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Original research

FOXO3A acts as immune response modulator in human virus-negative inflammatory cardiomyopathy

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ABSTRACT

Objective Inflammatory cardiomyopathy is characterised by inflammatory infiltrates leading to cardiac injury, left ventricular (LV) dilatation and reduced LV ejection fraction (LVEF). Several viral pathogens and autoimmune phenomena may cause cardiac inflammation.
The effects of the gain of function *FOXO3A* single-nucleotide polymorphism (SNP) rs12212067 on inflammation and outcome were studied in a cohort of patients with inflammatory dilated cardiomyopathy (DCMi) in relation to cardiac viral presence.
Methods Distribution of the SNP was determined in virus-positive and virus-negative DCMi patients and in control subjects without myocardial pathology. Baseline and outcome data were compared in 221 virus-negative patients with detection of cardiac inflammation and reduced LVEF according to their carrier status of the SNP.
Results Distribution of SNP rs12212067 did not differ between virus-positive (n=22, 19.3%), virus-negative (n=45, 20.4%) and control patients (n=18, 23.4%), indicating the absence of susceptibility for viral infection or inflammation per se (p=0.199). Patients in the virus-negative DCMi group were characterised by reduced LVEF 35.5% (95% CI 33.5 to 37.4) and increased LVEDD (LV end-diastolic diameter) 59.8 mm (95% CI 58.5 to 61.2). Within the group, SNP and non-SNP carriers had similarly impaired LVEF 39.2% (95% CI 34.3% to 44.0%) vs 34.5% (95% CI 32.4 to 36.5), p=0.083, and increased LVEDD 58.9 mm (95% CI 56.3 to 61.5) vs 60.1 mm (95% CI 58.6 to 61.6), p=0.702, respectively. The number of inflammatory infiltrates was not different in both SNP groups at baseline. Outcome after 6 months showed a significant improvement in LVEF and clinical symptoms in SNP rs12212067 carriers 50.9% (95% CI 45.4 to 56.3) versus non-SNP carriers 41.7% (95% CI 39.2 to 44.2), p≤0.01. The improvement in clinical symptoms and LVEF was associated with a significant reduction in cardiac inflammation (ΔCD45RO⁺ p≤0.05; ΔMac-1⁺ p≤0.05; ΔLFA-1⁺ p≤0.01; ΔCD54⁺ p≤0.01) in the SNP cohort versus non-SNP cohort, respectively. Subgroup analyses identified ΔMac-1⁺, ΔLFA-1⁺, ΔCD3⁺ and Δperforin⁺ as predictors for improvement in cardiac function in SNP-positive patients.
Conclusion FOXO3A might act as modulator of the cardiac immune response, diminishing cardiac inflammation and injury in pathogen-negative DCMi.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ FOXO3A is a transcription factor involved in immunoregulation.
⇒ That has been shown to suppress inflammation in viral inflammatory dilated cardiomyopathy (DCMi) leading to unfavourable outcome.

WHAT THIS STUDY ADDS

⇒ Gain of function FOXO3 single-nucleotide polymorphism rs12212067 is associated with attenuated inflammation and improvement of clinical symptoms, as well as remodelling in virus-negative DCMi.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Modulation of FOXO3 activity might be a useful therapeutic target for inflammatory heart disease.

INTRODUCTION

With its helix-loop-helix DNA-binding domain, *FOXO3A* is involved in an abundance of cellular processes, such as cell cycle regulation, apoptosis, oxidative stress, angiogenesis and immunity.[1] The activation of FOXO3A is regulated via post-translational modifications. Growth factors lead to phosphorylation/inactivation of FOXO3A via the phospho-inositol-3-kinase (PI3K/Akt) pathway inducing its cytoplasmic sequestration and degradation.[2] Oxidative stress or glucose phosphorylate FOXO3A via the AMP-activated protein kinase (AMPK) pathway on specific consensus sites leading to its activation and differential target gene expression. Moreover, sirtuins have been implicated in *FOXO3A* transcription. Although FOXO3A proteins are expressed by immune cells, their physiological role in immune responses during an infection is still not fully understood. Single nucleotide polymorphisms (SNPs) in the *FOXO3A* gene are associated with longevity, a better self-rated health and low prevalence of cardiovascular diseases in independent populations.[3] Importantly, the human SNP rs12212067 in *FOXO3A* was recently shown to be associated with an increased risk for pathogen-induced inflammatory disorders but with

a milder course in patients with autoimmune disease-induced inflammation.[4]

The immune-modulatory function of FOXO3A has been elucidated to some extent: studies in *Foxo3a*-deficient mice showed an association with sustained proliferation and survival of T cells, as well as spontaneous autoimmunity due to increased nuclear factor kappa B (NF-κB) activity, a downstream target of Foxo3a. Foxo3a acts as negative regulator of cell responses in CD8$^+$ T cells[5] and decreased expression of Foxo3a stimulates CD8$^+$ T cells to differentiate into cytotoxic CD8$^+$ T cells.[6] Cooperatively, Foxo3a and Foxo1 control the differentiation of regulatory T cells via the protein kinase C/Akt pathway and transforming growth factor beta (TGF-β), thereby maintaining immune tolerance. Moreover, Foxo3a prevents naive T cells to acquire T effector functions. In immature B cells, Foxo3a promotes apoptosis and diminishes auto-antibody generation. In macrophages, Foxo3a negatively regulates interleukin-10 (IL-10), which in turn regulates cell polarisation and subsequent adaptive immune responses.

Studies from our group and others have previously shown that Foxo3a plays an important role in cardiac hypertrophy, cardiomyocyte survival, cell differentiation and remodelling. Furthermore, the *FOXO3A* gain of function SNP rs12212067 diminished the innate immune response resulting in delayed viral clearance, increased cardiac inflammation and attenuated improvement of left ventricular ejection fraction (LVEF) indicating worsened prognosis in mice with coxsackievirus B3-induced myocarditis and patients with virus-positive inflammatory cardiomyopathy (DCMi).[7] These effects were at least in part due to modulation of natural killer (NK) cell differentiation and activation, and reduced interferon-γ (IFN-γ) expression leading to an attenuated innate immune response in wild-type mice and SNP carriers.[7]

Active chronic cardiac inflammation due to post-viral antigen mimicry or cardiac involvement in systemic autoimmune disease is often seen in patients presenting with virus-negative DCMi. In these patients, viral pathogens are not present in endomyocardial specimens characterised by mononuclear inflammatory infiltrates.[8 9]

We hypothesised that immune modulation by the *FOXO3A* gain of function SNP rs12212067 is associated with reduced cardiac inflammation and attenuated immune-mediated tissue injury resulting in improved outcomes in this patient cohort.

Materials and methods

Patients
Data of patients with DCMi were retrospectively collected from a tissue and biodata bank of the collaborative research network CRC Transregio 19 (NCT02970227).

Consecutively enrolled patients were included in the study who were older than 18 years, did undergo EMB (endomyocardial biopsies) and TTE (transthoracic echocardiography) at Charité - University Hospital between 2004 and 2010 (see online supplemental file 1 for more information).

(Immuno-)Histological and molecular diagnostics as well as determination of carrier status
EMBs were obtained in patients after exclusion of coronary artery disease and other possible causes for cardiac dysfunction. Then EMBs were stained and analysed with light and fluorescence microscopy. Immunoreactivity of the inflammatory cells was quantified by digital image analysis. Carrier status was determined by sampling DNA and using genotyping assays based on the principle of allelic discrimination PCR (see online supplemental file 1 for full description). The following criteria defined the virus-negative DCMi group: left ventricular (LVEF) <50%, increased LV end-diastolic diameter (LVEDD) >55 mm and a positive myocardial inflammation score, as well as absence of cardiac viral genomes in the PCR test (see online supplemental file 1).

