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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 ($\Delta CD45RO^+$ p≤0.05; $\Delta Mac\text{-}1^+$ p≤0.05; $\Delta LFA\text{-}1^+$ p≤0.01; $\Delta CD54^+$ p≤0.01) in the SNP cohort versus non-SNP cohort, respectively. Subgroup analyses identified $\Delta Mac\text{-}1^+$, $\Delta LFA\text{-}1^+$, $\Delta CD3^+$ and $\Delta perforin^+$ 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 $p \leq 0.05$ (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 non-carriers 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									
		All patients			SNP carrier Mm n=45, 20.4%			No SNP MM n=176, 79.6%	
Variable n=221		Mean	95% CI		Mean	95% CI		Mean	95% CI	P value
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

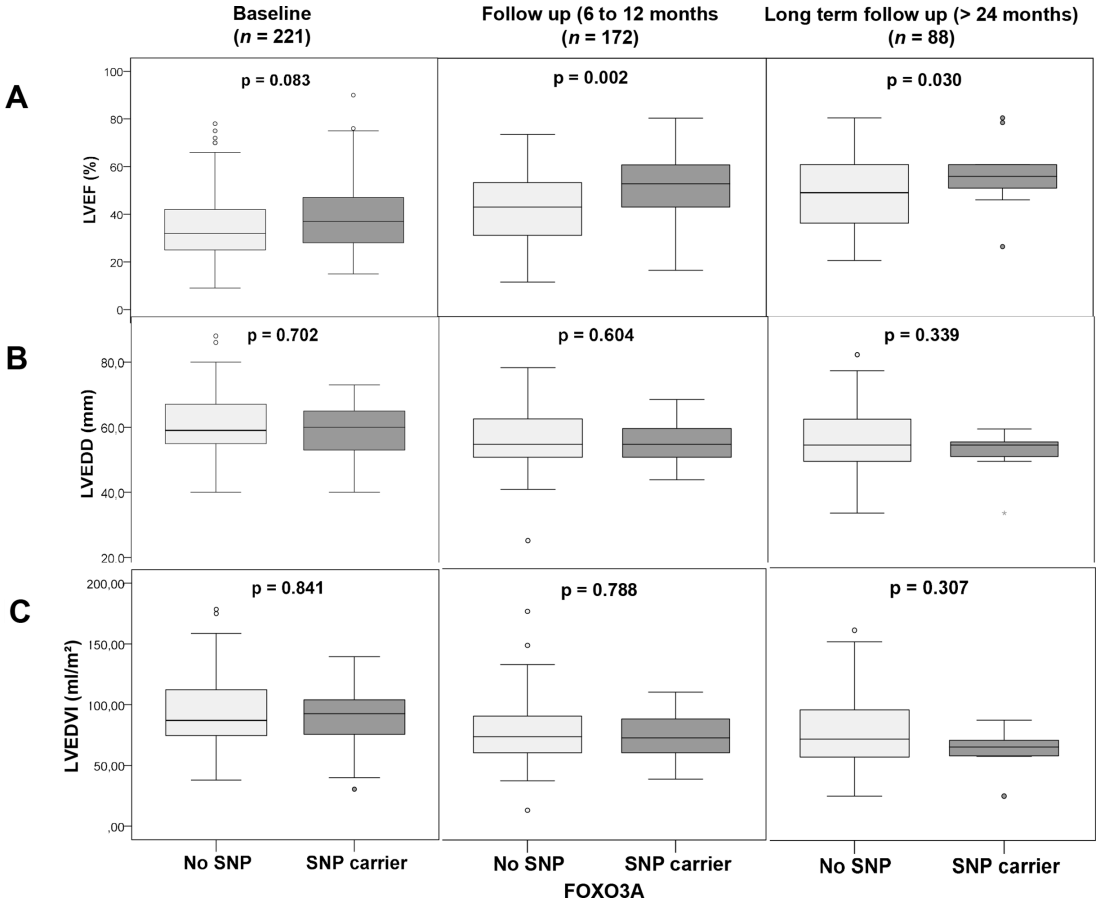

Figure 1 Echocardiographic changes of left ventricular ejection fraction (LVEF), left ventricular end-diastolic diameter (LVEDD) and left ventricular end-diastolic diameter volume index (LVEDVI) at short-term and long-term follow-up according to FOXO3A single-nucleotide polymorphism (SNP) status. Patients 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: $\Delta Mac\text{-}1^+$ (mm^2) p=0.04; $\Delta CD45RO^+$ (mm^2) p=0.034; $\Delta LFA\text{-}1^+$ (mm^2) p=0.004 and $\Delta CD54^+$ (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/

