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Myocardial gene expression profiles and cardiodepressant autoantibodies predict response of patients with dilated cardiomyopathy to immunoadsorption therapy

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Aims Immunoadsorption with subsequent immunoglobulin G substitution (IA/IgG) represents a novel therapeutic approach in the treatment of dilated cardiomyopathy (DCM) which leads to the improvement of left ventricular ejection fraction (LVEF). However, response to this therapeutic intervention shows wide inter-individual variability. In this pilot study, we tested the value of clinical, biochemical, and molecular parameters for the prediction of the response of patients with DCM to IA/IgG.

Methods and results Forty DCM patients underwent endomyocardial biopsies (EMBs) before IA/IgG. In eight patients with normal LVEF (controls), EMBs were obtained for clinical reasons. Clinical parameters, negative inotropic activity (NIA) of antibodies on isolated rat cardiomyocytes, and gene expression profiles of EMBs were analysed. Dilated cardiomyopathy patients displaying improvement of LVEF (≥20 relative and ≥5% absolute) 6 months after IA/IgG were considered responders. Compared with non-responders ($n = 16$), responders ($n = 24$) displayed shorter disease duration ($P = 0.006$), smaller LV internal diameter in diastole ($P = 0.019$), and stronger NIA of antibodies. Antibodies obtained from controls were devoid of NIA. Myocardial gene expression patterns were different in responders and non-responders for genes of oxidative phosphorylation, mitochondrial dysfunction, hypertrophy, and ubiquitin–proteasome pathway. The integration of scores of NIA and expression levels of four genes allowed robust discrimination of responders from non-responders at baseline (BL) [sensitivity of 100% (95% CI 85.8–100%); specificity up to 100% (95% CI 79.4–100%); cut-off value: −0.28] and was superior to scores derived from antibodies, gene expression, or clinical parameters only.

Conclusion Combined assessment of NIA of antibodies and gene expression patterns of DCM patients at BL predicts response to IA/IgG therapy and may enable appropriate selection of patients who benefit from this therapeutic intervention.

Keywords Dilated cardiomyopathy • Immunoadsorption • Gene expression • Negative inotropic activity of antibodies • Prediction of outcome • Biomarker signature • Pilot study

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Introduction

Dilated cardiomyopathy (DCM) is characterized by ventricular enlargement and impaired myocardial function and is one of the leading indications for heart transplantation.[1] Besides genetic predisposition, viral infection and myocardial inflammation play a causal role in the disease process of DCM.[1–3] Furthermore, autoimmune disorders with the activation of the cellular and humoral immune system have been implicated in the development of DCM.[3–5] Cardiac-specific antibodies have been reported in DCM patients.[4,6] Their pathogenic potential has been proved in animal models by active immunization or by transfer of antibodies against the corresponding epitopes, both leading to dilatation and dysfunction of the left ventricle.[7] Moreover, cardiac antibodies are independent predictors of disease development among healthy relatives of DCM patients.[8] Supporting the functional role of cardiac autoantibodies in DCM, the extraction of immunoglobulins from the plasma of DCM patients by immunoadsorption with subsequent immunoglobulin G substitution (IA/IgG) resulted in significant increase in cardiac index, left ventricular ejection fraction (LVEF), symptom relief,[9–12] and improvement of endothelial function.[13] Furthermore, a decrease of activated T-cells and an increase of regulatory T-cells have been shown to be associated with haemodynamic improvement after IA/IgG, revealing a link between cellular and humoral immunity.[14] However, response rates to this therapeutic intervention are characterized by a wide inter-individual variability.[12] The presence of cardiac antibodies with negative inotropic activity (NIA) has previously been shown to be associated with response to IA/IgG[15,16] and may predict the efficacy of IA/IgG. Given the high treatment costs and the invasive character of this therapeutic method, factors that predetermine differential outcome after IA/IgG are of particular interest. Therefore, a detailed analysis of patients and development of prognostic tests that may combine clinical and molecular information for the prediction of therapeutic efficacy remain important challenges.

In recent years, molecular classifiers for the prediction of outcome were primarily developed for cancer patients[17] but are not restricted to this disease entity. Predictors of outcome of patients with suspected myocarditis have been developed using a combination of different clinical parameters and gene expression patterns.[18] Furthermore, transcriptomic approaches have been used for the accurate diagnosis of myocarditis[19] and the identification of classifiers for individual risk assessment in new-onset heart failure.[20] The prediction of response to IA/IgG would enable selective treatment of a subgroup of DCM patients.

In this pilot study, we measured NIA of cardiac antibodies of 40 DCM patients on rat cardiomyocytes and profiled myocardial expression patterns using endomyocardial biopsies (EMBs) of the same patients at baseline (BL). Subsequently, the association of NIA and gene expression patterns with the improvement of LVEF (ΔLVEF) after IA/IgG was investigated.

Methods

Study design

Between January 2004 and May 2008, 162 DCM patients with LV dysfunction (LVEF $< 45\%$), as well as with symptoms of chronic heart failure according to New York Heart Association (NYHA) classifications II and III underwent IA/IgG in the University Hospital Greifswald. IA/IgG was not performed if patients suffered from acute infectious diseases, cancer, chronic alcoholism, postpartum cardiomyopathy, or heart failure due to other known origins (e.g. primary valvular disease). Coronary heart disease was excluded by angiography at BL before IA/IgG; acute myocarditis was excluded by histopathological analysis of EMBs in accordance with the Dallas criteria.[21]

From 40 DCM patients, sufficient EMB material obtained at BL was available for RNA extraction, and these patients received stable oral medication for at least 3 months before inclusion into the study and throughout the whole study period, comprising angiotensin-converting enzyme (ACE)-inhibitors or angiotensin receptor antagonists, beta-blockers, aldosterone antagonists, digitalis, and diuretics.