Statistical analysis
Descriptive statistics included frequencies and percentage or mean values with 95% CI). Based on normality, Mann-Whitney U test or t-test without Bonferroni correction was used. Multivariate linear regression analysis was performed at baseline and T1, adjusted for age, in a subgroup (see online supplemental file 1 for full information). In all cases, a p≤0.05 (with calculated effect size) was regarded as statistically significant. Statistical analyses were performed using SPSS Statistics software V23.0 (IBM, Armonk, New York, USA).

RESULTS

Distribution of *FOXO3A* SNP rs12212067 in different patient cohorts
FOXO3A SNP rs12212067 presence was determined in patients with virus-negative inflammatory cardiomyopathy (n=221), virus-positive DCMi (n=114) and control subjects (n=77) (online supplemental figure 1). Of note, SNP distribution did not differ in patients with autoimmune (virus-negative) inflammatory cardiomyopathy compared with controls with preserved LVEF and LV dimensions that were negative for intracardiac inflammation (p=0.199). Moreover, the carrier status for *FOXO3A* SNP rs12212067 was not different from patients with proven virus-positive DCMi. In both DCMi patient cohorts, approximately 20% of patients were identified as being carriers for the *FOXO3A* SNP rs12212067, numbers were comparable to control subjects. Moreover, there was no sex-specific distribution in *FOXO3A* SNP rs12212067 (online supplemental figure 2) in patients with virus-negative inflammatory cardiomyopathy.

Patient characteristics at baseline
Out of 221 consecutive patients (age: 50.9 years (95% CI 48.9 to 52.9)) with virus-negative DCMi analysed, 45 were identified as carriers of the *FOXO3A* SNP rs12212067 (table 1). LVEF was severely reduced with 39.2% (95% CI 34.3% to 44.0%) vs 34.5% (95% CI 32.4% to 36.5%), p=0.083, in the SNP carrier group versus non-SNP carrier group, respectively (figure 1A, figure 2A). Compared with normal subjects (<55 mm), the LVEDD was enlarged with 59.8 mm (95% CI 58.5 to 61.2) in accordance with increased LVEDVI (LV end-diastolic diameter volume index) (figure 1B,C, figure 2B,C, table 1). Baseline characteristics did not significantly differ in carriers versus noncarriers of the SNP. In line with these observations, vital parameters and clinical symptoms did not significantly differ. Analysis for cardiac viral genomes was negative for CoxB3, hepatitis B, enteroviruses and parvovirus B19. Both patient cohorts were characterised by cardiac immune cell infiltration indicating ongoing cardiac inflammation. In accordance with increased expression of immune cell surface or cytoplasmatic markers, immunohistochemical analysis showed increased Mac-1 positive infiltrates without significant differences in cardiac inflammatory marker expression. Comorbidities,[10] medication and number of cardiovascular risk factors did not differ among both groups (table 1, online supplemental figures 3 and 4).

Table 1 Baseline characteristics according to genotype of FOXO3A SNP rs12212067 in patients with non-viral cardiomyopathy

Baseline characteristics *FOXO3A* SNP rs12212067 in V- DCMi

| Variable n = 221	All patients			SNP carrier Mm n=45, 20.4%			No SNP MM n=176, 79.6%			P value
		Mean	95% CI		Mean	95% CI		Mean	95% CI	
Age (years)		50.9	48.9 to 52.9		50.2	45.9 to 54.6		51.1	48.9 to 53.3	0.547
Sex m/f (%)	74.7/25.3			77.8/22.2			73.9/26.1			0.59
Echocardiographic parameters										
LVEF (%)		35.5	33.5 to 37.4		39.2	34.3 to 44.0		34.5	32.4 to 36.5	0.083
LVEDD (mm)		59.8	58.5 to 61.2		58.9	56.3 to 61.5		60.1	58.6 to 61.6	0.702
LVEDV (mL)		184.4	175.2 to 193.5		176.5	159.8 to 193.1		186.3	175.6 to 197.1	0.702
LVEDVI (mL/m²)		93.3	88.5 to 98.2		89	80.2 to 97.8		94.5	88.8 to 100.2	0.841
LA (mm)		44.8	43.4 to 46.2		45.6	42.0 to 49.2		44.6	43.1 to 46.2	0.595
IVSd (mm)		11.5	11.1 to 11.9		12.3	11.1 to 13.4		11.3	10.9 to 11.7	0.18
LVPWs (mm)		10.8	10.5 to 11.1		11.4	10.5 to 12.3		10.7	10.4 to 11.0	0.185
FS (%)		22.2	20.4 to 24.0		25.2	20.7 to 29.8		21.3	19.4 to 23.2	0.104
Clinical parameters										
Dyspnoea (%)	81			15			66			0.138
NYHA class I (%)	19			6			13			0.138
NYHA class II (%)	24			5			19			0.616
NYHA class III (%)	39			6			33			0.129
NYHA class IV (%)	18			4			14			0.896
NYHA class I–IV total (n)	197			41			156			0.151
Angina pectoris (%)	16			5			11			0.598
CCS class 0 (%)	84			19			65			0.598
CCS class I (%)	5			2			3			0.243
CCS class II (%)	2			2			0			0.058
CCS class III (%)	2			1			1			0.396
CCS class IV (%)	8			0			8			0.061
CCS class 0–IV (n)	133			32			101			0.505
RR sys (mm Hg)		121.9	118.8 to 125.1		123.4	117.6 to 129.2		121.6	118.0 to 125.3	0.356
RR dia (mm Hg)		75.6	73.6 to 77.6		76.7	73.0 to 80.3		75.3	73.0 to 77.7	0.378
Pulse (n/min)		80.9	77.9 to 83.9		78.4	73.5 to 83.2		81.6	78.0 to 85.2	0.774
EMB analysis: Inflammatory infiltrates										
CD3+ (mm²)		14	11.9 to 16.2		14.7	8.6 to 20.8		13.9	11.7 to 16.1	0.855
CD45+ (mm²)		32.5	28.1 to 36.9		40.1	25.3 to 54.8		30.4	26.4 to 34.4	0.306
Perforin+ (mm²)		2.1	1.7 to 2.6		1.9	0.8 to 3.0		2.2	1.7 to 2.7	0.636
Mac-1+ (mm²)		52.3	46.1 to 58.5		59.2	40.9 to 77.5		50.5	44.1 to 56.8	0.088
HLA-1+/AF (%)		7.9	7.4 to 8.4		8.2	6.6 to 9.8		7.9	7.4 to 8.3	0.578
CD106+/AF (%)		0.08	0.07 to 0.09		0.09	0.07 to 0.11		0.07	0.06 to 0.08	0.086
LFA-1+ (mm²)		33.2	27.6 to 38.8		41.6	20.0–63.2		30.9	26.5 to 35.3	0.584
CD54+/AF (%)		2.7	2.5 to 2.8		2.9	2.5 to 3.3		2.6	2.4 to 2.8	0.222
Cardiomyocyte diameter (µm)		21.1	20.5 to 21.7		21.4	20.0 to 22.7		21	20.3 to 21.8	0.619
Inflammatory markers in blood										
Leucocytes (cells/µL)		8.1	7.6 to 8.5		7.8	7.0 to 8.6		8.1	7.6 to 8.7	0.655
CRP (mg/dL)		2	1.4 to 2.7		1	0.4 to 1.6		2.3	1.5 to 3.1	0.308
Comorbidity charackteristics										
CCI score adjusted for age		3.1	2.8 to 3.4		2.9	2.3 to 3.4		3.2	2.8 to 3.5	0.545
Predicted 10-year survival (%)		66.6	61.9 to 71.4		71.2	61.5 to 80.9		65.5	60.0 to 70.9	0.514

Data are expressed as *mean* with 95% CI.