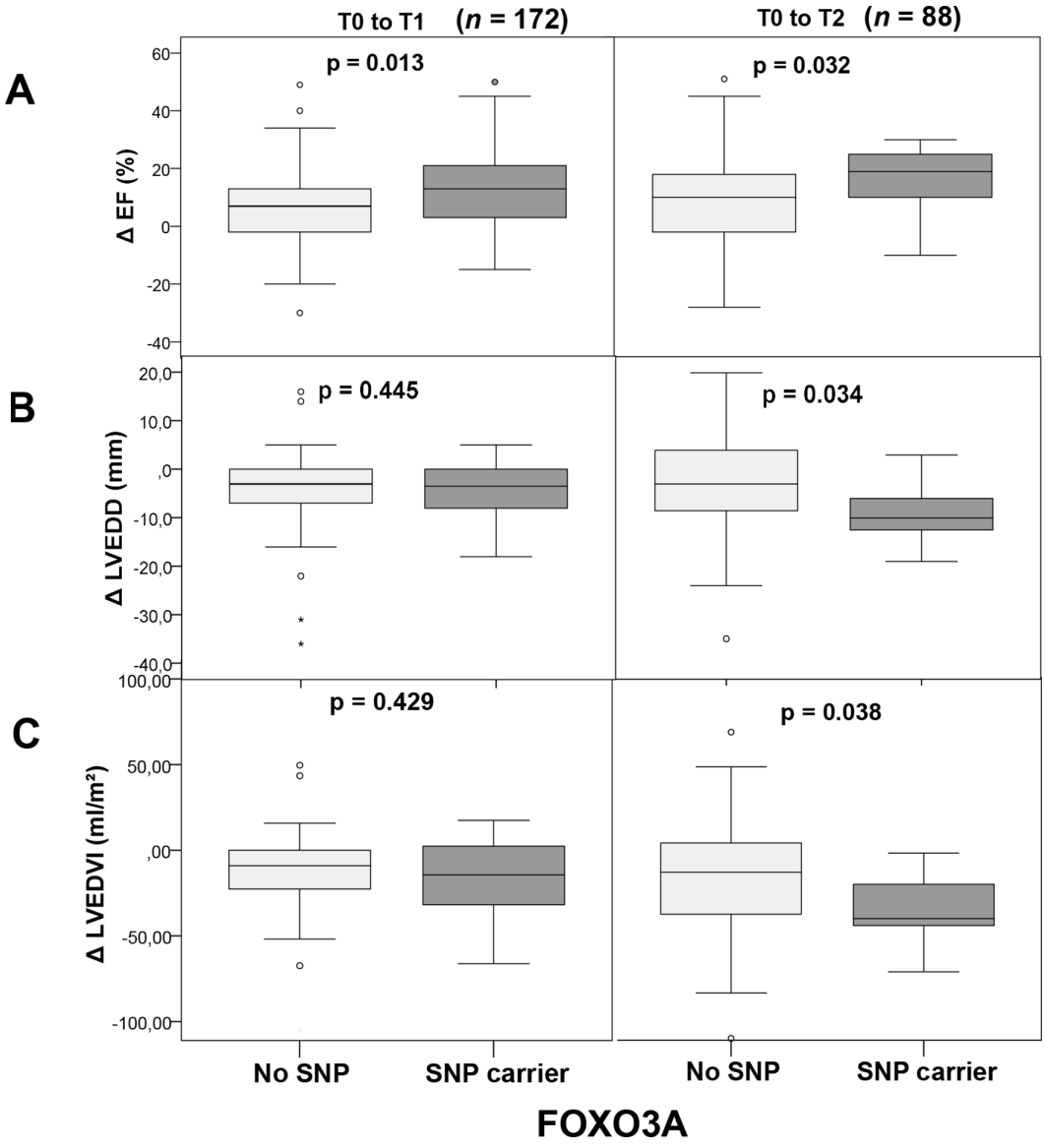

Figure 2 Probability of left ventricular ejection fraction (LVEF), left ventricular end-diastolic diameter (LVEDD) and left ventricular end-diastolic diameter volume index (LVEDVI) improvement according to *FOXO3A* single-nucleotide polymorphism (SNP) status. Patients were classified according to their 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									
	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
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
ΔCRP (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; Δ, 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

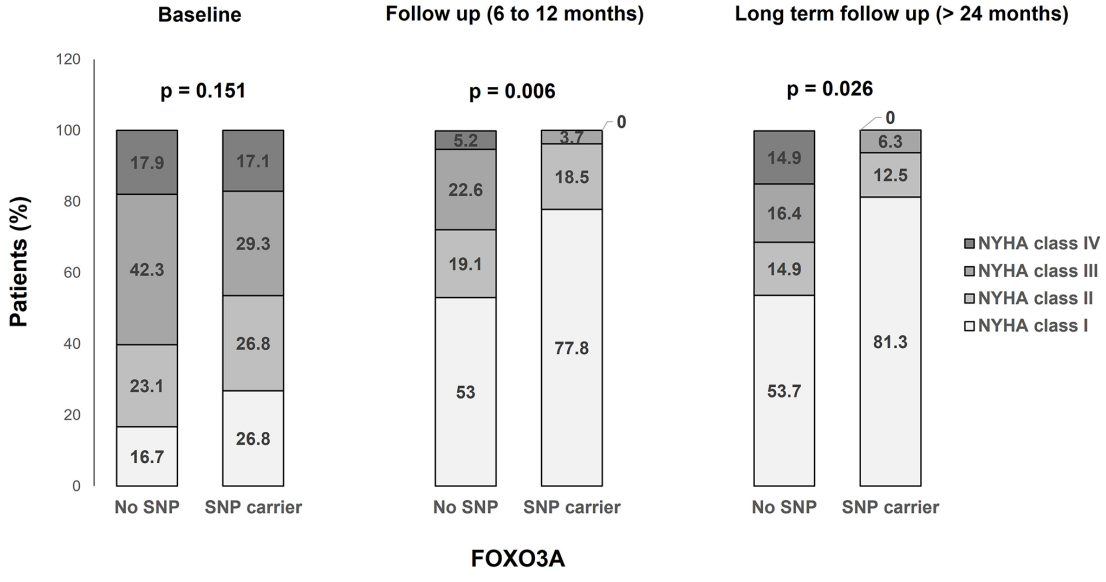

Figure 3 Comparison of New York Heart Association class distribution by FOXO3A genotype over time. Comparison of the distribution of NYHA functional classification (shown as percentage occurrence) according to FOXO3A single-nucleotide polymorphism (SNP) status over time. The differences between No SNP 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