The control group consisted of eight patients with normal LV systolic function from which EMBs were taken for clinical reasons due to suspicion of myocarditis. In these EMBs, myocardial inflammation and virus persistence were excluded. These patients also received echocardiography examinations, and coronary angiography, which yielded normal results and excluded significant cardiac disease.

The investigation conforms to the principles of the Declaration of Helsinki. Written informed consent was obtained from each patient, and the protocol was approved by the Ethics Committee of the University of Greifswald, Germany.

Immunoadsorption and subsequent immunoglobulin G substitution

Immunoadsorption was performed on five consecutive days using protein-A columns (Immunosorba®, Fresenius Medical Care AG, Bad Homburg, Germany) with an improved treatment regime for IgG-3 reduction as described elsewhere.[11,12] After the final immunoadsorption session, patients received 0.5 g/kg polyclonal IgG (Venimmun N®, Sandoglobulin®, CSL Behring, Germany) to restore IgG plasma levels.[11,12] Patients displaying an increase in LVEF $\geq 20\%$ relative to the BL value and, in addition, an increase of $\geq 5\%$ of the absolute value were classified as responders ($n = 24$).

Echocardiography

Echocardiographic parameters [LVEF according to Simpson's rule and LV internal diameter at diastole (LVIDd)] were determined by two-dimensional echocardiography at BL and follow-up (FU) 6 months after IA/IgG. The reading of the echocardiographic images was performed by two independent physicians who were unaware of the clinical variables of the patients. Intra-reader, intra-observer, inter-reader, and inter-observer agreements of all LVEF measurements revealed Spearman's correlation coefficients of >0.85 and differences in mean (± 2 SD) of $<5\%$ ($<25\%$).

Histological and immunohistological analyses and detection of viral genomes

For the detection of viral genomes in myocardial biopsies, nested PCR/RT-PCR was performed as described previously.[22] Myocarditis was diagnosed by routine histological staining according to the Dallas criteria. In addition, immunohistochemical analyses were performed for the identification of cardiac immune cells (CD3+ T lymphocytes and/or CD68+ macrophages) and measurement of human leucocyte antigen class II expression as described elsewhere.[12,18,22]

Preparation of plasma immunoglobulin G

Immunoglobulin G was isolated from serum samples at BL in case of DCM patients or at the time of presentation in case of controls as

described earlier.[15] Briefly, serum samples were filtered through anti-IgG Sepharose (PlasmaSelect, Teterow, Germany), dialysed against experimental buffer, and incubated for 30 min at 57°C for the denaturation of complement factors.

Detection of negative inotropic activity of cardiac autoantibodies by measurement of cell shortening in isolated rat cardiomyocytes

Ventricular cardiomyocytes from adult Wistar rats (RCM) were isolated as described elsewhere.[15] Single cardiomyocytes were field-stimulated (1 Hz, 5 ms) and superfused continuously with experimental buffer. Cell length of cardiomyocytes was continuously measured (120 images/s) by fluorescence microscopy (IonOptix, Milton, MA, USA). Inotropic activity of IgG from patients (0.3 g/L) was determined by measuring the change in maximum cell shortening of single cardiomyocytes during IgG superfusion compared with the BL value as described elsewhere.[15,16] Mean values were calculated from at least five independent measurements.

Transcriptome analyses

RNA was isolated from frozen EMBs (−80°C) following the manufacturer's instructions for total RNA isolation from fibrous tissues (RNeasy® Micro Kit, Qiagen, Inc., Valencia, CA, USA). After purification and quality assessment, transcriptional profiling of EMBs was performed with GeneChip-Human Genome-HG U133-Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA) and validated for a subset of genes by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Extensive validation of array data by qRT-PCR was not possible because of limited RNA availability (see Supplementary material online, *Figures S1–S3* and *Table S1*). Expression data have been submitted to GEO.

Statistical analyses

Data are expressed as mean values with standard deviation. The Mann–Whitney test, Fisher's exact test, the Wilcoxon signed rank test, Pearson's chi-squared test, and Spearman's correlation were used for appropriate comparisons. A *P*-value of <0.05 was considered significant for comparisons and correlations.

Multivariate linear regression analysis was performed using the *lm* function of software R 2.4.1 (http://www.R-project.org). All available clinical parameters known to potentially influence the outcome were added to the model (*Table 3*).