CCI, Charlson comorbidity index; CRP, C- reactive protein; DCMi, dilated inflammatory cardiomyopathy; EMB, endomyocardial biopsy;[10] FS, fractional shortening; IVSd, intraventricular septum diameter (diastole); LA, left atrium; LVEDD, left ventricular end-diastolic diameter; LVEDV, left ventricular end-diastolic diameter volume; LVEDVI, left ventricular end-diastolic diameter volume index; LVEF, left ventricular ejection fraction; LVPWs, left ventricular posterior wall (systole); MM, major; Mm, heterozygote; NYHA, New York Heart Association; RR, blood pressure; SNP, single-nucleotide polymorphism; V- DCMi, patients with non-viral cardiomyopathy.

Patient characteristics at 6–12 months' follow-up

Patients still symptomatic while on a guideline-directed medical therapy underwent follow-up endomyocardial biopsy (figure 2). Virus-negative patients carrying the *FOXO3A* SNP rs12212067 showed a significant improvement in LVEF: ΔLVEF 13 % (95 % CI 7.6 to 18.5) vs 6.5 % (95 % CI 4.3 to 8.7), p=0.013, SNP versus no SNP group, respectively (table 2), which resulted in significantly increased LVEF in carriers
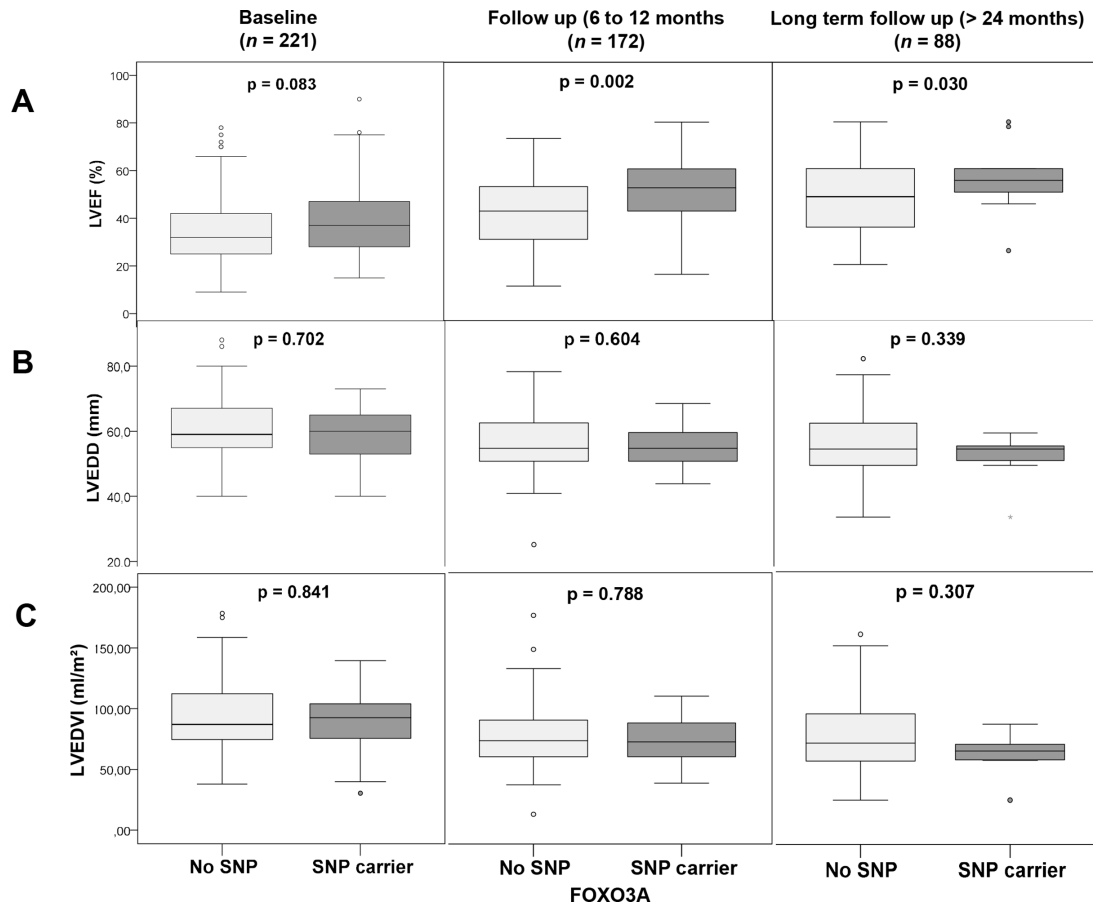
Figure 1 Echocardiographic changes of left ventricular ejection fraction (LVEF), left ventricular end-diastolic diameter (LVEDD) and left ventricular end-diastolic diameter volume index (LVEDVI) at short-term and long-term follow-up according to FOXO3A single-nucleotide polymorphism (SNP) status. Patients underwent serial echocardiographic examinations for LVEF (A), LVEDD (B) and LVEDVI (C) at baseline, short-term and long-term follow-up. Data are shown as a boxplot. Number of patients characterised is indicated.

50.9% (95% CI 45.4 to 56.3) vs 41.7% (95% CI 39.2 to 44.2), p=0.002 (figure 2A). The fractional shortening (FS) and LVEDD were not different, while LA diameters were significantly smaller in SNP carriers (p=0.028). These data implicate FOXO3A in the remodelling response in virus-negative patients with DCMi. FOXO3A has been shown to dampen immune cell function.[7 11] To get more insight into the immune-modulating effects of FOXO3A, the extent of cardiac inflammation was determined in follow-up biopsies. As shown in table 2, there was a trend towards reduced inflammation in the overall SNP carrier cohort. Although no significant absolute numbers in specific inflammatory markers were determined, *FOXO3A* SNP rs12212067 carriers showed distinct changes in cardiac immune marker expression for: ΔMac-1$^+$ (mm^2) p=0.04; ΔCD45RO$^+$ (mm^2) p=0.034; ΔLFA-1$^+$ (mm^2) p=0.004 and ΔCD54$^+$ (mm^2) p=0.009, favouring the SNP carrier cohort. These data implicate FOXO3A activity in resolution of intracardiac inflammation in virus-negative patients. In line with these observations, SNP carriers showed significant improvement in clinical symptoms (figure 3).

### Patient characteristics at long-term follow-up (>24–36 months)

n=88 patients were followed up for more than 24 months. Patients' characteristics are given in table 3. In line with our data characterising the disease state at 6–12 months, patients carrying the SNP were characterised by a significant improvement of LVEF: ΔLVEF 17.3% (95% CI 11.3 to 23.2) vs 9.4% (95% CI 5.6

to 13.2), p=0.033, SNP carriers versus non-carriers, respectively. LVEF was significantly enhanced in SNP carriers: 55.1% (95% CI 48.4 to 61.8) vs 46.7% (95% CI 42.9 to 50.4), p=0.030 (figure 2A). In accordance, reductions in LVEDD were significantly pronounced in this cohort (p=0.034). In line with these observations, LA size trended to be smaller and FS trended to be higher (table 3). In line with improvement of clinical symptoms, the rate of hospitalisations was significantly lower in carriers of the SNP (p=0.035, table 3).

With an exploratory intent, analyses with paired methods were used to account for changes in time per patient (online supplemental table 2).

