SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Study population - MYCPEDIG cohort

Clinical data from patients under 18 years of age with suspected myocarditis were extracted from medical records at the Pediatric Cardiology Departments of the Charité - Universitätsmedizin Berlin and the German Heart Center Berlin, Berlin, Germany between December 2006 and March 2019. Inclusion criteria were the following: presentation of new-onset cardiac symptoms (chest pain, dyspnea, and/or congestive heart failure); recent history of febrile illness (respiratory, gastrointestinal, or other suspected infection with time from onset of symptoms to admission ≤ 6 weeks); absence of structural congenital heart defects, syndromic disorder, metabolic, mitochondrial or neuromuscular disease, or known family history of cardiomyopathy (CMP). Clinical and diagnostic assessments including laboratory parameters and cardiac imaging were performed as described previously [21] and depicted in the Flow Chart (Figure 1). DCM was diagnosed by echocardiography according to standard definitions, with left ventricular (LV) systolic dysfunction and dilatation greater than two standard deviations above the mean of a normal population [22].

In 47 of 55 patients with clinically suspected myocarditis coronary angiography and EMB was performed as the gold standard for the diagnosis of myocarditis in explained CMP, new-onset heart failure or ventricular arrhythmia [4,38]. In 42 patients, defined as the MYCPEDIG (Genetics in PEDIatric MYoCarditis) cohort, the diagnosis of myocarditis was confirmed according to established histological and immunohistochemical criteria, and through viral genome detection (Suppl. Figure I) [4,2,39,40]. Deoxyribonucleic and ribonucleic acid (DNA, RNA) was detected in the myocardium and EDTA blood by nested (RT) or quantitative PCR of the following pathogens: Parvovirus B19 (PVB19), enteroviruses, adenoviruses, human

herpesvirus 6 (HHV6) and 7 (HHV7), cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus type 1 (HSV1) and type 2 (HSV2), varicella zoster virus (VZV), and mycoplasma pneumoniae (Suppl. Figure I). Only patients with biopsy-proven myocarditis and available DNA samples from the Competence Network for Congenital Heart Defects, Germany, were subsequently included in the analysis, and underwent genetic testing and clinical follow-up.

The study was approved by the institutional ethics committee (Charité - Universitätsmedizin Berlin, ID EA2/083/13, EA2/131/10, EA2/074/13), following the Declaration of Helsinki. All parents/guardians of patients <18 years gave written informed consent.

Starting with the year 2013 patients of the MYCPEDIG cohort were also enrolled in the prospective German multicenter registry for suspected myocarditis (MYKKE), which was set up to improve knowledge on diagnostics, clinical course and management in pediatric myocarditis (ClinicalTrials.gov Identifier: NCT02590341). Within the MYKKE registry, patient data are entered in an online database, hosted by the Competence Network for Congenital Heart Defects, Germany [41].

RIKADA cohort

The prospective RIKADA (Risk Stratification in Children and Adolescents with Primary Cardiomyopathy) study [21] is conducted at our institutions (ClinicalTrials.gov Identifier: NCT03572569). Between February 2014 and January 2017, 20 patients with primary DCM (RIKADA-DCM) were included. Patients with syndromic disorders, secondary DCM (specifically myocarditis), and mitochondrial, metabolic or neuromuscular disease were excluded from this cohort [22]. In 55% of DCM patients in RIKADA, myocarditis was excluded by EMB, and in 20% familial DCM was present. In another 25% of DCM patients in RIKADA, primary DCM was diagnosed by cardiovascular magnetic resonance imaging

(CMR) or patients were unsuitable for EMB because of young age, but had no history of viral infection.

Follow-up

All patients were monitored at the same two institutions. The follow-up for occurrence of adverse events started with the date of presentation. Mechanical circulatory support (MCS), heart transplantation (HTx), and/or all-cause death, were defined as a combined endpoint.