Differentially expressed genes were determined in Rosetta Resolver® 7.2 (Ceiba Solutions, Seattle, WA, USA) by comparison of each

Table 1 Baseline characteristics of heart failure patients

	Control ($n = 8$)	Responder ($n = 24$)	Non-responder ($n = 16$)	Responder vs. non-responder *P*-value
Age (years) ± SD[a]	43 ± 14	49 ± 10	52 ± 9	0.391[b]
Gender (♂/♀)	6/2	16/8	12/4	0.729[c]
LVEF (%) ± SD[a]	60 ± 8	33 ± 6	34 ± 7	0.719[b]
LVIDd (mm) ± SD[a]	51 ± 3	67 ± 7	74 ± 8	0.014[b]
NYHA classification (*n*)				0.755[c]
II	0	12	9	
III	0	12	7	
Disease duration (months) ± SD[a]		16 ± 18	52 ± 49	0.006[b]
Body mass index (kg/m^2) ± SD[a]	26.3 ± 5	27.9 ± 5	27.9 ± 4	0.858[b]
Inflammation positive[d] (*n*)	0	17	10	0.733[c]
Virus PVB19/PVB19+HHV6/other[e] (*n*)	0/0/0	4/1/8	4/0/5	
Medication (*n*)				
β-Blocker (% of optimal doses)		24 (65.4 ± 9.4)	16 (61.9 ± 10.0)	1.00[c] (0.81[b])
ACE-inhibitors and/or AT1 antagonists (% of optimal doses)		19 and/or 7 (69.9 ± 6.9)	14 and/or 5 (70.5 ± 7.0)	0.681[c] and/or 1.00[c] (0.92[b])
Aldosterone antagonists (% of optimal doses)		16 (62.5 ± 5.7)	9 (64.3 ± 5.6)	0.527[c] (0.80[b])
Diuretics		24	16	1.00[c]
Digitalis		2	6	0.04[c]

LVEF, left ventricular ejection fraction; LVIDd, left ventricular inner diameter at diastole; NYHA, New York Heart Association; PVB19, parvo virus B19; ACE, angiotensin-converting enzyme; AT1, angiotensin 1.
[a]Mean values with standard deviation (SD) are shown.
[b]The Mann–Whitney test, two-tailed.
[c]Fisher's exact test, two-tailed.
[d]Myocardial biopsies were considered to be inflamed if immunohistochemistry revealed focal or diffuse mononuclear infiltrates with more than 14 leucocytes per square millimetre (CD3+ T-lymphocytes and/or CD68+ macrophages) in addition to enhanced expression of HLA class II molecules.[18,22]
[e]Other virus types: HHV6, human herpes virus 6; EBV, Epstein–Barr virus; Enterovirus; ACE, angiotensin-converting enzyme; AT1, angiotensin II receptor subtype 1.

Table 2 Longitudinal characteristics of IA/IgG population

	Responder ($n = 24$)			Non-responder ($n = 16$)		
	BL	FU	*P*-value[a]	BL	FU	*P*-value[a]
LVEF (%) ± SD[b]	33 ± 6	46 ± 7	<0.001	34 ± 7	34 ± 9	0.689
LVIDd (mm) ± SD[b]	67 ± 7	62 ± 7	<0.001	74 ± 8	73 ± 8	0.408
ΔLVEF (%) ± SD[b]		13 ± 6			0.3 ± 4	<0.001[c]
NYHA classification (*n*)			0.007[d]			0.238[d]
I	0	7		0	2	
II	12	12		9	10	
III	12	5		7	4	

LVEF, left ventricular ejection fraction; LVIDd, left ventricular inner diameter at diastole; NYHA, New York Heart Association.
[a]*P*-value baseline (BL) vs. follow-up (FU) of responders and non-responders is based on the Wilcoxon signed rank test, two-tailed.
[b]Mean values with standard deviation (SD) are shown.
[c]*P*-value of ΔLVEF of responders vs. non-responders is based on the Mann–Whitney test, two tailed.
[d]Pearson's chi-square test.

subgroup (responder, non-responder) vs. control using *t*-test and multiple test correction (Benjamini–Hochberg) ($q < 0.05$). Ingenuity Pathway Analysis Version 8.6 (Ingenuity Systems, Redwood City, CA, USA) was used for functional assignments of differentially expressed genes.

For the development of a predictive signature, we used two independent approaches relying on a support vector machine (SVM) and a random forest (RF) analysis.[23] The top 25 genes of the two independent approaches were compared and the 4 overlapping genes were used as a molecular signature for the prediction of responders to IA/IgG. Based on the prediction of these four genes, NIA of antibodies and their combination was checked for robustness by adding random noise of various magnitudes to the original data (see Supplementary material online).

Results

Forty patients undergoing IA/IgG were examined at BL and FU. Patients were classified as responders (LVEF ≥20% relative to the BL and ≥5% absolute, $n = 24$) and non-responders according to the improvement of myocardial function after IA/IgG. Clinical BL characteristics of all patients are summarized in *Table 1*. Disease duration ($P = 0.006$) and LVIDd ($P = 0.014$) were higher in non-responders than in responders (*Table 1*). The immunohistochemistry and virology findings from EMBs of responders and non-responders did not differ significantly.

Follow-up characteristics of dilated cardiomyopathy patients

Responders exhibited an increase in LVEF from 33 ± 5.7 to 46 ± 6.7% ($P < 0.001$) and a decrease in LVIDd from 67 ± 6.8 to 62 ± 7.4 mm ($P < 0.001$) (*Table 2*). Left ventricular ejection fraction and LVIDd did not change significantly in non-responders during FU (see Supplementary material online, *Figure S6*). The NYHA classification improved in both subgroups. However, the improvement was stronger in responders ($P < 0.007$) compared with non-responders ($P = 0.238$) (*Table 2*).