Analysis of subgroups

The extent of inflammation and its effect on improvement of left ventricular function depending on carrier status of SNP rs12212067 is depicted in figure 4. For the subgroup of patients characterised by decreasing numbers of cardiac perforin-positive cells during the disease course, a difference in LVEF of 3.9% (95% CI −1.4 to 9.2) without SNP vs 20.7% (95% CI 3.3 to 38.0) in SNP carriers (p=0.013) was depicted. In this line of evidence, the patient cohort with decreasing mononuclear infiltration, that is, with declining numbers of CD3$^+$ cells and CD45RO$^+$ cells at follow-up biopsies, showed a significant improvement of cardiac function (CD3$^+$ cells/mm^2 with: ΔLVEF 5.3% (95% CI 1.0 to 9.7) without SNP vs 17.2% (95% CI 6.9 to 27.4) in SNP carriers (p=0.023) and CD45RO$^+$ cells/
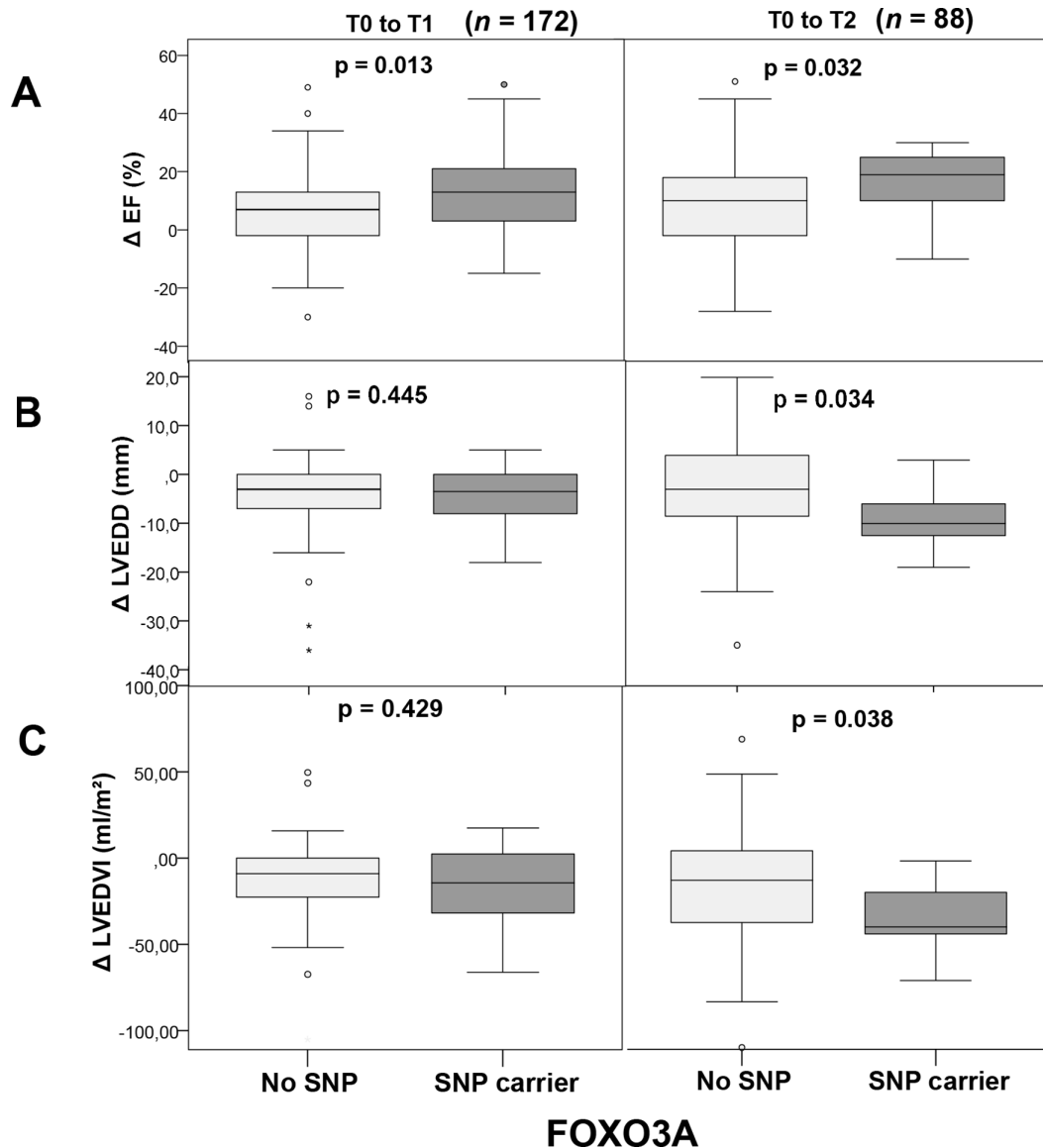
Figure 2 Probability of left ventricular ejection fraction (LVEF), left ventricular end-diastolic diameter (LVEDD) and left ventricular end-diastolic diameter volume index (LVEDVI) improvement according to *FOXO3A* single-nucleotide polymorphism (SNP) status. Patients were classified according to their carrier status. The boxplots show changes in LVEF (ΔLVEF) from baseline (T0) to short-term follow-up (T1) and baseline (T0) to long-term follow-up (T2), respectively, (A) as well as changes in LVEDD (ΔLVEDD) (B) and LVEDVI (ΔLVEDV Index) (C) from baseline (T0) to short-term (T1) and baseline (T0) to long-term follow-up (T2). Number of patients for each group and timepoint are indicated.

mm² with: ΔLVEF 1.1% (95% CI −5.1 to 7.3) without SNP vs 20.2% (95% CI 7.0 to 33.4) in SNP carriers (p=0.003) as well as in patients with decreasing lymphocytic LFA-1+ expression (CD11a+) with: ΔLVEF 6.3% (95% CI 1.5 to 11.1) without SNP vs 17.9% (95% CI 7.5 to 28.4) in SNP carrier (p=0.02). Moreover, attenuation of monocyte marker Mac-1+ expression in the follow-up biopsies was associated with better improvement of LVEF in SNP carriers: ΔLVEF 6.4% (95% CI 2.0 to 10.8) without SNP vs 17.7% (95% CI 6.2 to 29.3) in SNP carriers (p=0.023), an effect, that was not significantly associated with age (online supplemental table 1).

In another subgroup with detectable decreased endothelial activation in follow-up biopsies, a significant improvement in LVEF was observed in SNP carriers, both for ICAM (intercellular adhesion molecule) CD54+ with: ΔLVEF 6% (95% CI 1.5 to 10.5) without SNP vs 17.2% (95% CI 6.6 to 27.9) in SNP carriers (p=0.02) as well as for VCAM (vascular cell adhesion molecule)

CD106+ with: ΔLVEF 0.27% (95% CI −6.6 o 7.2) without SNP vs 18.3% (95% CI −0.98 to 37.7) in SNP carriers (p=0.024). Fittingly, the decreasing expression of HLA-1+ in cardiac tissue was also associated with the same significant effect: ΔLVEF 5.5% (95% CI 0.8 to 10.1) without SNP vs 21% (95% CI 3.8 to 38.2) in SNP carriers (p=0.03).

Similarly, reduction in cardiomyocyte size over time was associated with significant improvement in LVEF by SNP carriers: ΔLVEF 2% (95% CI −4.8 to 8.8) without SNP vs 15.7% (95% CI 2.4 to 29.0) in SNP-carriers (p=0.031).

