevant, suggested by associations with disease severity that were dependent on the level of inflammation and myocardial damage. Their functional activity, or relation to development of autoimmunity, remains undecided.

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Conflicts of interest

Dr. Harald Heidecke is an owner of CellTrend Gmbh and holds patented rights to the analyses. CellTrend was blinded to patients IDs and other variables and did not participate in data-analysis.

Appendix A

Appendix Table A.1
Antibody abbreviations and levels.

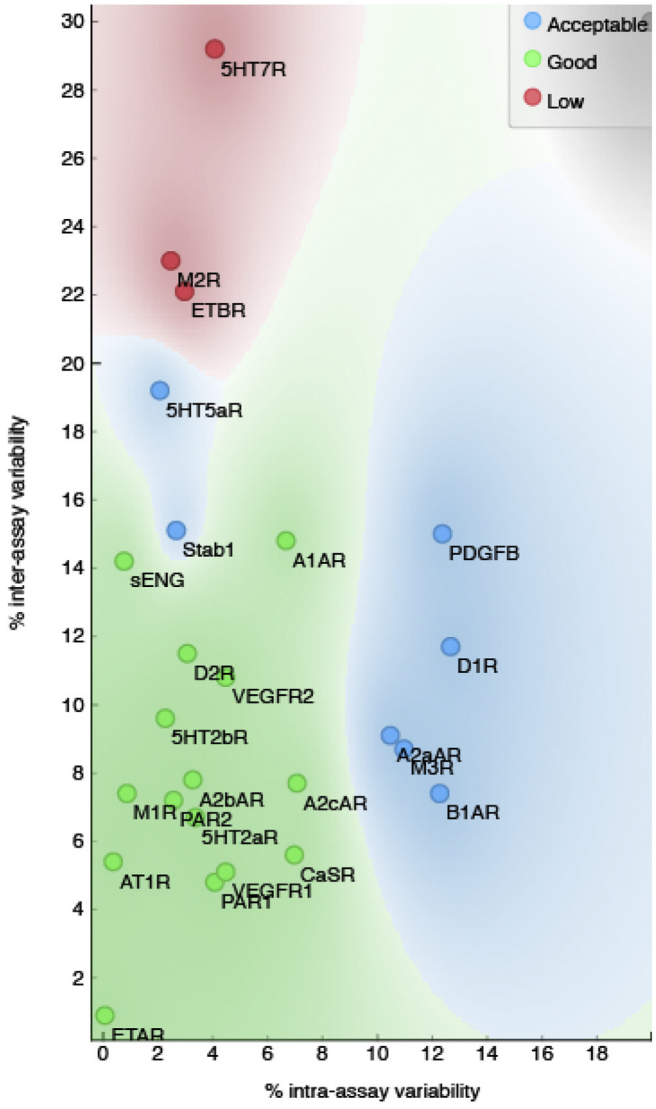
Abbreviation in text	Full name	Median (IQR)
VEGFR1	Vascular endothelial growth factor receptor 1	11.8 (6.9)
VEGFR2	Vascular endothelial growth factor receptor 2	7.6 (7.9)
PAR1	Protease activated receptor 1	0.6 (1.2)
PAR2	Protease activated receptor 2	5.4 (8.2)

(continued on next page)

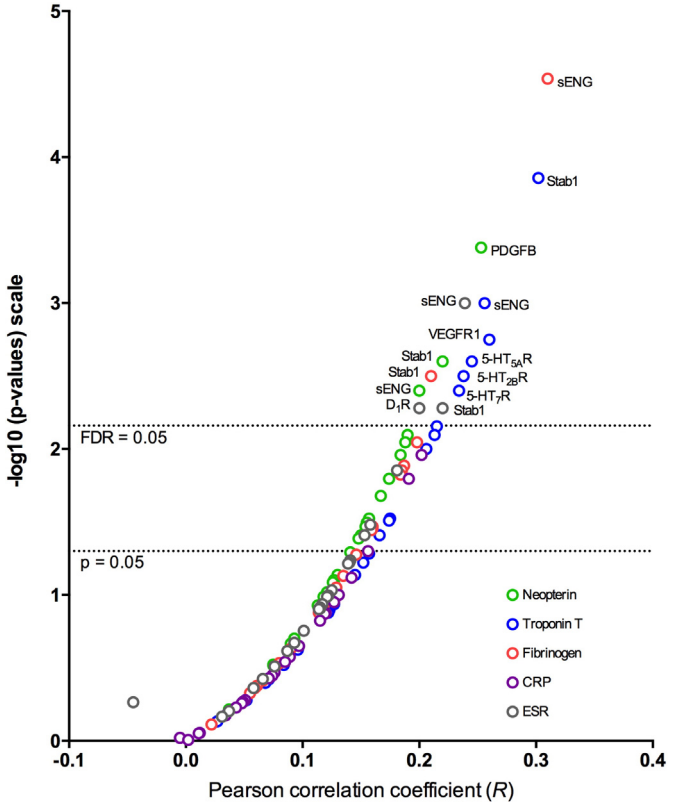
Appendix Table A.1 (*continued*)