Table 3 Association of haemodynamic improvement (ΔLVEF) with clinical parameters calculated by multivariate regression analysis[a]

Parameter	β	SE	*P*-value
Gender (♂/♀)	2.10	6.78	0.759
Age (years)	0.19	0.33	0.573
Body mass index (kg/m^2)	−0.48	0.71	0.513
Disease duration (months)	−0.26	0.08	0.002
Inflammation (positive)	0.27	0.13	0.049
LVIDd (mm)	−1.36	0.41	0.003
LVEF (%)	−1.53	0.49	0.004

Linear regression models with the change in left ventricular ejection fraction (ΔLVEF) as a dependent variable. Adjustments were made for gender, age, body mass index, disease duration, presence of inflammation, left ventricular inner diameter at diastole (LVIDd), and LVEF.
β, effect size; SE, standard error.
[a]Before multiple regression analysis was performed, residuals were tested for outliers, which however were not detected (see Supplementary material online).

Clinical parameters at baseline and association with haemodynamic improvement

Only a subgroup (60%) of patients demonstrated a significant improvement of LVEF after IA/IgG, which is in agreement with a previous study of a larger cohort.[15] Multiple regression analysis revealed that disease duration ($P = 0.002$), inflammation ($P = 0.049$), LVIDd ($P = 0.003$), and LVEF at BL ($P = 0.004$) were significant determinants of ΔLVEF after adjustment for all other covariates (*Table 3*).

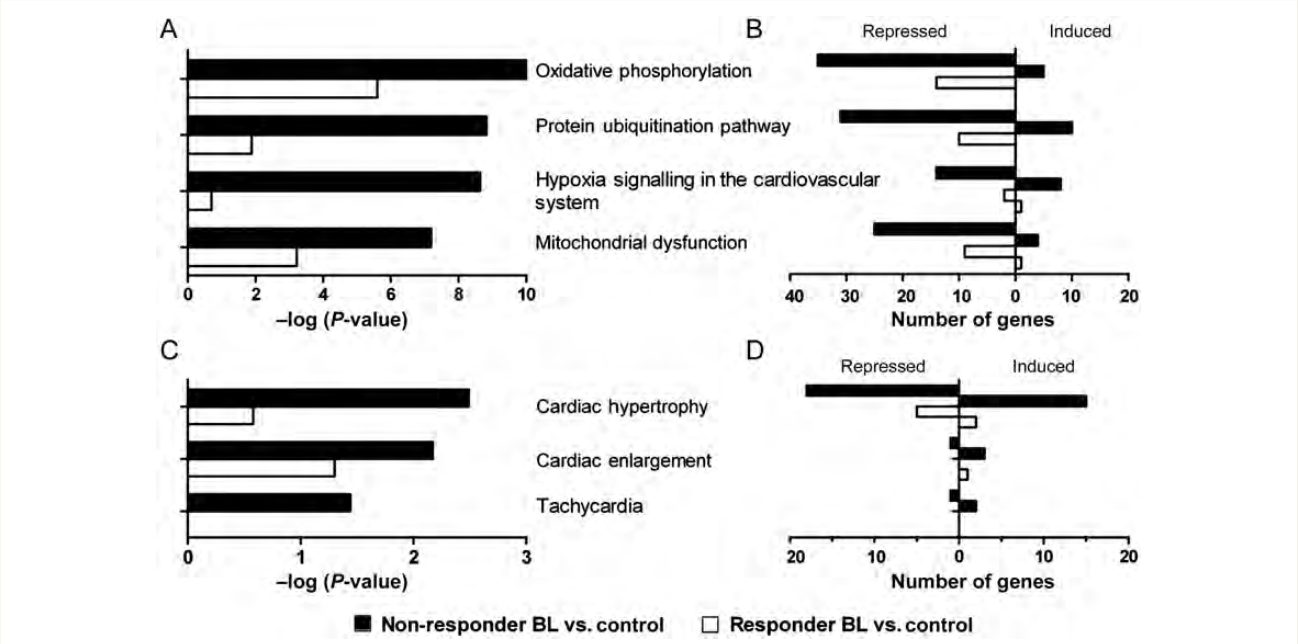

Figure 1 Functional assignment of genes differentially expressed in responders and non-responders compared with control individuals with normal left ventricular ejection fraction. Significance ($-\log P$-value) of the association, which is dependent on the number of genes in the class, for canonical pathways (*A*) and toxic functions in the heart (*C*) as assigned by Ingenuity Pathway Analysis version 8.6. Numbers of genes repressed and induced in comparison with the control group are displayed (*B* and *D*).

Myocardial gene expression in responders and non-responders in comparison with control individuals

Compared with the control group of subjects with normal LVEF, the expression profile of responders differed in 208 genes ($q < 0.05$) (see Supplementary material online, *Table S2* and *Figure S7*), whereas non-responders were characterized by more extensive differences (867 genes, $q < 0.05$, see Supplementary material online, *Tables S3* and *S5* and *Figure S7*). In comparison with controls, elevated expression of genes coding for common heart failure markers such as natriuretic peptides NPPB and NPPA as well as endothelin (EDN1) or angiotensin I-converting enzyme 2 (ACE2) (see Supplementary material online, *Figure S8*) were found in both responders and non-responders.

Functional assignment of genes displaying different expression in responders and non-responders compared with controls revealed major changes in genes involved in oxidative phosphorylation/mitochondrial dysfunction, the protein ubiquitination pathway, and hypoxia (*Figure 1A*). However, in all these categories, more genes displayed altered expression levels in non-responders at BL compared with responders (*Figure 1B*). Likewise, alterations in expression levels of genes associated with hypertrophy were more pronounced in non-responders than in responders (*Figure 1C* and *D*).