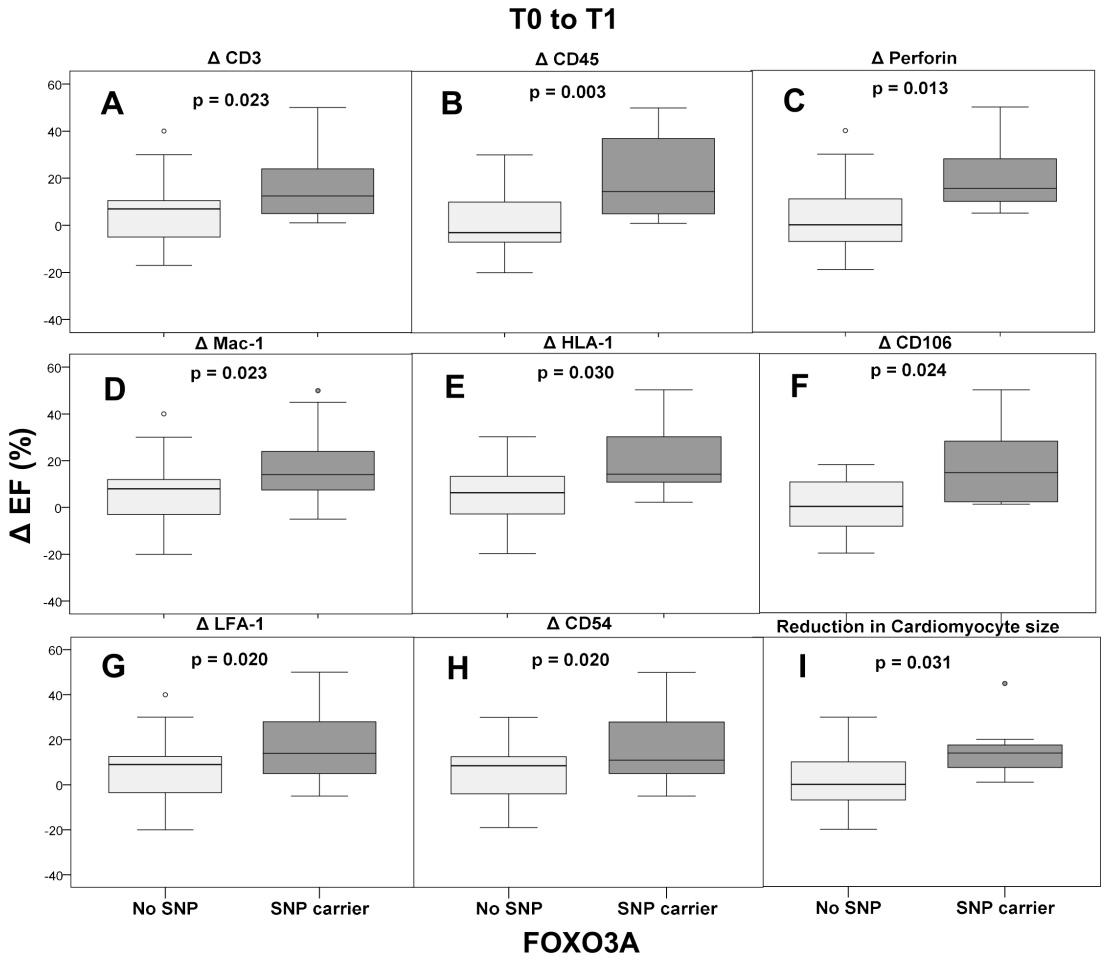

Figure 4 Effects of intracardiac inflammation on improvement of left ventricular ejection fraction (LVEF) in relation to carrier status of *FOXO3A* single-nucleotide polymorphism (SNP) rs12212067. Patients with declining inflammation levels during follow-up were studied. Endomyocardial biopsy parameters were determined as follows: $CD3^+$, $CD45RO^+$, $perforin^+$, $Mac\text{-}1^+$, $HLA\text{-}1^+$, $CD106^+$, $LFA\text{-}1^+$, $CD54^+$ 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

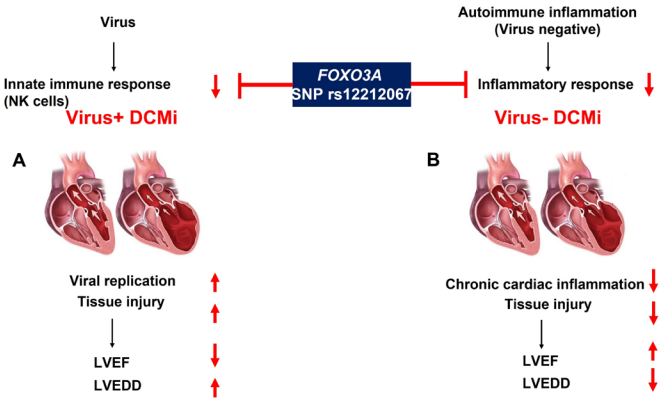

Figure 5 Proposed effects of FOXO3A in cardiac inflammation. Summary of a working model of FOXO3A effects in cardiac inflammation: (A) in virus-induced inflammation, FOXO3A inhibits innate immune response by attenuating natural killer (NK) cell activation leading to delayed viral clearance and tissue 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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Supplemental Material

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

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Materials and methods

Patients

Patients were admitted to our hospital with symptoms and signs of heart failure (HF) in all of whom EMBs were obtained by standard procedure following exclusion of coronary artery disease and other possible causes for cardiac dysfunction for histological, immunohistological, and molecular virological analyses [1]. Each patient received a transthoracic echocardiography (TTE) examination on hospital admission and after cardiac catheterization to evaluate cardiac pump function and determine structural cardiac pathologies. Further TTEs were performed on an outpatient basis at 3, 6, 12 18 and 24 months. During the initial hospital admission, endomyocardial biopsies were taken and analysed. SNP carrier status was determined from DNA retrievd from PBMC collected at time of biopsy. Control patients were admitted to our clinic to evaluate suspected cardiomyopathy, in whom the diagnostic workup finally revealed that their complaints were of non-cardiac in origin. Virus-negative DCMi patients were compared with virus-positive DCMi patients [2] as well as healthy volunteers without cardiac disease to determine the distribution of the SNP. The following criteria defined the virus-negative DCMi group of $n = 221$ patients: reduced LVEF < 50 %, increased LVEDD > 55 mm and a positive myocardial inflammation score ($CD3^+$ lymphocytes > 7 per mm^2, or $CD45RO^+$ memory T cell lymphocytes > 14 per mm^2, or perforin$^+$ cytotoxic T lymphocytes > 2.9 per mm^2, or $CD11b^+$/Mac-1^+ macrophages > 35 per mm^2, or $CD11a^+$/LFA-1^+ lymphocytes > 9 per mm^2) as well as absence of cardiac viral genomes as defined by negative PCR testing. A follow-up examination of all patients in the DCMi groups for determination of LVEF by a transthoracic echocardiographic examination was performed at approximately 6 months after enrollment into the study registry and serially every 6 months thereafter. The last obtainable echocardiographic study was used for long-term follow-up examination. The following periods were defined for the patient, follow up with 6 to 12 months (short observation) and long term follow up from 24 to 36 months (long observation). All patients were treated according to the present guidelines for medical treatment of heart failure [3, 4]. Moreover, some patients that still showed clinical symptoms despite optimal heart failure therapy underwent follow-up EMBs according to the present consensus recommendations [5].

Histological and immunohistological studies

At least five EMBs were taken from the septum of the left ventricle in all patients using a Cordis^{TM} bioptome. EMBs were analyzed histologically and immunohistologically in the *CAP*-accredited laboratory IKDT (Institute for Cardiac Diagnostic and Therapy Berlin, Germany).