Statistical analysis for clinical and outcome data

Categorical variables were summarized by frequencies and percentages. For continuous measures data were presented as median values with interquartile range (IQR). Pearson's chi-square test was used to compare dichotomous variables, and where applicable Fisher´s exact test. For comparison of independent groups, the Mann-Whitney U and Kruskal-Wallis tests were applied. Kaplan-Meier curves and log rank tests were used for survival analysis. Data were analyzed using IBM SPSS Statistics version 24.0 (Armonk, NY, IBM Corp). A probability value of <0.05 was considered statistically significant.

Next-Generation Sequencing (NGS) and variant calling

For NGS analysis the Illumina TruSight Cardio Sequencing Kit was used (Illumina, USA). Alignment of NGS raw data sets and variant calling was performed to the GRCh37 (hs37d5.fa) reference genome [31]. Briefly, the called variants were evaluated with Variant Studio (Illumina, USA) for their minor allele frequency (MAF) <0.0001 and mutation specification. We used Genome Aggregation Database (gnomAD v.2.) as genetic reference database for unaffected individuals (http://gnomad.broadinstitute.org/) [42]. We evaluated 89 CMP disease genes [31], which were clustered into functional groups [28,31] according to their specific molecular function.

Gene List of 89 CMP disease genes

Variants in the following genes were bioinformatically evaluated and classified: *ABCC9, ACTA1, ACTC1, ACTN2,* ***ALMS1****, ANKRD1, BAG3, BRAF, CALR3, CAV3, CBL,* ***COX15****, CRYAB, CSRP3, DES,* ***DMD****,* ***DNAJC19****,* ***DOLK****, DSC2, DSG2, DSP, DTNA,* ***EMD****, EYA4, FBN1,* ***FHL1****, FHL2,* ***FKRP, FKTN, FXN, GAA, GATAD1****, GLA,* ***HADHA****, HCN4,* ***HFE****, HRAS, HSPB8, JPH2, JUP, KRAS,* ***LAMA2****, LAMA4, LAMP2, LDB3/ZASP, LMNA, MAP2K1, MAP2K2, MIB1, MYBPC3, MYH6, MYH7, MYL2, MYL3, MYLK2, MYOZ2, MYPN, NEXN, NKX2-5, NRAS, PDLIM3, PKP2, PLN, PRDM16, PRKAG2, PTPN11, RAF1, RBM20, RYR2, SCN5A,* ***SCO2, SDHA, SGCB****, SGCD,* ***SGCG****, SHOC2, SOS1,* ***TAZ****, TBX20, TCAP, TGFB3, TMEM43, TNNC1, TNNI3, TNNT2, TPM1, TTN, TTR, VCL*. Genes in bold were considered as disease causing only with recessive inheritance.

Genetic analysis and variant classification

All filtered genetic variants were classified as pathogenic (P), likely pathogenic (LP), or variant of uncertain significance (VUS) according to the guidelines of the American College of Medical Genetics and Genomics (ACMG) [43]. The used MAF was <0.0001 as recommended for CMP. Novel LP/P variants from the RIKADA-DCM cohort were previously published [31,44] and are deposited in the National Center for Biotechnology (NCBI) database ClinVar [18], available at: https://www.ncbi.nlm.nih.gov/clinvar/submitters/506935/. Genetic data generated from this study will also be made available in ClinVar.

Variant classification was performed according to ACMG guidelines [43]. The term PVS1 was applied for variants in genes where loss of function (LOF)/truncating variants are a proven CMP disease mechanism. PVS1 was applied for *DSP*, *BAG3*, and *TNNI3,* only. For *TTN* truncating variants we did not apply PVS1. In pediatric DCM TTN variants are rarely associated with disease compared with adult patients [45]. The ACMG terms PM5 or PS1 were