Negative inotropic activity of cardiac autoantibodies

The presence of negative inotropic cardiac antibodies has previously been shown to be associated with response to IA/IgG.[15,16]

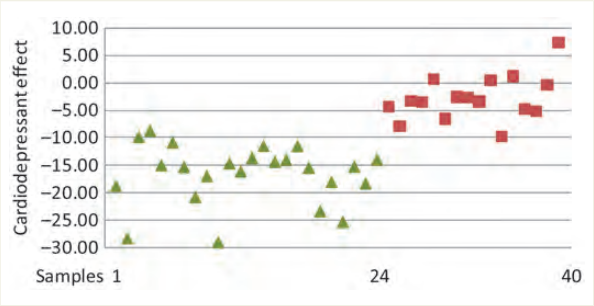

Figure 2 Negative inotropic activity of cardiac autoantibodies in responders and non-responders. Negative inotropic activity was determined by measuring percentage change of maximum cell shortening of RCM during immunoglobulin G superfusion compared with the baseline value. Green, responders; red, non-responders.

Thus, NIA of antibodies was determined in this patient cohort and the respective controls. Immunoglobulin G purified from plasma of controls did not induce a negative inotropic reaction in isolated RCM (relative change to the BL value of cell shortening: $2.0 \pm 5.7\%$), whereas IgG of DCM patients showed a significant NIA (range: -29.2 to 7.5; mean: $-11.1 \pm 8.4\%$, $P < 0.001$ vs. controls). Furthermore, stronger NIA was observed after the treatment of isolated RCM with antibodies from responders ($-16.7 \pm 5.3\%$) than with those from non-responders ($-2.8 \pm 4.0\%$, *Figure 2*, responders vs non-responders $P < 0.0001$).

Identification of predictive parameters for response to IA/IgG

In order to assess the relevance of clinical, biochemical, and molecular parameters for classification at BL, the correlation of the values of the individual patient to the average of responders (responder template) and non-responders (non-responder template) was determined using the leave one out method[17] (see Supplementary material online). The validity of the classification of patients increases with the degree of: (i) positive correlation to the template of the group the patient belongs to (maximum value 1) and (ii) negative correlation with the other template (minimum value −1).

A combination of the four clinical parameters (disease duration, inflammation, LVIDd, and LVEF) which significantly determined ΔLVEF did not allow reliable discrimination between responders and non-responders at BL (cut-off value 1) because similar correlation patterns were generated irrespective of the use of the responder or non-responder template (*Figure 3A*).

Since gene expression was distinctively different in responders and non-responders when compared with controls, we used two independent methods, an SVM algorithm and an RF analysis to develop a robust classifier which might distinguish responders and non-responders before the start of therapy. Four genes [ras-related nuclear binding protein 1 (RANBP1), regulator of G-protein signaling 10 (RGS10), ubiquitin protein ligase E3B