Formalin fixed and paraffin embedded endomyocardial biopsy sample tissue sections were prepared by cutting with the rotary microtome. The paraffin sections were then stained using

standard procedures such as formaldehyde or RNA*later* fixation staining with haematoxylin and eosin, PAS reaction, Elastica van Gieson (EvG) and Azan stain as described previously [6]. The stained endomyocardial tissue prepared in this manner was then assessed for inflammation, histological and morphologic characteristics (e. g. diameter of cardiomyocytes, size and quality of biopsy, fibrosis, fatty tissue, and capillaries) by light and fluorescence microscopy and saved digitally as colour photographs. The EMB diagnosis of active myocarditis was based on the histomorphologic criteria according to the Dallas Classification [7].

Myocardial inflammation was diagnosed by 14.0 lymphocytes/mm² according to the European Society of Cardiology (ESC) position statement [8], Furthermore, we analysed macrophages (threshold > 40.0 $CD11b^+$/Mac-1^+ macrophages/mm²), $CD45RO^+$ T memory cells (threshold > 40 cells/mm²), and perforin-positive cytotoxic cells (threshold > 2.9 cells/mm²) [9].

The following antibodies were used: $CD3^+$ T lymphocytes (Dako, Glostrup, Denmark, dilution 1:700), $CD45RO^+$ T memory cells (Dako, Glostrup, Denmark, dilution 1:300), $CD11b^+$/Mac-1^+ macrophages (ImmunoTools, dilution 1:500) and $CD11a^+$/LFA-1^+ lymphocytes (ImmunoTools, Friesoythe, Germany, dilution 1:250). There was an association between cellular infiltrates and expression of CAMs: human leucocyte antigen-1 (HLA-1^+; Dako, dilution 1:2000) as well as intercellular adhesion molecule 1: $CD54^+$ (ICAM-1; ImmunoTools, dilution 1:800) and vascular cell adhesion molecule 1: $CD106^+$ (VCAM-1, ImmunoTools, dilution 1:800). Perforin-positive cellular infiltrates were also detected by immunohistochemistry (clone δG9, BD Bioscience, San Jose, CA, U.S.A., dilution 1:150). Enhancing EnVision™ peroxidase-conjugated anti mouse antibody (Dako Cytomation, Hamburg, Germany) was used as a secondary antibody. Immunohistological staining was visualized using a chromogenic substrate and counter-stained with haematoxylin. Immunoreactivity of the inflammatory cells was quantified by digital image analysis. Intramyocardial inflammation was categorized according to the European Society of Cardiology guidelines [8].

Molecular diagnostics

Qualitative detection of cardiotropic viruses was based on (reverse transcription)-PCR and nested-PCR. Quantification of all viral genomes was determined by (reverse transcription) real-time PCR (RT-qPCR) and fluorescence signals were captured by a QuantStudio 12k flex thermocycler (TaqMan, Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA U.S.A.). Depending on the type of viral nucleic acid, the isolation of DNA and RNA from the endomyocardial biopsies were performed in separate extraction procedures. The DNA was extracted from the biopsies using PUREGENE isolation kit according to the manufacturer´s protocol (Qiagen, Hilden, Germany) and was used to detect Adenovirus, Epstein-Barr virus,

Parvovirus B19 and human herpesvirus 6 genomes as described previously [2]. Total RNA was isolated for detection of enterovirus and influenza virus genomes as described [2] during routine endomyocardial biopsy diagnostics using Trizol reagent (QIAzol Lysis Reagent, QIAGEN, Hilden, Germany) and then treated with DNase (PerfeCTa® DNase I (RNase-free), Quanta BioSciences, Inc. Beverly, MA U.S.A.) to remove any traces of genomic DNA. RNA was reverse transcribed into cDNA by the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA U.S.A.) using random hexamer primers according to the manufacturer´s instructions. The DNA and cDNA concentrations were quantified using the real-time PCR-based Quantifiler™ Human DNA Quantification Kit or expression analysis of HPRT gene for the latter (Thermo Fisher Scientific, Waltham, MA U.S.A.). Subsequently, all amplified viral genomes were sequenced for determination of existing viral subtypes or infectious variants.

Determination of *FOXO3A* SNP carrier status

Genomic deoxyribonucleic acid (DNA) from the EMB samples was isolated using PUREGENE DNA isolation kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. Afterwards, DNA concentration was analysed using a NanoDrop®-ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.) and adjusted to 5 ng/µl with DEPC-treated water. Samples pre-prepared for the TaqMan SNP Genotyping Assay were stored at - 20 °C until use. Analysis of the *FOXO3A* SNP rs12212067 in the isolated genomic DNA was performed according to the manufacturer's instructions (Applied Biosystems™, Foster City, U.S.A.) using commercially available TaqMan PCR kits for SNP Genotyping (for SNP ID: rs12212067: C__30780203_10, cat. no 4351379, Thermo Fischer SCIENTIFIC). The method is based on the principle of Allelic discrimination PCR (discrimination of single base) and requires the enzyme Taq Polymerase, two specific primers and two TaqMan probes. One is labelled with a VIC fluorophore and one with a FAM fluorophore to specifically detect one of the two SNP alleles. It is a qualitative assay and provides the ability to distinguish homozygous from heterozygous as well as between the two homozygous samples.

Statistical analysis

Descriptive statistics included absolute and relative frequencies and percentage for categorical variables. Pearson's chi-squared test was applied to sets of categorical data and tested for significant results. Quantitative measurements were expressed as mean values with 95 % confidence interval (*95 % CI*) or as Boxplots, showing median, interquartile range and Whiskers (Tukey). Comparisons between two independent groups were done using two- or hypothesis-driven one-tailed test. According to the Shapiro-Wilk test, part of the data was either normally or non-normally distributed. Since for some statistical comparisons between

groups, the distribution was skewed in at least one of the groups, the nonparametric Mann-Whitney *U* test was utilized for group comparisons. Equivalent, for normally distributed data the parametric unpaired *t* test without Bonferroni correction was used to compare two independent groups. Longitudinal analyses using paired methods as the paired t test and Wilcoxon test to compare the paired data, were included to account for changes in time per patient. Multivariate linear regression analysis was performed at baseline and T1, adjusted for age, in a subgroup. In all cases, a value of *p* less or equal than 0.05 (with calculated effect size) was regarded as statistically significant. Statistical analyses were performed using version 23.0 of the SPSS Statistics software (IBM Corp. Armonk, NY, U.S.A.).

Supplemental Tables

We have included multivariate linear regression analyses between FOXO3A SNP carrier status on change in left-ventricular ejection fraction (Δ LVEF) adjusted for age in subgroups stratified by Mac-1 positive and Mac-1 negative status. In Mac-1 positive patients, SNP-positive carrier status was associated with 19.42 % (*95 % CI* 2.64 - 36.20) improvement in LVEF, while patients with Mac-1 negative status had an LVEF improvement of only 10.89 % (*95 % CI* 1.14 - 20.65). Age was not significantly associated with Δ LVEF across subgroups (each $p > 0.05$).