Abbreviation in text	Full name	Median (IQR)
ETAR	Endothelin A receptor	6.0 (7.4)
ETBR	Endothelin B receptor	6.3 (4.3)
PDGFB	Platelet derived growth factor subunit beta	2.6, (4.4)
sENG	Soluble endoglin	2.8 (4.4)
CaSR	Calcium sensing receptor	2.5 (2.8)
Stab1	Stabilin-1 receptor	10.4 (15.7)
AT1R	Angiotensin II type 1 receptor	3.1 (4.6)
α_1AR	Alpha 1 adrenoceptor	5.2 (6.2)
α_{2a}AR	Alpha 2a adrenoceptor	6.3 (4.3)
α_{2b}AR	Alpha 2b adrenoceptor	6.8 (3.9)
α_{2c}AR	Alpha 2c adrenoceptor	10.5 (5.9)
β_1AR	Beta 1 adrenoceptor	18.3 (9.5)
M_1R	Muscarinic acetylcholine receptor M_1	6.7 (7.2)
M_2R	Muscarinic acetylcholine receptor M_2	7.6 (6.2)
M_3R	Muscarinic acetylcholine receptor M_3	13.7 (13.4)
5-HT_{2A}R	5-hydroxytryptamine receptor 2A	1.9 (2.0)
5-HT_{2B}R	5-hydroxytryptamine receptor 2B	3.7 (3.3)
5-HT_{5A}R	5-hydroxytryptamine receptor 5A	2.9 (3.6)
5-HT_7R	5-hydroxytryptamine receptor 7	2.3 (2.3)
D_{1a}R	Dopamine receptor D1a	3.1 (2.8)
D_{2s}R	Dopamine receptor D2s	2.8 (1.9)

Abbreviations: IQR = interquartile range.



Appendix Fig. B.1. Inter- and intra-assay variability in antibody measurements. Inter- and intra-assay variability shown in %. Performance was evaluated as good below 10%, acceptable below 20%, low over 20%.



Appendix Fig. C.1. Univariate correlations between antibodies, inflammation markers and troponin-T. The negative log of the q-value is on the y-axis and the r of the Pearson correlation on the x-axis. The levels of a p-value of 0.05, the FDR at Q = 0.05 are indicated. This plot illustrates the relative strength of association for antibodies with the biomarkers measured. The strongest correlations can be identified in the upper and outer corner of the graph.

Appendix Table D.1
Principal components[a] of antibodies.

Component 1		Component 2		Component 3		Component 4	
27.9%[b]		19.5%[b]		16.8%[b]		8.9%[b]	
Abs	RCL	Abs	RCL	Abs	RCL	Abs	RCL
ETBR	0.681	PAR1	0.749	VEGFR1	0.723	ETAR	0.857
α_1AR	0.776	Stab1	0.621	PAR2	0.656	AT1R	0.850
α_{2a}AR	0.723	M_3R	0.657	PDGFB	0.640		
α_{2b}AR	0.646	D_{1a}R	0.822	sENG	0.636		
α_{2c}AR	0.768	D_{2s}R	0.842	5-HT_{5A}R	0.670		
β_1AR	0.876						
M_1R	0.792						
M_2R	0.692						
5-HT_{2A}R	0.674						
5-HT_7R	0.715						

Abbreviations: Component = principal component, abs = antibodies to antigens as listed, RCL = rotated component loadings (>0.6 displayed).
[a] Categorical principal component analysis by optimal scaling with rank-discretization, varimax rotation.
[b] Percentage explained variance of each principal component.

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