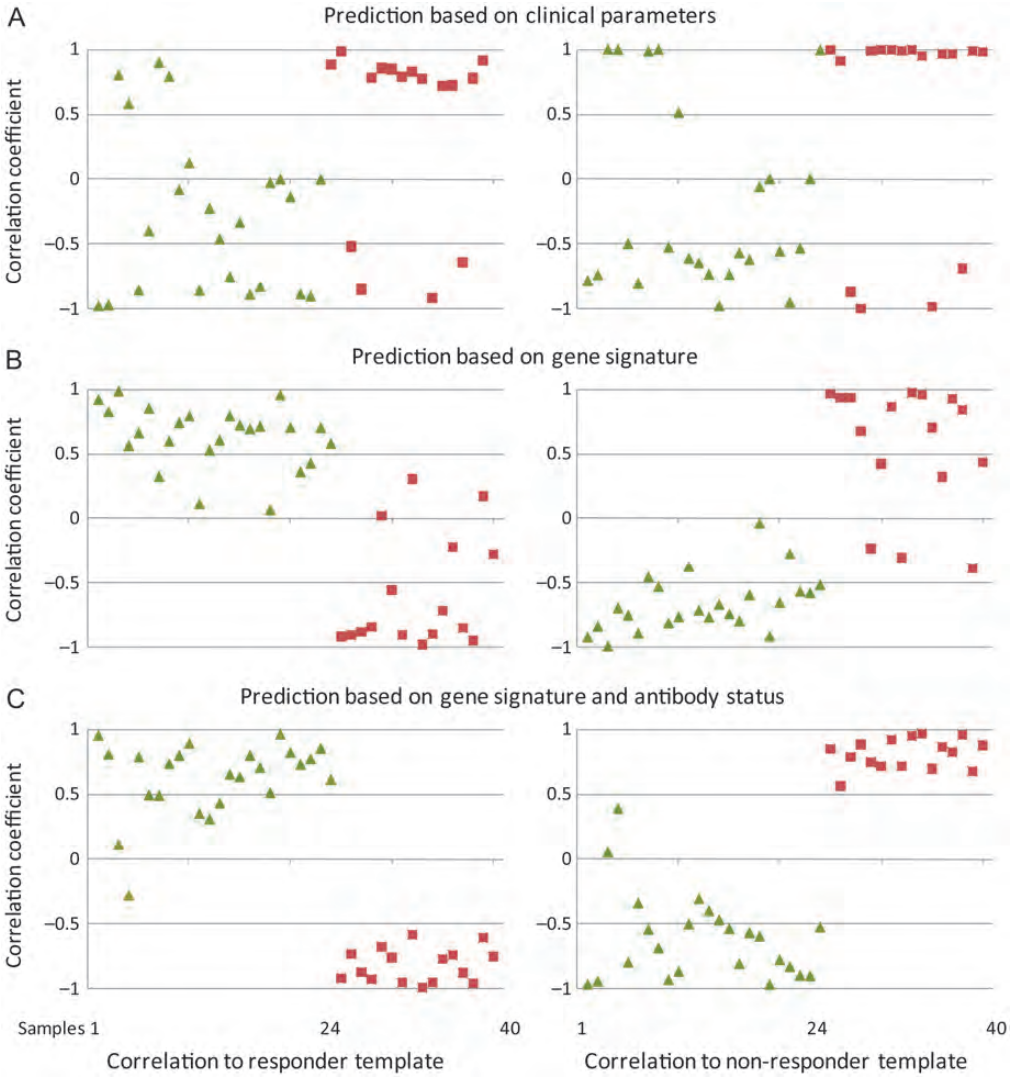

Figure 3 Assessment of the value of clinical parameters (*A*), gene signature (*B*), and a combination of gene signature and antibody status (*C*) for the classification of responders and non-responders at BL. The correlation of the individual patients to the responder template is displayed in the left column, and that to the non-responder template in the right column. Green, responders; red, non-responders. Validity of the classification of patients into responders or non-responders increases with the degree of positive correlation to the corresponding template (maximum value 1) and negative correlation with the other template (minimum value −1).

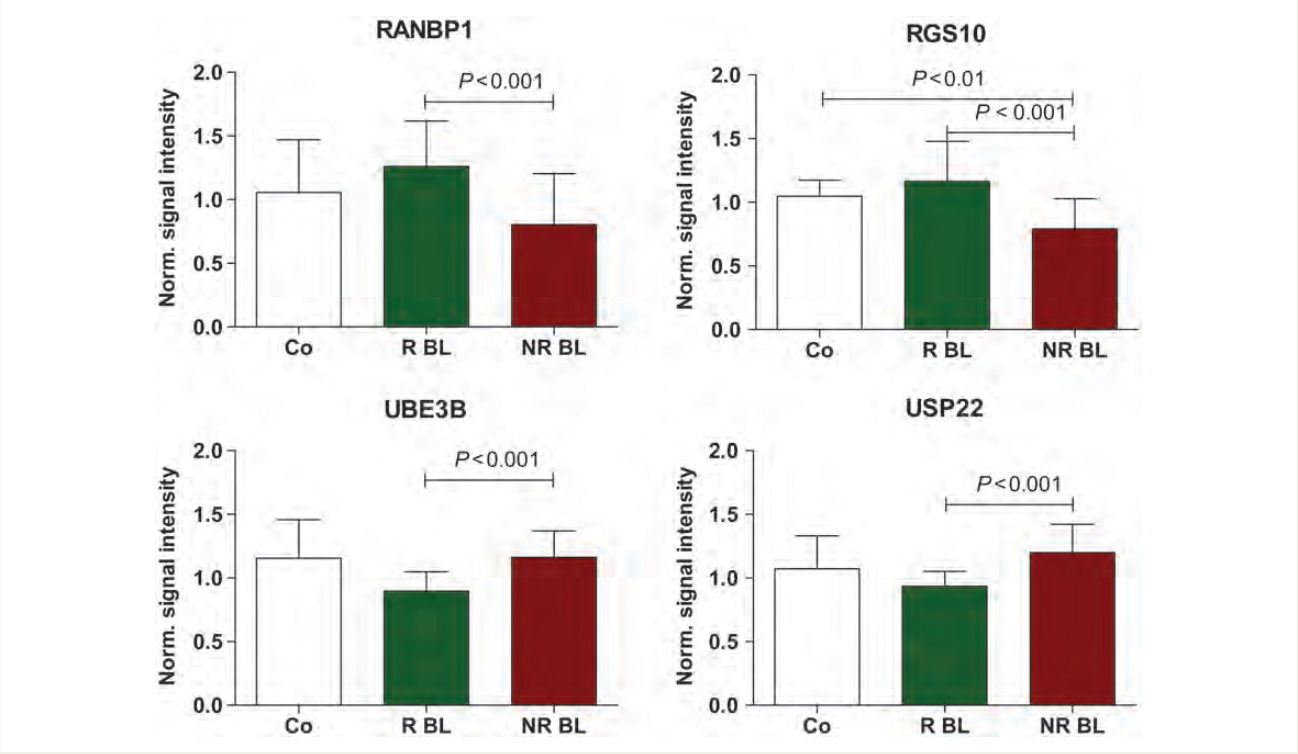

Figure 4 Expression patterns of the four signature genes commonly identified with the support vector machine and random forest analysis. The mean of normalized signal intensities and the standard deviation of expression values of genes coding for RANBP1 (ras-related nuclear binding protein 1), RGS10 (regulator of G-protein signaling 10), UBE3B (ubiquitin protein ligase E3B), and USP22 (ubiquitin specific peptidase 22) (*P*-value, the Mann–Whitney test) are displayed for controls (Co, $n = 8$, open bars), responders (R, $n = 24$, green bars), and non-responders (NR, $n = 16$, red bars) at baseline (BL).