DISCUSSION

FOXO3A has been shown to act as a gatekeeper for homeostasis in different biological processes. In our study, virus-negative patients with cardiac inflammation and reduced ejection fraction carrying the gain of function SNP rs12212067 showed a better

Table 2 Follow-up (6–12 months) characteristics according to genotype of FOXO3A SNP rs12212067 in patients with non-viral cardiomyopathy

Follow-up characteristics (6–12 months) *FOXO3A* SNP rs12212067 in V- DCMi

Variable n = 172	All patients		SNP carrier Mm n=33, 19.2%		No SNP MM n=139, 80.8%		Effect size	P value
	Mean	95% CI	Mean	95% CI	Mean	95% CI		
Age (years)	51.5	49.2 to 53.8	51.4	45.9 to 56.9	51.5	48.9 to 54.1		0.765
Δtime (months)	10.6	8.7 to 12.4	13.8	8.3 to 19.3	9.8	7.9 to 11.7		0.027
Echocardiographic parameters							*r*	
LVEF (%)	43.5	41.2 to 45.8	50.9	45.4 to 56.3	41.7	39.2 to 44.2	0.24	0.002
ΔLVEF (%)	7.8	5.7 to 9.8	13	7.6 to 18.5	6.5	4.3 to 8.7	0.19	0.013
LVEDD (mm)	55.1	53.6 to 56.6	54.3	51.6 to 56.9	55.3	53.5 to 57.0		0.604
ΔLVEDD (mm)	−19.6	(−5.2) to (−2.7)	−4.8	(−7.6) to (−2.0)	−3.8	(−5.2) to (−2.4)		0.445
LVEDV (mL)	153.1	143.7 to 162.5	145.3	129.0 to 161.6	154.7	143.8 to 165.7		0.66
ΔLVEDV (mL)	−25.6	(−33.9) to (−17.3)	−32.5	(−51.3) to (−13.6)	−24.1	(−33.5) to (−14.8)		0.429
LVEDVI (mL/m²)	76.8	72.1 to 81.6	73.7	65.2 to 82.2	77.5	72.0 to 83.1		0.788
ΔLVEDVI (mL/m²)	−14.2	(−19.0) to (−9.3)	−17.2	(−27.5) to (−7.0)	−13.5	(−19) to (−7.9)		0.429
LA (mm)	41.6	40.2 to 43.1	38.7	36.8 to 40.6	42.3	40.5 to 44.0	0.21	0.028
ΔLA (mm)	−1.9	(−3.4) to (−0.3)	−4.2	(−10.0) to 1.5	−1.4	(−2.9) to 0.1		0.164
IVSd (mm)	10.9	10.6 to 11.3	10.8	9.9 to 11.6	11	10.6 to 11.4		0.695
ΔIVSd (mm)	−0.4	(−0.7) to (−0.1)	−0.7	(−1.6) to 0.2	−0.3	(−0.7) to 0.02		0.328
LVPWs (mm)	10.5	10.3 to 10.8	10.3	9.7 to 10.8	10.6	10.3 to 10.9		0.444
ΔLVPWs (mm)	−0.1	(−0.4) to 0.2	−0.3	(−1.1) to 0.5	−0.1	(−0.3) to 0.2		0.424
FS (%)	28.6	26.3 to 30.8	29.9	24.4 to 35.5	28.3	25.7 to 30.8		0.582
ΔFS (%)	5.4	3.1 to 7.7	3.8	(−1.1) to 8.7	5.9	3.2 to 8.5		0.285
Clinical parameters							*Φ*	
Dyspnoea (%)	42		4		38		0.20	0.019
NYHA class I (%)	58		15		43		0.20	0.019
NYHA class II (%)	19		3		16			0.942
NYHA class III (%)	19		1		18		0.19	0.024
NYHA class IV (%)	4		0		4			0.225
NYHA class I–IV total (n)	142		27		115		0.24	0.006
Angina pectoris (%)	2		0		2			0.676
CCS class 0 (%)	98		15		83			0.676
CCS class I (%)	0		0		0			
CCS class II (%)	0		0		0			
CCS class III (%)	0		0		0			
CCS class IV (%)	2		0		2			0.676
CCS class 0–IV (n)	68		10		58			0.678
RR sys (mm Hg)	123	119.4 o 126.6	120.7	114.0 to 127.5	123.5	119.3 to 127.6		0.517
RR dia (mm Hg)	73.6	71.5 to 75.7	75.3	70.2 to 80.4	73.3	71.0 to 75.7		0.487
Pulse (n/min)	72.5	70.0 to 75.0	72.6	67.0 to 78.2	72.5	69.6 to 75.4		0.965
EMB analysis: Inflammatory infiltrates							*r*	
CD3+ (mm²)	10	7.7 to 12.3	8.3	4.7 to 11.8	10.5	7.7 to 13.2		0.698
ΔCD3+ (mm²)	−10.4	(−15.6) to (−5.2)	−19	(−36.2) to (−1.7)	−7.9	(−12.7) to (−3.2)		0.176
CD45+ (mm²)	39.5	24.9 to 54.2	28.3	18.5 to 38.1	42.9	24.0 to 61.9		0.985
ΔCD45+ (mm²)	−8.1	(−31.3) to 15.1	−51.4	(−100.4) to (−2.5)	6.7	(−19.1) to 32.5	0.31	0.034
Perforin+ (mm²)	2.2	1.1 to 3.3	1.9	(−0.3) to 4.1	2.3	1.0 to 3.6		0.868
ΔPerforin+ (mm²)	−0.6	(−2.1) to 0.9	−0.6	(−4.5) to 3.3	−0.6	(−2.2) to 1.1		0.432
Mac-1+ (mm²)	47.3	34.0 to 60.6	37.1	24.2 to 50.0	50	33.5 to 66.6		0.637
ΔMac-1+ (mm²)	−15.7	(−36.9) to 5.5	−49.7	(−103.8) to 4.4	-6	(−28.9) to 16.8	0.24	0.04
HLA-1+/AF (%)	7.5	6.8 to 8.3	8	7.0 to 9.0	7.4	6.5 to 8.4		0.396
ΔHLA-1+/AF (%)	−1.5	(−3.0) to (−0.01)	−2.9	(−7.9) to 2.2	−1.1	(−2.6) to 0.3		0.876
CD106+/AF (%)	0.08	0.04 to 0.11	0.07	0.02 to 0.11	0.08	0.03 to 0.12		0.623
ΔCD106+/AF (%)	−0.01	(−0.05) to 0.03	−0.05	(−0.09) to 0.004	0.002	(−0.05) to 0.05		0.102
LFA-1+ (mm²)	30.2	20.4 to 40.1	16.7	10.5 to 22.8	33.9	21.6 to 46.2		0.212
ΔLFA-1+ (mm²)	−17.9	(−36.9) to 1.1	−62	(−123.9) to (−0.07)	−5.1	(−21.9) to 11.7	0.34	0.004
CD54+/AF (%)	2.6	2.2 to 3.1	2.3	1.9 to 2.7	2.7	2.2 to 3.3		0.748
ΔCD54+/AF (%)	−0.3	(−0.8) to 0.3	−1.4	(−2.0) to (−0.8)	0.05	(−0.6) to 0.7	0.31	0.009
Cardiomyocyte diameter (µm)	22.4	21.3 to 23.6	20.5	17.2 to 23.9	23	21.9 to 24.1		0.067
ΔCardiomyocyte diameter (µm)	1.5	0.1 to 2.8	−0.6	(−3.6) to 2.4	2	0.5 to 3.5	0.24	0.049
Inflammatory markers in blood								
Leucocytes (cells/µL)	7.5	7.0 to 7.9	7.7	6.5 to 8.8	7.5	6.9 to 8.0		0.589
ΔLeucocytes (cells/µL))	−0.8	(−1.3) to (−0.3)	−1.1	(−2.0) to (−0.1)	−0.8	(−1.4) to (−0.2)		0.733

