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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