(UBE3B), and ubiquitin specific peptidase 22 (USP22), *Figure 4*] were consistently identified as good predictors by the two different algorithms. This discriminating four-gene signature revealed a much better prediction performance than clinical parameters (correlation coefficient cut-off value 0.33 instead of 1 for clinical parameters, *Figure 3B* and Supplementary material online, *Figure S9*). Here, responders displayed a good correlation to the responder template and, as expected, anti-correlation to the non-responder templates, respectively. However, prediction performance was lower for non-responders, because a subgroup of those patients did not display the expected correlation with the non-responder template and anti-correlation with the responder template. By far, the best prediction was accomplished when gene signature and NIA of autoantibodies were combined (*Figure 3C*), because clear assignments to the groups of responders and non-responders could be accomplished with both templates for all but one patient (correlation coefficient cut-off value −0.28).

Furthermore, NIA of antibodies showed strong positive correlation to the expression level of genes encoding proteins involved in ubiquitination, i.e. two of the four predictive genes USP22 (rho = 0.54, $P = 0.001$) and UBE3B (rho = 0.59, $P = 7.8E-5$).

Prediction values were calculated for all analyses in comparison with the responder template and plotted in an ROC curve (see Supplementary material online, *Figure S9*). At a sensitivity of 100% (95% CI 85.8−100%), which ensured the selection of all responders among the patients, specificity up to 100% (95% CI 79.4−100%) was achieved when information on molecular signature and NIA of antibodies was combined (see Supplementary material online, *Figure S9*).

The evaluation of the robustness of the prediction by an independent test set was not feasible due to limited number of EMBs, which could not be increased given the invasiveness of this procedure. Therefore, the variation of values in the population was simulated by adding incrementally increasing measurement errors to the data available and by assessing the effect of these errors on classification. *Figure 5* illustrates that the sensitivity was more error-tolerant when the combined information of molecular signature and NIA of antibodies were exposed to increasing variations in values. The combined score allowed much better assessment of responders than molecular signature or NIA of antibodies alone, which is important in order to assure appropriate identification of patients who could benefit from IA/IgG.

Discussion

In this pilot study, we related the response to IA/IgG treatment of DCM patients to whole-genome expression profiles of myocardial biopsies in addition to common clinical BL characteristics. The extensive alterations in the gene expression of non-responders compared with control individuals and the specific classes of genes

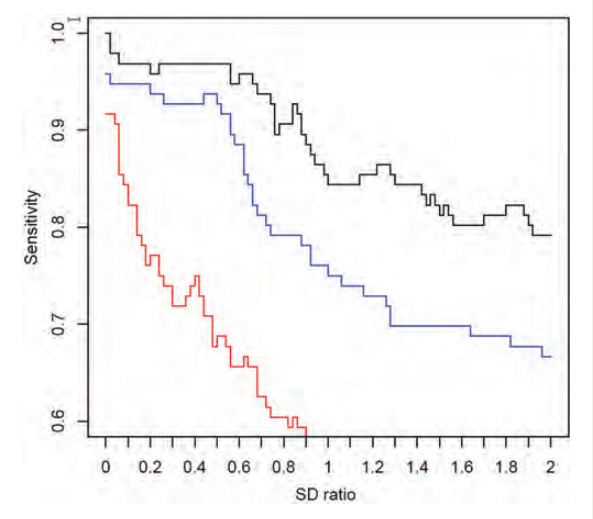

Figure 5 Simulation of robustness of the prediction of therapy outcome. The robustness of the prediction based on the expression of the four signature genes, negative inotropic activity of antibodies, and their combination was determined by adding a random noise to the parameter values of each sample prior to the classification to simulate the variation of values in the population. Added random noise is displayed on the *X*-axis as fold-values of the standard deviation (SD). Predictions are based on the expression level of four signature genes (red line), negative inotropic activity of antibodies (blue line), and a combination of both values (black line).

identified fit well with more advanced disease stages in non-responders compared with responders. Furthermore, the combination of individual profiles of gene expression and NIA of antibodies predicts IA/IgG outcome. These findings may facilitate early identification of individuals with high odds of response to IA/IgG treatment and thus may permit individualized therapeutic approaches.

Potential beneficial effects by various treatment strategies such as immunosuppression or immunomodulation with IgG have been reported for the therapy of DCM and heart failure.[24,25] Furthermore, several randomized pilot studies showed that the removal of cardiac autoantibodies with IA/IgG may be an effective therapeutic principle for DCM treatment.[9–11] With respect to invasiveness, high treatment costs, and wide variability in response to IA/IgG,[15,16] the identification of patient-specific factors that may predict clinical efficacy of this treatment was of particular interest in the current study. We have previously reported that the presence of negative inotropic cardiac antibodies in the plasma of DCM patients is associated with the beneficial response to IA/IgG.[15,16] Clinical parameters may be used as prognostic factors of heart failure patients as well. Interestingly, Kindermann *et al.*[18] have shown that NYHA functional class or immunohistological evidence of myocardial inflammation and lack of β-blocker therapy are good predictors of outcome for patients with suspected myocarditis. In agreement with a previous report,[12] our study showed that parameters such as LVIDd, LVEF, and the presence of inflammation at BL, as well as disease duration were also associated with LVEF improvement. However, even if disease duration clearly correlated with LVEF improvement (rho = 0.4; $P = 0.012$) and responders displayed shorter mean disease duration than non-responders (16 ± 18 months vs. 52 ± 49 months), non-responders also include patients with short disease duration (see Supplementary material online, *Figure S10*). Accordingly, neither disease duration alone (Supplementary material online, *Figure S11*) nor combination with the presence of inflammation, LVIDd, and LVEF at BL permitted reliable classification of patients to the groups of responders or non-responders (*Figure 3A*).