Suppl. Table 1
Δ LVEF change in *FOXO3A* SNP carriers depending on Mac-1 status adjusted for age

Subgroups	FOXO3A SNP carrier positive	Age
Mac-1 positive	19.42 (2.64 – 36.20), p = 0.026	-0.27 (-0.65 – 0.11), p = 0.149
Mac-1 negative	10.89 (1.14 – 20.65), p = 0.030	0.11 (-0.13 – 0.37), p = 0.352

Values are displayed as coefficients (95% Confidence Intervals), p value.

Subgroup analysis of the effect of FOXO3A SNP carrier status-positive on the change in left ventricular pump function (Δ LVEF in %) adjusted for age, depending on Mac-1 positive and Mac-1 negative status. Data are expressed as *Mean* with *95 % Confidence Interval* (*CI*).

In our study, cross-sectional methods were utilized to compare the effects between SNP carriers and non-SNP carriers. We now have included additional analyses on paired methods to account for changes in time. We used either the paired t test or the Wilcoxon test according to the normal distribution of the paired data for the longitudinal analysis to compare patients over time, according to FOXO3A SNP carrier status.

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Suppl. Table 2
The changes in short-term and long-term follow-up per patient depending on *FOXO3A*SNP rs12212067 carrier status

Patient changes over time T0 to T1 and T0 to T2 by *FOXO3A* SNP rs12212067 in V- DCMi

	All patients								SNP carrier *Mm*								No SNP MM							
	T0 to T1				T0 to T2				T0 to T1				T0 to T2				T0 to T1				T0 to T2			
Variable *n* = 221	*Mean*	*95 % CI*	*r*-effect size	*p*-value	*Mean*	*95 % CI*	*r*-effect size	*p*-value	*Mean*	*95 % CI*	*r*-effect size	*p*-value	*Mean*	*95 % CI*	*r*-effect size	*p*-value	*Mean*	*95 % CI*	*r*-effect size	*p*-value	*Mean*	*95 % CI*	*r*-effect size	*p*-value
Echocardiographic parameters																								
LVEF (%)	-7.8	(-9.8)-(-5.7)	0.33	<0.001	-10.9	(-14.2)-(-7.6)	0.31	<0.001	-13	(-18.5)-(-7.6)	0.44	<0.001	-17.3	(-23.2)-(-11.3)	0.43	0.001	-6.5	(-8.7)-(-4.3)	0.30	<0.001	-9.4	(-13.2)-(-5.6)	0.28	<0.001
LVEDD (mm)	4	2.7-5.2	0.49	<0.001	4.1	1.8-6.4	0.21	0.001	4.8	2.0-7.6	0.40	0.002	8.9	5.4-12.3	0.45	0.001	3.8	2.4-5.2	0.47	<0.001	2.8	0.1-5.6	0.27	0.045
LVEDV (ml)	25.6	17.3-33.9	0.35	<0.001	25.4	9.5-41.3	0.20	0.001	32.5	13.6-51.3	0.62	0.002	58.3	34.0-82.7	0.45	0.001	24	14.8-33.5	0.34	<0.001	16.6	(-2.1)-35.2		0.062
LVEDVI (ml/m²)	14.2	9.3-19.0	0.36	<0.001	14.8	5.9-23.8	0.22	0.001	17.2	7.0-27.5	0.63	0.002	32	20.5-43.6	0.47	0.001	13.5	7.9-19.0	0.35	<0.001	9.9	(-0.8)-20.7		0.061
LA (mm)	1.9	0.3-3.4	0.15	0.029	2.7	(-1.3)-6.7		0.227	4.2	(-1.5)-10.0		0.135	5.3	(-0.9)-11.6		0.08	1.4	(-0.1)-2.9		0.071	1.9	(-3.1)-6.9		0.438
IVSd (mm)	0.4	0.1-0.7		0.061	0.7	0.2-1.1	0.16	0.009	0.7	(-0.2)-1.6		0.121	0.6	(-0.2)-1.4		0.16	0.3	(-0.2)-0.7		0.161	0.7	0.1-1.2	0.16	0.024
LVPWs (mm)	0.1	(-0.2)-0.4		0.478	0.3	(-0.1)-0.7		0.167	0.3	(-0.5)-1.1		0.48	-0.2	(-1.3)-0.9		0.678	0.07	(-0.2)-0.3		0.629	0.4	(-0.05)-0.9		0.069
FS (%)	-5.4	(-7.7)-(-3.1)	0.31	<0.001	-8.2	(-14.6)-(-1.7)	0.19	0.027	-3.8	(-8.7)-1.1		0.112	-4.3	(-28.5)-19.8		0.52	-5.9	(-8.5)-(-3.2)	0.32	<0.001	-9.1	(-16.8)-(-1.4)	0.21	0.031
Clinical parameters																								
RR sys (mmHg)	2	(-2.4)-6.3		0.414	-0.6	(-8.1)-7.0		0.735	3	(-4.2)-10.2		0.384	-6	(-17.2)-5.2		0.31	1.7	(-3.5)-6.9		0.55	0.5	(-8.4)-9.4		0.92
RR dia (mmHg)	2.4	(-0.6)-5.4		0.179	3.7	(-1.2)-8.6		0.177	2.5	(-2.9)-7.9		0.371	1.9	(-11.2)-14.9		0.779	2.4	(-1.2)-6.0		0.272	4.1	(-1.5)-9.6		0.184
Pulse (n/min)	7.9	3.8-12.0	0.26	0.001	7.3	1.0-13.6	0.14	0.026	8.2	0.04-16.4	0.46	0.049	0.5	(-9.6)-10.7		0.912	7.8	3.1-12.6	0.20	0.004	9.1	1.6-16.6	0.16	0.02