activated when published LP/P variants occurred at the same amino acid position or amino acid exchange, respectively. PS3 was applied when database knowledge e.g. PubMed (https://pubmed.ncbi.nlm.nih.gov), Ensembl (http://www.ensembl.org/index.html), UniProt (https://www.uniprot.org/), or ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) provided a clear pathological impact of the variant. PM1 was used when variants in proximity affect the functional domain or LP/P variants accumulate close to the analyzed variant. The ACMG term PM2 was activated at a gnomAD MAF <0.0001. PM4 was applied for protein length changing variants due to coding sequence changes. PM6 was activated when a *de novo* variant was detected without confirmation of the paternity and maternity. PP3 was activated when *in silico* prediction tools e.g. MT2 (http://www.mutationtaster.org/), Polyphen (http://genetics.bwh.harvard.edu/pph2/), or Provean (http://provean.jcvi.org/index.php) provide negative impact of the variant. For classification the National Center for Biotechnology (NCBI) database ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) was used [18]. Missense variants in *TTN* were not further evaluated. The impact of *TTN* length changing variants was validated according to splice pattern displayed at cardiodb (https://www.cardiodb.org/titin) [46]. If applicable, genetic variants detected in index patients were traced in first degree family members to determine *de novo* mutation or segregation.

Rare variant enrichment analysis

From the International Genome Sample Resource (IGSR) collection 503 control individuals of European descent [47,48] and all patients from the MYC-NonDCM, MYC-DCM, and RIKADA-DCM cohorts were automatically filtered with the following parameters: variant is heterozygous, homozygous, or hemizygous; MAF <0.0001%, 89 CMP genes, and maximum 4 carriers are present in cohort. Classification of variants occurred with the Combined Annotation Dependent Depletion (CADD) score v1.6 [49]. Enrichment of CADD >30 variants was tested

with Wilcoxon rank sum test with continuity correction and Fisher´s exact test for count data using R version 6.2. R Foundation for Statistical Computing, Vienna, Austria (https:// www.R-project.org/). A probability value of <0.05 was considered statistically significant.

Gene-based burden testing

For the burden testing, variants were processed according to Guo et al. [50] and Mazzarotto et al. [30]. Briefly, variants were called with Varfish, quality filtered, filtered for MAF <0.0001%, and the individual burden of truncating/missense disease variants was calculated for each subgroup MYC-NonDCM, MYC-DCM, and RIKADA-DCM (Suppl. Figure IIIA) [51]. Variants were filtered to MAF <0.0001 derived from gnomAD and variant sites were marked by the variant caller with read depth (DP >10), depth quality score (QD ≥5), genotype quality (QG ≥80). A calibration QQ-plot was computed using equidistantly values between 0 and 1 for the expected P-value because of the low sample size using all 174 genes of the panel (Suppl. Figure IIIB). P-values for burden testing were computed using the dominant/recessive model with non-/truncating variants accordingly for the 89 CMP disease genes. P-values were subjected to Bonferroni correction for 89 tests. A probability value of <0.05 was considered statistically significant.

Protein Expression Analysis

Human ventricular biopsies obtained at time of cardiac catheterization or surgery were subjected to paraformaldehyde fixation and paraffin embedding. Paraffin sections were cut with 5 µm thickness and processed according to standard protocols [52]. For immunofluorescence analysis, tissue sections were probed with the following primary antibodies: anti-BAG3 (Sigma-Aldrich, HPA020586) and anti-TNNT2 (ThermoFisher, MA5-12960). Nuclei and plasma membranes were stained with DAPI Alexa Flour 405 and wheat germ agglutinin (WGA) Alexa Flour 488, respectively. Imaging of immunofluorescence

staining occurred with a four-channel laser scanning microscope (LSM700, Zeiss, Germany) under identical conditions.