Extending the application of transcriptional analyses for accurate diagnosis of myocarditis[19] and differentiation of patients with several diseases,[26,27] we used gene expression profiles to predict response to IA/IgG therapy in DCM patients. Molecular classifiers have already been developed by genome-wide transcriptional profiling with rather small sample sets[19,20,28] but are always challenging because of overfitting. In accordance with previous studies showing that classifiers become more robust if more than one bioinformatic tool is used for the selection of prognostic genes,[19,29] we combined SVM and RF analysis and identified a molecular classifier based on the expression of the four discriminating genes RANBP1, RGS10, UBE3B, and USP22. The expression pattern of this molecular signature did not correlate with disease duration (see Supplementary material online, *Figure S12*), but strongly with the autoantibody status of the patients. Consequently, the most distinct discrimination of responders and non-responders was accomplished when a combined score of this molecular classifier and NIA of antibodies was used (*Figure 3C*). The increased value of combined biomarkers compared with single ones is already well recognized, e.g. for the prognosis of Alzheimer disease.[30] The bioactivity of cardiodepressant antibodies had a substantial impact on the classifier (*Figures 2* and *3*), which is in agreement with previous observations demonstrating that this parameter is associated with a better response to IA/IgG.[12,15] Especially in the staging of autoimmune diseases, the possible role of autoantibodies as biological markers is under investigation.[31] Negative inotropic activity of antibodies was a very potent predictor of response to IA/IgG emphasizing the functional role of antibodies in a subset of DCM patients. The beneficial effects of IA/IgG might be not directly associated with the selective elimination of autoantibodies directed against a particular cardiac epitope but with the removal of IgG3 subclass antibodies in general.[32] In this context, Baba *et al.*[33] postulated a relation of LVEF improvement to the degree of autoantibody elimination in DCM patients who underwent IgG3 removal.

Whole-genome expression patterns cannot only be used for classification but additionally provide insights into the molecular differences between responders and non-responders. This is particularly important because in our study common heart failure markers such as natriuretic peptides (NPPB, NPPA) displayed diminished expression in both subgroups. However, in non-responders a considerably larger number of genes displayed differential expression compared with controls than in responders. Particularly, low expression of genes encoding subunits of different respiration complexes was observed and might indicate more

pronounced energy limitation due to impaired ATP synthesis in non-responders compared with responders, which has been described in rat models with ongoing heart failure.[34] Also, hypertrophy-associated gene expression was profoundly influenced in non-responders, but only to a minor degree in responders. These expression patterns probably reflect advanced disease states in patients who seem to be related to a lower likelihood to benefit from IA/IgG. Thus, compared with responders, the molecular changes in non-responders seem to resemble, to a greater extent, those described for heart failure patients who display impairment of energy metabolism, changes of the extracellular matrix, hypertrophy, and altered Ca^{2+} handling.[35]

A direct comparison of responders and non-responders revealed significant differences in expression for a large set of genes encoding ligases and proteases of the ubiquitin–proteasome system (UPS), probably indicating differences in the protein turnover of both subgroups. Eighty to 90% of intracellular proteins are degraded by the UPS, and precise tuning of protein turnover seems to be pivotal for normal cardiac function.[36] Increased levels of mRNA and proteins of the UPS have been reported in animal models, human DCM hearts,[37] and in end-stage heart failure.[36] These alterations correlated to oxidative stress and misfolded proteins[38] which require adjustments in proteolysis mechanisms. In non-responders, we found higher expression of some E2 components and a set of ubiquitin-specific proteases compared with responders, probably indicating the requirement for increased proteolysis. The deregulation of UPS components also seems to play an important role in heart failure progression by regulating the stability of apoptosis regulators such as p53.[39] Two genes of this functional class, UBE3B and USP22, belong to the predictive gene signature.

Another molecule of the classifier, RGS10, has recently been shown to act as GTPase-activating protein on G-protein species that mediates the activation of atrial G protein-coupled inwardly rectifying potassium channels. Moreover, RGS10, via protein kinase A-dependent phosphorylation, enables a crosstalk between beta-adrenergic and muscarinic cholinergic signalling.[40] The lower expression in non-responders in comparison with responders suggests a disturbance in these signalling pathways.

Clinical implications

Molecular and biochemical analyses such as those illustrated in this study may, on one side, provide new insights in disease pathophysiology and, on the other, allow the prediction of potential myocardial recovery of patients with DCM and thus constitute important components for the development of therapeutic approaches in individualized medicine.

Limitations

This study was designed as a pilot study. The authors are aware that microarrays will likely not be applicable in the daily clinical practice. However, microarray- or, in the future, RNA-Seq-based technologies constitute powerful screening approaches for unbiased identification of candidates, which will subsequently have to be validated by other techniques more suitable for standard clinical screening of small numbers of molecules such as qRT-PCR (Supplementary online material, *Figure S1*). Owing to the invasiveness of myocardial biopsies, the study population is confined to a limited sample size and no replication cohort is currently available.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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