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EMB analysis: Inflammatory infiltrates																				
CD3+ (mm²)	10.4	5.2-15.6	0.25	<0.001				19	1.7-36.2	0.34	0.008					7.9	3.2-12.7	0.23	0.001			
CD45+ (mm²)	8.1	(-15.1)-31.3	0.13	0.034				51.4	2.5-100.4	0.34	0.012					-6.7	(-32.5)-19.1		0.351			
Perforin+ (mm²)	0.6	(-0.9)-2.1		0.109				0.63	(-3.3)-4.5		0.826					0.57	(-1.1)-2.2		0.102			
Mac-1+ (mm²)	15.7	(-5.5)-36.9	0.17	0.003				49.7	(-4.4)-103.8	0.33	0.01					6.04	(-16.8)-28.9		0.058			
HLA-1+ /AF (%)	1.5	0.01-3.0		0.069				2.9	(-2.2)-7.9		0.278					1.1	(-0.3)-2.6		0.148			
CD106+ /AF (%)	0.01	0.1-0.02		0.21				0.05	(-0.004)-0.1		0.08					-0.002	(-0.05)-(-0.08)		0.686			
LFA-1+ (mm²)	17.9	(-1.1)-36.9	0.18	0.002				62	0.07-123.9	0.42	0.001					5.1	(-11.7)-21.9		0.108			
CD54+ /AF (%)	0.3	(-0.3)-0.8	0.14	0.019				1.4	0.8-2.0	0.77	<0.001					-0.05	(-0.7)-0.6		0.41			
Cardiomyocyte diameter (µm)	-1.5	(-2.8)-(-0.1)	0.12	0.048				0.6	(-2.4)-3.5		0.247					-2.02	(-3.5)-(-0.5)	0.17	0.01			
Inflammatory markers in blood																				
Leucocytes (cells/µl)	0.8	0.3-1.3	0.20	0.001	0.4	(-0.3)-1.2	0.161	1.1	0.1-2.0	0.34	0.021	1.7	0.5-3.05	0.32	0.036	0.8	0.2-1.4	0.19	0.006	0.1	(-0.8)-1.04	0.67
CRP (mg/dl)	0.8	(-0.8)-2.4		0.159	-0.3	(-1.9)-1.3	0.633	1	(-1.2)-3.2		0.345	1.6	(-0.5)-3.8		0.068	0.8	(-1.05)-2.65		0.26	-0.6	(-2.4)-1.2	0.241

Longitudinal analysis with changes in time: T0 to T1 as short-term follow-up and T0 to T2 as long-term follow-up per patient, depending on *FOXO3A* SNP rs12212067 carrier status in non-viral cardiomyopathy patients. Data are expressed as *Mean* with *95 % Confidence Interval* (*CI*); effect size when $p \leq 0.05$.

Supplemental Figures

Suppl. Figure 1

Distribution of the *FOXO3A* SNP rs12212067 in different patient cohorts

Patients were characterized according to reduced LVEF, increased LVEDD, detection of cardiac inflammation and presence or absence of viral genomes within the myocardium in one of the following groups: controls (LVEF > 55 %, LVEDD < 50 mm, $CD3^+$ cells < 5 /mm^2), virus-negative DCMi (LVEF < 50 %, LVEDD > 55 mm, $CD3^+$ cells > 15 /mm^2, exclusion of viral genomes) and virus-positive DCMi (LVEF < 50 %, LVEDD > 55 mm, $CD3^+$ cells > 15 /mm^2, detection of cardiac viral genomes). The allelic distribution for the SNP rs12212067 was determined by RT-PCR in each group.

Suppl. Figure 2

Sex Distribution in FOXO3A SNP rs1221206 by non-viral cardiomyopathy patients

In nonviral cardiomyopathy in our population, the percentage distribution of sexes according to FOXO3A SNP status is as follows. The sexes are equally distributed here; no significant affinity is seen between FOXO3A genotype and one sex.

Suppl. Figure 3

Medical heart failure therapy at baseline according to FOXO3A SNP status (not significantly different)

During the initial hospitalization, patients were initiated on heart failure medical therapy. This is the percentage distribution of separately mentioned medications in all patients and in both patients' groups according to FOXO3A SNP status. There are no significant differences by FOXO3A at baseline in comparison; (ACE inhibitors = angiotensin-converting enzyme inhibitors; AT1 receptor blockers = angiotensin II type 1 receptor blockers).

Suppl. Figure 4

Cardiovascular risk factors in the initial patient group according to FOXO3A SNP status (not significantly different)

The following graph shows the percentage distribution of the selected diagnoses for the characterization of the population at baseline. The distribution of cardiovascular risk factors is shown here: in the group of all patients and in the groups according to FOXO3A SNP status.