SUPPLEMENTAL TABLES

Supplemental Table I. Histological, Immunhistochemical and Viral PCR Results of Endomyocardial Biopsies

	All	MYC-NonDCM	MYC-DCM	P-Value
Patients	42	22	20	
Histology - Lymphocyte Infiltrate				
None	1 (3)	1 (5)	0 (0)	**0.007**
Normal	2 (5)	1 (5)	1 (6)	
Mild	12 (30)	9 (41)	3 (17)	
Moderate	19 (48)	11 (50)	8 (44)	
Severe	6 (15)	0 (0)	6 (33)	
Histology - Necrosis				
Positive	12 (29)	3 (14)	9 (47)	**0.04**
Histology - Interstitial Fibrosis				
None	3 (8)	1 (5)	2 (11)	0.894
Mild	14 (35)	8 (38)	6 (32)	
Moderate	23 (58)	12 (57)	11 (58)	
Severe	0 (0)	0 (0)	0 (0)	
Immunhistochemistry - CD3+ T Cell Detection				
None	1 (3)	0 (0)	1 (6)	**0.003**
Normal	8 (23)	6 (32)	2 (13)	
Mild	12 (34)	10 (53)	2 (13)	
Moderate	9 (26)	3 (16)	6 (38)	
Severe	5 (14)	0 (0)	5 (31)	
Immunhistochemistry - CD68+ Macrophage Detection				
None	0 (0)	0 (0)	0 (0)	**0.002**
Normal	3 (9)	1 (5)	2 (14)	
Mild	8 (24)	8 (42)	0 (0)	
Moderate	18 (55)	10 (53)	8 (57)	
Severe	4 (12)	0 (0)	4 (29)	
Myocardial Virus Detection (PCR)				
Positive	17 (43)	6 (29) *	11 (58) †	0.109

Values are given as n (%). DCM = dilated cardiomyopathy; MYC-DCM = Myocarditis with phenotype of dilated cardiomyopathy; MYC-NonDCM = Myocarditis without phenotype of dilated cardiomyopathy; PCR = polymerase chain reaction. * In 6/21 patients (29%) a myocardial virus was detected (PVB19 n=4; HHV7 n=1; enterovirus n=1). † In 11/19 patients of the MYC-DCM subgroup (58%) a virus could be detected within the myocardium (PVB19 n=4; HHV6 n=4; CMV n=2; Mycoplasma pneumoniae n=1). Within the MYC-NonDCM cohort, a moderate or high viral DNA/RNA load was only seen in 3 patients of the MYC-DCM cohort.

Supplemental Table II. Genetic Variants of 42 Patients with Myocarditis and 20 Patients with Primary DCM from the RIKADA-Study

	MYC-NonDCM + MYC-DCM N=42	MYC-NonDCM N=22	MYC-DCM N=20	RIKADA-DCM* N=20
Patients with Variants				
Patients with 0 variant, n	22	14	8	9
Patients with 1 variant, n	16	6	10	9
Patients with ≥2 variants, n	4	2	2	2
Patients with pathogenic variant, n	2	0	2	1
Patients with likely pathogenic variant, n	7	2	5	3
Category of Genetic Variants				
Total variants, n	25	11	14	15
Pathogenic variants†, n	2	0	2	1
Likely pathogenic variants†, n	7	2	5	3
Variants of unknown significance (VUS) †, n	16	9	7	11
De novo, n	2	0	2	2
Not *de novo* (inherited), n	5	3	2	10
Novel, n	17	9	8	9
Not novel (known), n	8	2	6	6
Missense, n	15	5	10	11
Indel/frameshift, n	4	1	3	3
Stop gain, n	2	1	1	1
Splice site, n	4	4	0	0

* Parts of the data have been published [21,31]. † Classification according to Richards et al., Genetics in Medicine, 2015 [43].