Continued

Table 2 Continued

Follow-up characteristics (6–12 months) *FOXO3A* SNP rs12212067 in V- DCMi									
	All patients			SNP carrier Mm n=33, 19.2%			No SNP MM n=139, 80.8%		
Variable n = 172	Mean	95% CI		Mean	95% CI		Mean	95% CI	Effect size	P value
CRP (mg/dL)	1.6	0.7 to 2.5		0.4	0.07 to 0.8		1.8	0.7 to 2.8		0.825
△CRP (mg/dL)	−0.8	(−2.4) to 0.8		−1	(−3.2) to 1.2		−0.8	(−2.6) to 1.0		0.79

Data are expressed as mean with 95% CI; effect size when p≤0.05.
DCMi, dilated inflammatory cardiomyopathy; EMB, endomyocardial biopsy; FS, fractional shortening; IVSd, intraventricular septum diameter (diastole); LA, left atrium; LVEDD, left ventricular end-diastolic diameter; LVEDV, left ventricular end-diastolic diameter volume; LVEDVI, left ventricular end-diastolic diameter volume index; LVEF, left ventricular ejection fraction; LVPWs, left ventricular posterior wall (systole); MM, major; Mm, heterozygote; SNP, single-nucleotide polymorphism; V- DCMi, patients with non-viral cardiomyopathy; △, difference between the individual values in the time interval T1 - T0.

outcome characterised by enhanced improvement of LVEF, FS and attenuation of LVEDD/LVEDVI, and LA sizes. In follow-up biopsy specimens, this improvement over time was associated with significant reduction in inflammatory infiltrates within the myocardium and improvement of clinical symptoms.

Myocardial inflammation has been shown to be a predictor for outcome in inflammatory cardiomyopathy.[12] Infections by cardiotropic viruses might trigger chronic immune processes frequently followed by heart-specific autoimmunity.[13] Moreover, cardiac involvement in autoimmune disease has been observed.[14] A breakdown in the control mechanisms protecting against autoimmune reactions by both, presentation of normally not accessible self-antigens and bystander-activation, induced by the pathogen, leads to the formation of autoreactive antibodies and T cells.[15] Chronic autoimmune inflammation leads to cardiomyocyte destruction, reparative fibrosis and heart failure.[16]

Our previous data in pathogen-induced, that is, viral myocarditis, implicate the role of FOXO3A in differentiation and activation of NK cells.[7] In an animal model of CVB3-induced myocarditis, lack of *Foxo3a* in mice resulted in enhanced NK cell activation, improved viral clearance, attenuated cardiac inflammation and preserved left ventricular function. In accordance with these findings, patients with a gain of function *FOXO3A* SNP rs12212067 were characterised by attenuation of NK cell function resulting in decreased viral clearance associated with prolonged cardiac inflammation and attenuated improvement of cardiac functional parameters suggesting a more severe course of

virus-induced DCMi.[7] These outcome data might be explained by viral persistence, ongoing inflammation and tissue injury in SNP carriers (ie, FOXO3A activation) due to diminished innate immune responses. In contrast, in the present study, non-carriers of the SNP were characterised by perforin persistence, a marker for NK cell accumulation, that was associated with decreased LV function and larger left ventricular end-diastolic diameters at follow-up indicating worse outcome in patients with virus-negative chronic (ie, autoimmune) cardiac inflammation. Carriers of the gain of function *FOXO3A* SNP, however, showed lower perforin levels and better outcome at follow-up (figure 5). These data are in accordance with observations of perforin as a marker cytokine for worse prognosis in DCMi.[17] FOXO3A has been recently shown to act as an immunosuppressor.[18] The attenuation of cardiac inflammation by FOXO3A might be caused by several immunological actions of the transcription factor. Besides modulating NK cell activity, FOXO3A has been shown to inhibit monocyte activation[4] and phagocytic activity.[4] Monocytes are modulators of innate and adaptive immune responses. In our study, the number of Mac1-positive cells delineating monocytes and macrophages was significantly reduced at 6 months follow-up in carriers of the SNP and associated with a significant increase in cardiac function at follow-up. Taken together, in contrast to pathogen-induced inflammation, where a robust inflammatory and innate immune response is warranted in order to clear the pathogen,[8] virus-negative (ie, autoimmune) chronic inflammation might lead to unwarranted cell and tissue
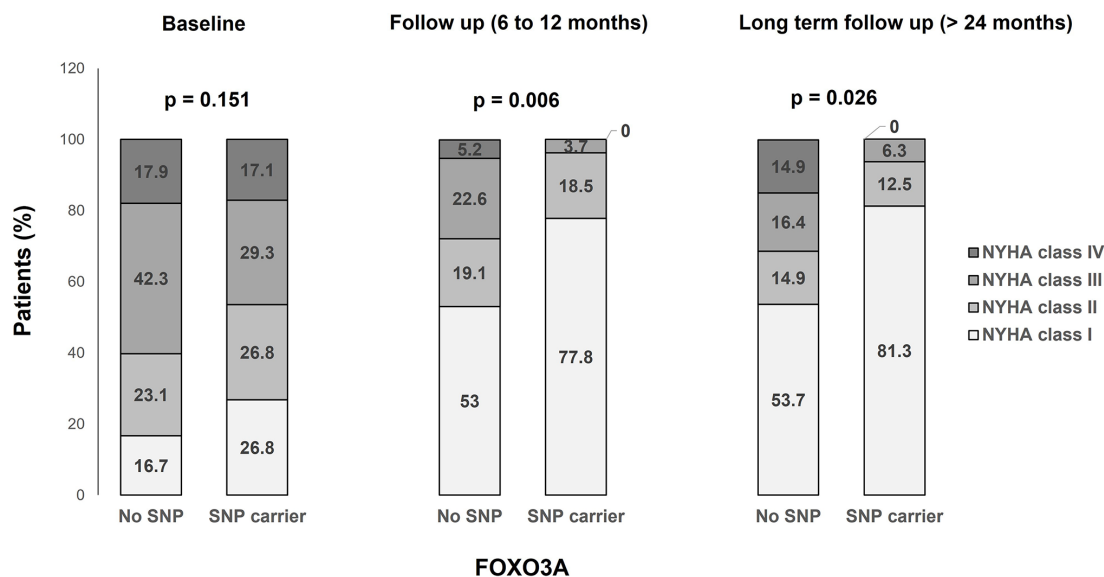
Figure 3 Comparison of New York Heart Association class distribution by FOXO3A genotype over time. Comparison of the distribution of NYHA functional classification (shown as percentage occurrence) according to FOXO3A single-nucleotide polymorphism (SNP) status over time. The differences between No SNP and SNP carrier are shown at baseline, follow-up and long-term follow-up.