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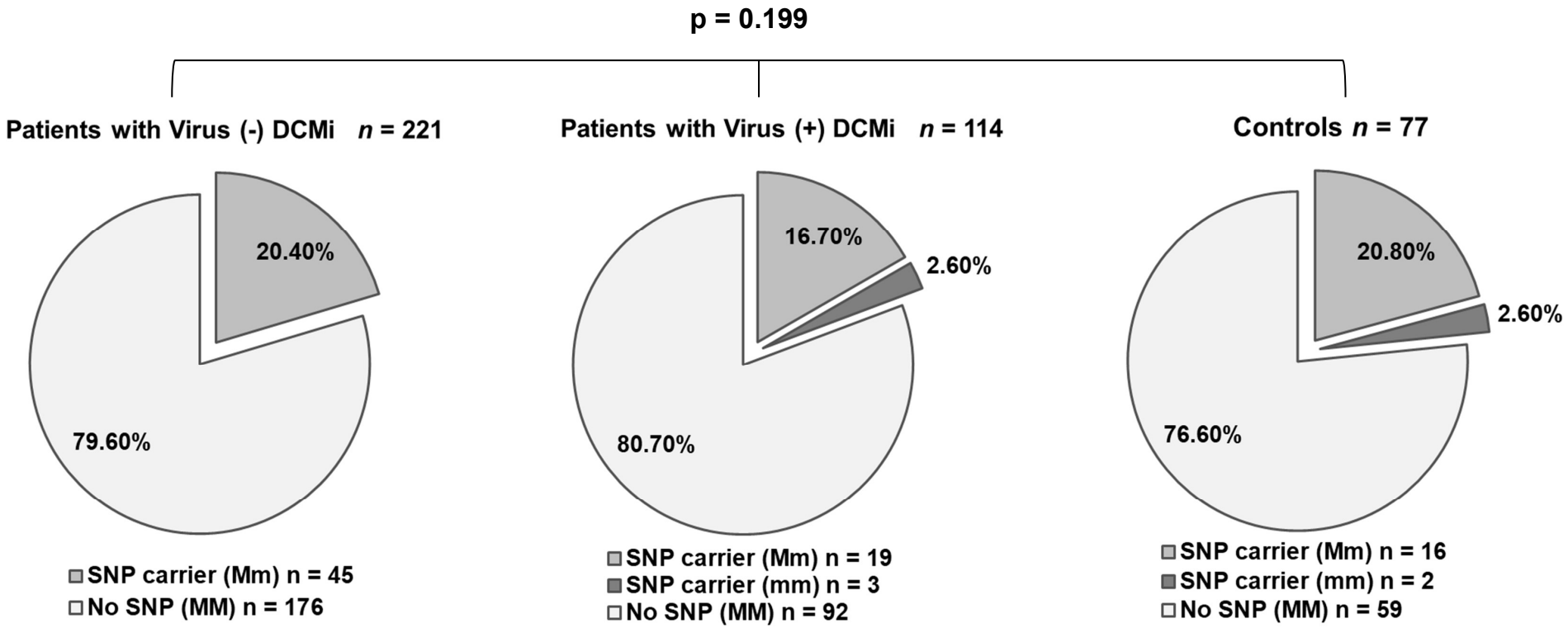
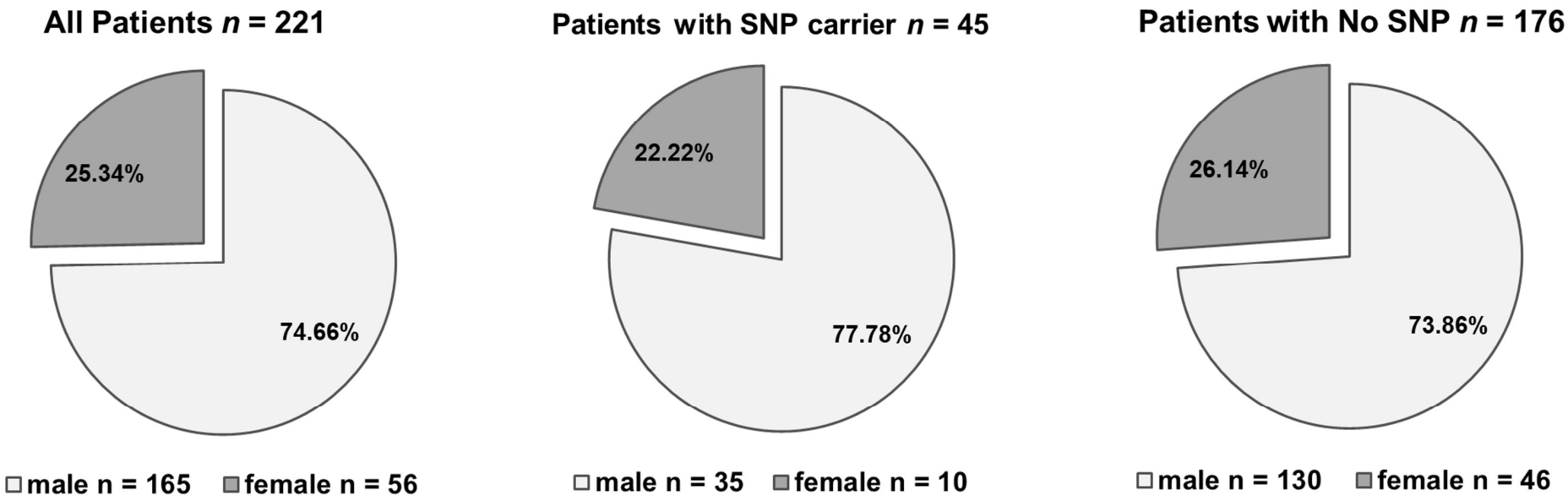
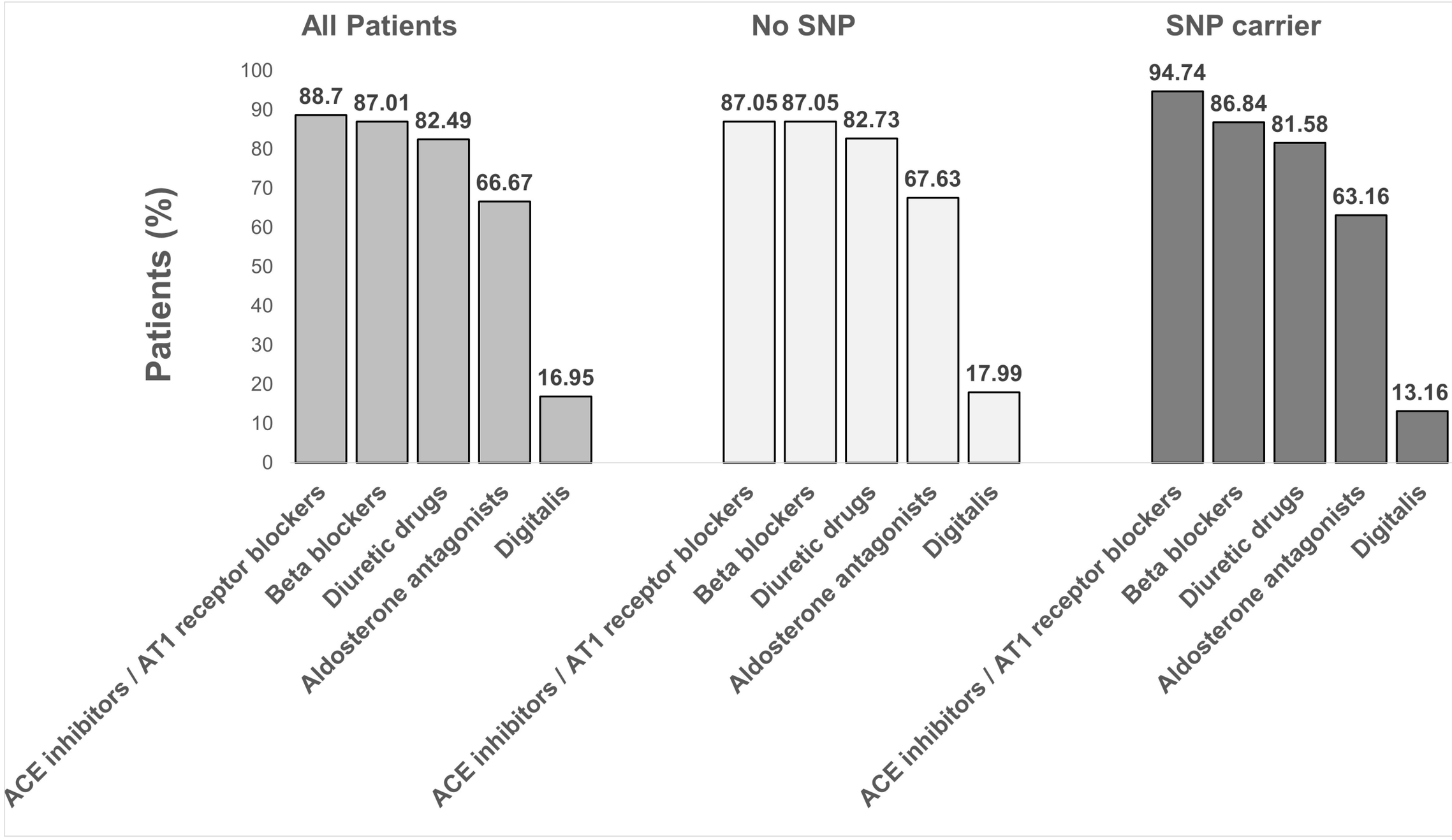
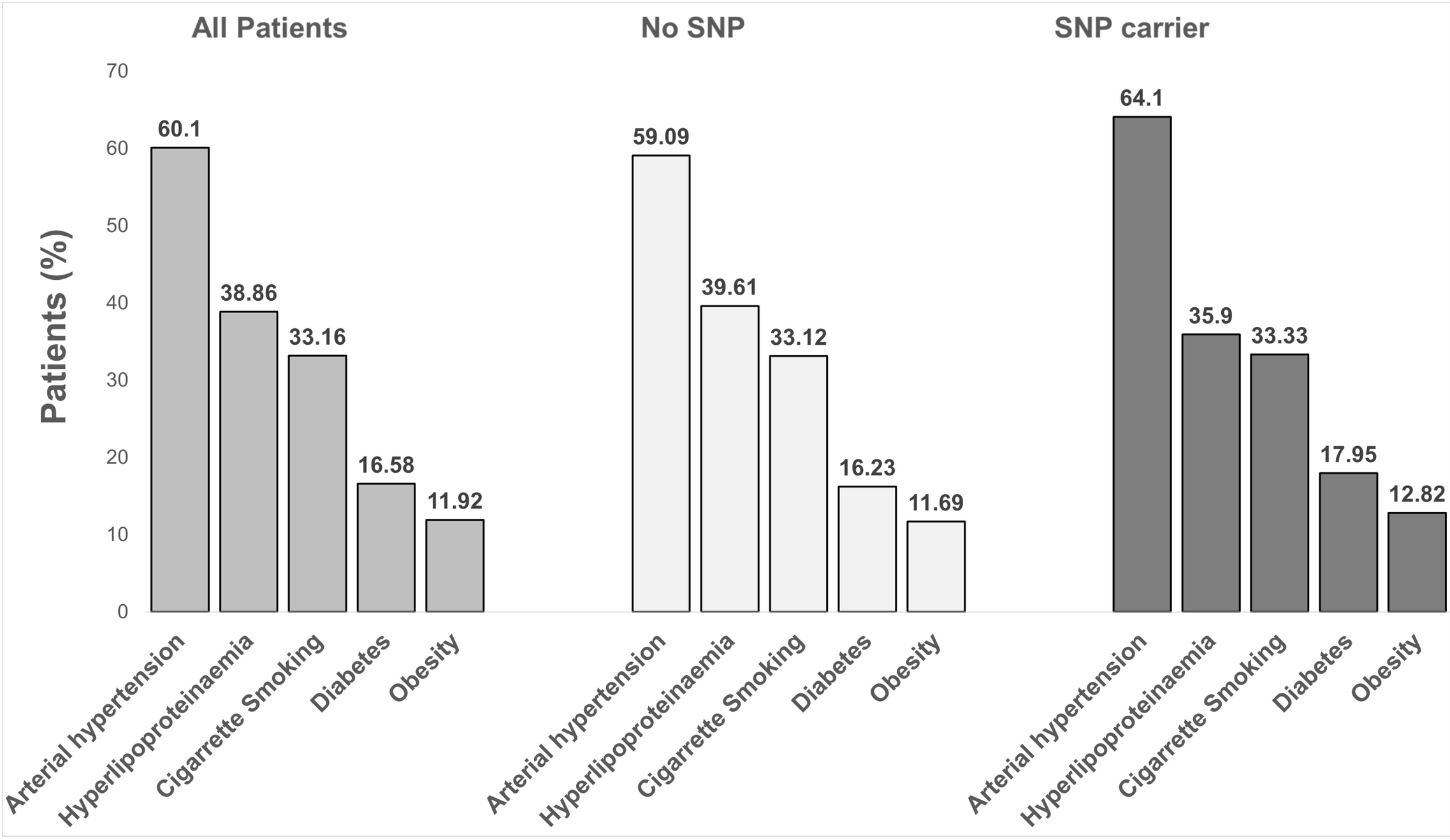