Supplemental Table III. Genetic variants in 42 index patients with myocarditis (MYC-NonDCM and MYC-DCM)

Gene	Transcript	cDNA alteration	Protein alteration	Diagnosis	Variant ID	GnomAD MAF	Pathogenicity	de novo	novel	CADD analysis§	Gene-based burden analysis‖
Pathogenic and likely pathogenic genetic variants											
BAG3	NM_004281.3 ENST00000369085	c.608delG	p.Tyr205Thrfs*6	MYC-DCM	CMP-100-01	0	Likely pathogenic (PM2, PVS1)	no	yes	yes	yes
BAG3	NM_004281.3 ENST00000369085	c.925C>T	p.Arg309*	MYC-DCM	CMP-99-01	0	Pathogenic (PM2, PVS1, PM6)	yes	no	yes	yes
DSP	NM_004415.2 ENST00000379802	c.2200A>del	p.Arg734Glufs*31	MYC-NonDCM	CMP-105-01	0	Likely pathogenic (PM2, PVS1)	no	yes	yes	yes
DSP †	NM_004415.2 ENST00000379802	c.4372C>T	p.Arg1458*	MYC-NonDCM	CMP-81-02	0.000008129	Likely pathogenic (PM2, PVS1)	no	yes	yes	yes
LMNA	NM_005572.3 ENST00000368300.4	c.868G>A	p.Glu290Lys	MYC-DCM	CMP-89-01	0	Likely pathogenic (PM1, PM2, PS1, PP3)	?	no	yes	yes
MYH7	NM_000257.2 ENST00000355349	c.644C>T	p.Thr215Ile	MYC-DCM	CMP-87-01	0	Likely pathogenic (PM1-2, PM6, PP3)	yes	yes	no	yes
TNNI3 *	NM_000363.4 ENST00000344887	c.204delG (hom)	p.Arg69Alafs*9	MYC-DCM	CMP-84-01	0.00003436	Pathogenic (PM2, PS3, PVS1)	no	no	no	yes
TNNT2	NM_001276345.1 ENST00000455702.1	c.460C>T	p.Arg154Trp	MYC-DCM	CMP-83-03	0.000036	Likely pathogenic (PM2, PS1, PP3)	?	no	no	yes
TTN	NM_133378.4 ENST00000342992	c.25889_25892del	p.Glu8630Glyfs*28	MYC-DCM	CMP-90-01	0.00000713	Likely pathogenic (PM1-2, PM4)	?	no	yes	yes
Genetic variants of uncertain significance (VUS)											
ACTN2	NM_001103.3	c.590T>C	p.Leu197Pro	MYC-DCM	CMP-85-01	0.000004061	Uncertain significance (PP3, PM2)	?	yes	yes#	yes
EYA4	NM_172105.3	c.59A>G	p.Asp20Gly	MYC-DCM	CMP-106-01	0.000008132	Uncertain significance (PM2)	?	yes	no	yes
FHL1	ENST00000394155	c.944C>T (hem)	p.Thr315Ile	MYC-NonDCM	CMP-94-01	0.0000293	Uncertain significance (PM2)	?	no	no	yes
MIB1	NM_020774.3	c.1371+5G>A	-	MYC-NonDCM	CMP-82-01	0.000007319	Uncertain significance (PM2)	?	yes	no	no‡