Table 3 Long-term follow-up (>24–36 months) characteristics according to genotype of *FOXO3A* SNP rs12212067 in patient with non-viral cardiomyopathy

Long term follow-up characteristics (>24–36 months) *FOXO3A* SNP rs12212067 in V- DCMi									
		All patients		**SNP carrier Mm n=16, 18.2%**		**No SNP MM n=72 81.8%**			
Variable n=88		**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**Effect size**	**P value**
Age (years)		56.6	53.4 to 59.8	52.5	44.9 to 60.2	57.5	54.0 to 61.1		0.23
Δtime (months)		66.2	58.5 to 74	59.5	42.3 to 76.7	67.8	59.0 to 76.5		0.433
Echocardiographic parameters								*r*	
LVEF (%)		48.2	44.9 to 51.5	55.1	48.4 to 61.8	46.7	42.9 to 50.4	0.41	0.03
ΔLVEF (%)		10.9	7.6 to 14.2	17.3	11.3 to 23.2	9.4	5.6 to 13.2	0.23	0.033
LVEDD (mm)		54.9	52.6 to 57.1	51.8	48.6 to 55.0	55.7	53.0 to 58.4		0.339
ΔLVEDD (mm)		−4.1	(−6.4) to (−1.8)	−8.9	(−12.3) to (−5.4)	−2.8	(−5.6) to (−0.1)	0.25	0.034
LVEDV (mL)		152.8	137.8 to 167.8	130.5	114.6 to 146.4	158.6	140.3 to 176.8		0.339
ΔLVEDV (mL)		−25.4	(−41.3) to (−9.5)	−58.3	(−82.7) to (−34.0)	−16.6	(−35.2) to (−2.1)	0.26	0.031
LVEDVI (mL/m²)		77.5	69.0 to 86.0	64.1	56.0 to 72.3	81.2	70.6 to 91.7		0.307
ΔLVEDVI (mL/m²)		−14.8	(−23.8) to (−5.9)	−32	(−43.6) to (−20.5)	−9.9	(−20.7) to 0.8	0.26	0.038
LA (mm)		43.4	40.3 to 46.4	39.3	33.2 to 45.4	44.3	40.7 to 47.9		0.195
ΔLA (mm)		−2.7	(−6.7) to 1.3	−5.3	(−11.6) to 0.9	−1.9	(−6.9) to 3.1		0.464
IVSd (mm)		10.7	10.2 to 11.2	10.9	9.8 to 12.0	10.6	10.0 to 11.2		0.698
ΔIVSd (mm)		−0.7	(−1.1) to (−0.2)	−0.6	(−1.4) to 0.2	−0.7	(−1.2) to (−0.1)		0.904
LVPWs (mm)		10.2	9.8 to 10.6	10.5	9.5 to 11.4	10.1	9.6 to 10.6		0.453
ΔLVPWs (mm)		−0.3	(−0.7) to 0.1	0.2	(−0.9) to 1.3	−0.4	(−0.9) to 0.05		0.242
FS (%)		27.8	22.4 to 33.1	33.3	7.5 to 59.1	26.4	21.0 to 31.8		0.293
ΔFS (%)		8.2	1.8 to 14.6	4.3	(−19.8) to 28.5	9.1	1.4 to 16.8		0.558
Clinical parameters								*Φ*	
Dyspnoea (%)	40			4		36		0.21	0.049
NYHA class I (%)	59			16		43		0.22	0.044
NYHA class II (%)	15			3		12			0.804
NYHA class III (%)	14			1		13			0.299
NYHA class IV (%)	12			0		12			0.099
NYHA class I–IV total (n)	83			16		67		0.25	0.026
Angina pectoris (%)	14			0		14			0.298
CCS class 0 (%)	86			12		74			0.298
CCS class I (%)	2			0		2			0.712
CCS class II (%)	0			0		0			
CCS class III (%)	0			0		0			
CCS class IV (%)	12			0		12			0.341
CCS class 0–IV (n)	51			6		45			0.326
RR sys (mm Hg)		126.9	121.4 o 132.5	131.8	117.4 to 146.2	125.9	119.7 to 132.1		0.577
RR dia (mm Hg)		73.5	69.9 to 77.2	75.7	67.8 to 86.6	73.1	68.9 to 77.3		0.546
Pulse (n/min)		75.7	72.0 to 79.3	79.9	73.0 to 86.7	74.6	70.4 to 78.9		0.128
Inflammatory markers in blood								*r*	
Leucocytes (cells/µL)		7.4	6.7 to 8.1	7.1	5.5 to 8.6	7.5	6.7 to 8.3		0.968
Δ Leucocytes (cells/µL)		−0.4	(−1.2) to 0.3	−1.7	(−3.0) to (−0.5)	−0.1	(−1.0) to 0.8	0.48	0.035
CRP (mg/dL)		2.1	1.2 to 3.0	0.2	0.07 to 0.3	2.5	1.4 to 3.5	0.44	0.005
ΔCRP (mg/dL)		0.3	(−1.3) to 1.9	−1.6	(−3.8) to 0.5	0.6	(−1.2) to 2.4	0.38	0.043
Hospitalisation rate (n)		1.7	1.5 to 1.9	1.3	1.0 to 1.7	1.8	1.6 to 2.0	0.14	0.035

Data are expressed as mean with 95% CI; effect size when p≤0.05.
CCS, Canadian Cardiovascular Society; CRP, C-reactive protein; DCMi, dilated inflammatory cardiomyopathy; FS, fractional shortening; IVSd, intraventricular septum diameter (diastole); LA, left atrium; LVEDD, left ventricular end-diastolic diameter; LVEDV, left ventricular end-diastolic diameter volume; LVEDVI, left ventricular end-diastolic diameter volume index; LVEF, left ventricular ejection fraction; LVPWs, left ventricular posterior wall (systole); MM, major; Mm, heterozygote; NYHA, New York Heart Association; RR, blood pressure; SNP, single-nucleotide polymorphism; V-DCMi, patients with non-viral cardiomyopathy; Δ, difference between the individual values in the time interval T2 - T0.

injury resulting in reduced LV function and worsening of heart failure symptoms.