p = 0.199
Patients with Virus (-) DCMi n = 221
20.40%
79.60%
SNP carrier (Mm) n = 45
No SNP (MM) n = 176
Patients with Virus (+) DCMi n = 114
16.70%
2.60%
80.70%
SNP carrier (Mm) n = 19
SNP carrier (mm) n = 3
No SNP (MM) n = 92
Controls n = 77
20.80%
2.60%
76.60%
SNP carrier (Mm) n = 16
SNP carrier (mm) n = 2
No SNP (MM) n = 59


All Patients n = 221
25.34%
74.66%
male n = 165
female n = 56
Patients with SNP carrier n = 45
22.22%
77.78%
male n = 35
female n = 10
Patients with No SNP n = 176
26.14%
73.86%
male n = 130
female n = 46


All Patients
No SNP
SNP carrier
Patients (%)
100
90
80
70
60
50
40
30
20
10
0
88.7
87.01
82.49
66.67
16.95
87.05
87.05
82.73
67.63
17.99
94.74
86.84
81.58
63.16
13.16
ACE inhibitors / AT1 receptor blockers
Beta blockers
Diuretic drugs
Aldosterone antagonists
Digitalis


All Patients
No SNP
SNP carrier
Patients (%)
70
60
50
40
30
20
10
0
60.1
38.86
33.16
16.58
11.92
59.09
39.61
33.12
16.23
11.69
64.1
35.9
33.33
17.95
12.82
Arterial hypertension
Hyperlipoproteinaemia
Cigarrette Smoking
Diabetes
Obesity