Here, we provide further data for an immunosuppressive function of active FOXO3A in virus-negative DCMi. The SNP, a non-coding polymorphism in *FOXO3A* (rs12212067: T>G), does not lead to differential gene expression under unstimulated conditions but allele-specific expression occurs under inflammatory conditions leading to enhanced activity of the transcription factor. LFA-1 and CD3 are expressed on lymphocytes that characterise the adaptive immune response. Expression of both markers was attenuated at short-term and long-term follow-up in SNP carriers indicating resolution of inflammatory infiltrates associated with improvement of LV function and clinical symptoms as well as fewer hospitalisations. In accordance with the hypothesis of inhibition of chronic inflammation and attenuation of tissue injury, LV and atrial dimensions were significantly
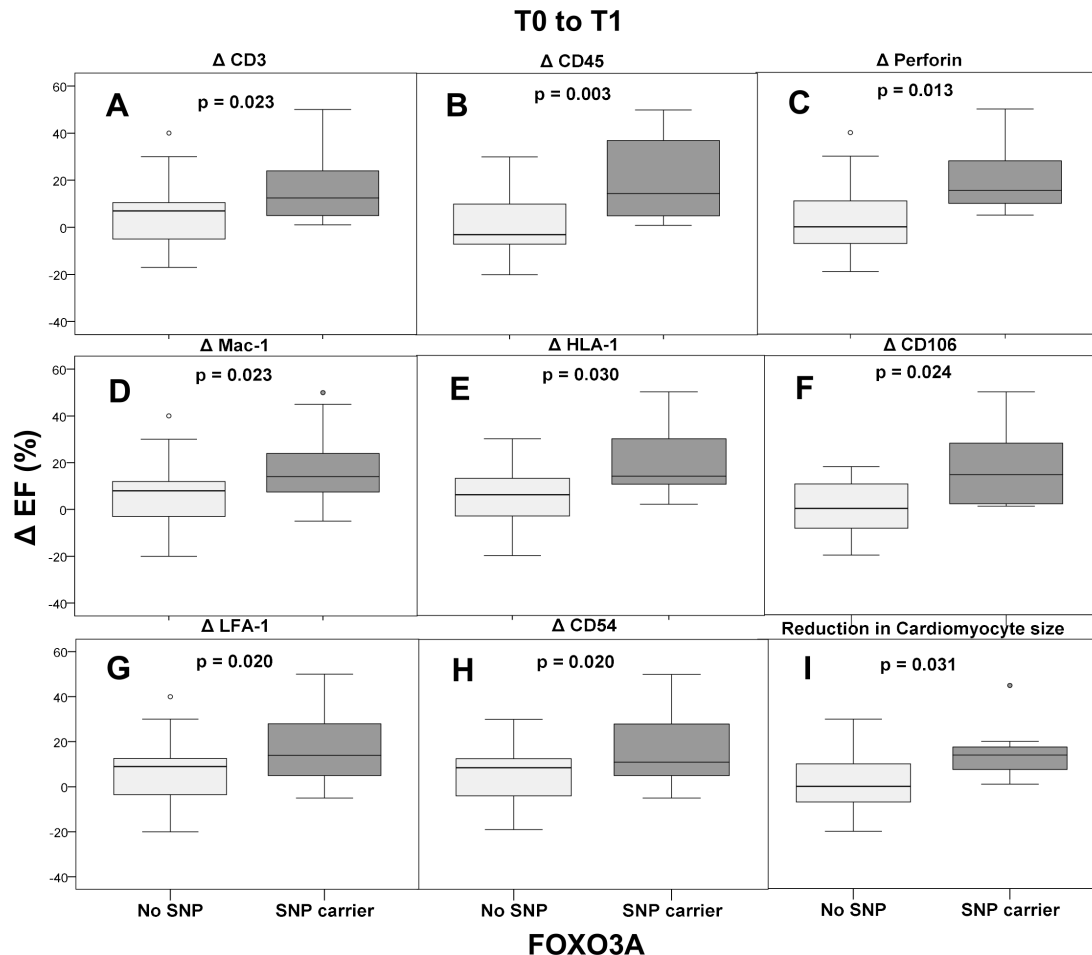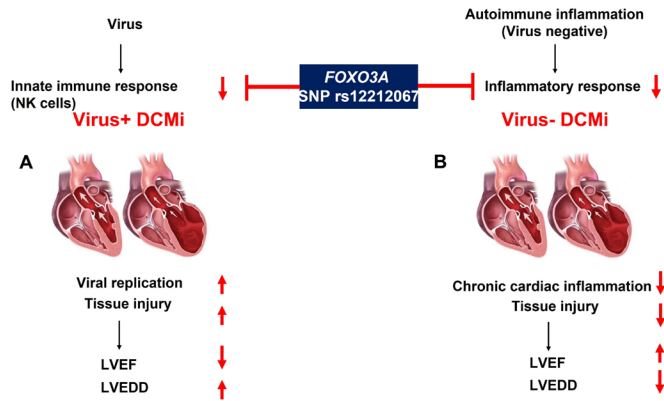
Figure 4 Effects of intracardiac inflammation on improvement of left ventricular ejection fraction (LVEF) in relation to carrier status of *FOXO3A* single-nucleotide polymorphism (SNP) rs12212067. Patients with declining inflammation levels during follow-up were studied. Endomyocardial biopsy parameters were determined as follows: CD3^{+}, CD45RO^{+}, perforin^{+}, Mac-1^{+}, HLA-1^{+}, CD106^{+}, LFA-1^{+}, CD54^{+} and cardiomyocyte diameter. Results are shown as boxplots with LVEF changes over time. EF, effect size.

smaller in SNP carriers in line with observations of a milder course of chronic autoimmune diseases such as rheumatoid arthritis or chronic bowel disease.[4] These observations are in contrast to viral cardiomyopathy[7] or pathogen-induced inflammation such as malaria,[4] where carriers of the SNP exhibited more severe disease and decreased clearance of pathogens.[4] The dichotomy of FOXO3A action in pathogen-induced[7 18] and autoimmune inflammation (our results[18]) is easy to ascertain. In virus infection, a strong innate immune response will be able to inhibit viral replication thereby preventing chronic inflammation and virus-inflicted tissue injury.[9] Priming of the adaptive immune response will then clear the virus. In contrast, in autoimmune disease, tight control of innate and adaptive immune responses will diminish inflammatory cell-mediated tissue injury. There are still other mechanisms how FOXO3A activity is capable of attenuating the inflammatory process. FOXO3A modulates the function of T cells, regulatory T cell development and dendritic cell activation,[19] and suppression of their activity by FOXO3A would attenuate the inflammatory process. FOXO3A can regulate the inflammatory and immune responses through targeting central transcription factors involved in self-tolerance such as NF-κB, as well as upregulating anti-inflammatory cytokines (eg, IL-10) while downregulating proinflammatory cytokines such as TNF-α in macrophages.[4] These cytokines, together with antibodies against viral and cardiac proteins, further exacerbate the

damage to the heart and impairment of systolic function due to changes of the contractile apparatus and matrix proteins.[20–22] Taken together, several lines of evidence received from knockout studies in animals and associative studies in humans implicate FOXO3A in immunoregulation, homeostasis and attenuation of inflammation.

Endomyocardial biopsy is considered the gold standard for the diagnosis of acute or chronic inflammatory heart disease for identifying the aetiology of cardiac inflammation.[23] MRI with T1 and T2 sequencing and late gadolinium enhancement is important for visualising structural changes, infiltration, inflammation, fibrosis and scarring. Although MRI provides non-invasive tissue characterisation, it is unable to identify infectious agents, or the degree and quality of inflammation but might be used for therapy monitoring if necessary.[24] Immunosuppressive therapies have been shown to prevent later immune-mediated myocardial injury in patients with myocardial inflammation or persisting systemic autoimmunity despite virus elimination.[25 26] Treatment approaches for these patients with post infectious chronic myocarditis/inflammatory cardiomyopathy consist of corticosteroids, azathioprine, mycophenolate, ciclosporin A or immunoadsorption with subsequent intravenous immunoglobulin therapy in addition to optimal heart failure medication.[27 28]

Modulation of FOXO3A activity might be a promising novel therapeutic approach, since activation of the transcription factor

Figure 5 Proposed effects of FOXO3A in cardiac inflammation. Summary of a working model of FOXO3A effects in cardiac inflammation: (A) in virus-induced inflammation, FOXO3A inhibits innate immune response by attenuating natural killer (NK) cell activation leading to delayed viral clearance and tissue injury.[7] Virus-positive SNP carriers show reduced left ventricular ejection fraction (LVEF)/enlarged left ventricular end-diastolic diameter (LVEDD) and enhanced cardiac inflammatory activation at follow-up. (B) SNP carriers with virus-negative (autoimmune) cardiac inflammation show improved outcome due to immunosuppressive functions of FOXO3A. NK cell function and monocyte activation are reduced in SNP carriers.[4] Inhibition of chronic cardiac inflammation leads to attenuated myocardial injury with positive remodelling resulting in better LVEF and reduced LVEDD.

would benefit patients with autoimmune inflammation while inhibition of FOXO3A could provide viral clearance. The identification of the FOXO3A SNP might serve as one example for the combination of a genetic factor modulating the susceptibility, penetrance and severity of DCMi.

Several limitations apply to our study. First, although 221 patients were enrolled, only a small group of SNP rs12212067 carriers was investigated. Moreover, a multivariate analysis could only be performed in a subgroup of patients due to small patient numbers. However, it would have been difficult to recruit more patients with serial endomyocardial biopsy specimens for this disease entity given the rare distribution of the SNP in the whole cohort.

In conclusion, our study allows for new insights into genetically determined clinical course and eventual outcome or prognosis of cardiac inflammation. Enhancement of FOXO3A activity and resulting immunomodulation might afford protection in chronic autoimmune disease where inflammation should be suppressed but is less beneficial during infectious disease, where a proper immune response is needed to control rapid pathogen clearance and tissue injury. Further studies are needed to clarify the therapeutic potential of targeting FOXO3A activity with specific inhibitors presently in development.

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