MYH6	NM_002471.3	c.4883A>T	p.Glu1628Val	MYC-NonDCM	CMP-98-02	0.000004061	Uncertain significance (PP3, PM2)	?	yes	no	yes
MYPN	ENST00000354393	c.1027G>A	p.Val343Met	MYC-DCM	CMP-97-02	0	Uncertain significance (PM2)	?	yes	no	yes
NEXN	NM_144573.3	c.154G>C	p.Asp52His	MYC-NonDCM	CMP-81-03	0.000008152	Uncertain significance (PM2)	no	yes	no	yes
	NM_144573.3	c.1789G>A	p.Glu597Lys	MYC-DCM	CMP-102-01	0.000004069	Uncertain significance (PP3, PM2)	?	yes	no	yes
RYR2	ENST00000360064	c.13771T>C	p.Phe4591Leu	MYC-NonDCM	CMP-88-01	0	Uncertain significance (PP2, PM2)	?	yes	no	yes
	NM_001035.2	c.9655G>A	p.Val3219Met	MYC-DCM	CMP-103-02	0.00007581	Uncertain significance (PP2, PS4)	?	no	no	yes
SOS1	NM_005633.3	c.3220G>C	p.Glu1074Gln	MYC-DCM	CMP-85-02	0	Uncertain significance (PP3, PM2)	?	yes	no	yes
TNNI3	ENST00000344887	c.178G>A	p.Glu60Lys	MYC-DCM	CMP-89-02	0	Uncertain significance (PP3, PM2)	?	yes	no	yes
TNNT2	ENST00000236918	c.128C>T	p.Thr43Ile	MYC-NonDCM	CMP-96-01	0	Uncertain significance (PM2)	?	yes	no	yes
TTN	NM_001267550.1	c.68225-5T>C	-	MYC-NonDCM	CMP-82-02	0.00003707	Uncertain significance (PM2)	?	no	no	no‡
	NM_001267550.1 ENST00000589042	c.44282-7C>G	-	MYC-NonDCM	CMP-86-01	0	Uncertain significance (PM2)	?	yes	no	no‡
VCL	NM_014000.2	c.874+5C>T	-	MYC-NonDCM	CMP-82-03	0.000004061	Uncertain significance (PM2)	?	yes	no	no‡

Codes for pathogenicity prediction were used according to American College of Medical Genetics (ACMG) guidelines [43]. * This genetic variant was published previously as p.Arg69Alafs*8 in Kühnisch et al., Clinical Genetics, 2019 [31]. † This genetic variant was published previously as p.Arg1458* in Poller et al., JAHA, 2020 [17]. ‡ These splice variants were excluded from gene-wise burden testing by the algorithm. § Inclusion of the variant for CADD based rare variant enrichment analysis when CADD score >30. ‖ Inclusion of the variant in gene-based burden testing according to Guo et al. and Mazzarotto et al. [30,50]. # *ACTN2* is not a validated DCM disease gene according to Mazzarotto et al. [30].

Supplemental Table IV. Selected complex genotypes in 42 index patients with myocarditis (MYC-NonDCM and MYC-DCM)

Gene	Transcript	cDNA Position	Protein position	Diagnosis	Variant ID	gnomAD allele frequency	Pathogenicity	de novo	novel
Truncating TTN variants									
TTN	NM_001267550.1 ENST00000589042	c.44282-7C>G	-	MYC-NonDCM	CMP-86-01	0	Uncertain significance (PM2)	?	yes
	ENST00000342992	c.25889_25892del	p.Glu8630Glyfs*28	MYC-DCM	CMP-90-01	0.00000713	Likely pathogenic (PM1-2, PM4)	?	no
	NM_001267550.1	c.68225-5T>C	-	MYC-NonDCM	CMP-82-02	0.00003707	Uncertain significance (PM2)	?	no
Index patients with >1 variant									
DSP	NM_004415.2 ENST00000379802	c.4372C>T	p.Arg1458*	MYC-NonDCM	CMP-81-02	0.000008129	Likely pathogenic (PM2, PVS1)	no	yes
NEXN	NM_144573.3	c.154G>C	p.Asp52His	MYC-NonDCM	CMP-81-03	0.000008152	Uncertain significance (PM2)	no	yes
MIB1	NM_020774.3	c.1371+5G>A	-	MYC-NonDCM	CMP-82-01	0.000007319	Uncertain significance (PM2)	?	yes
TTN	NM_001267550.1	c.68225-5T>C	-	MYC-NonDCM	CMP-82-02	0.00003707	Uncertain significance (PM2)	?	no
VCL	NM_014000.2	c.874+5C>T	-	MYC-NonDCM	CMP-82-03	0.000004061	Uncertain significance (PM2)	?	yes
ACTN2	NM_001103.3	c.590T>C	p.Leu197Pro	MYC-DCM	CMP-85-01	0.000004061	Uncertain significance (PP3, PM2)	?	yes
SOS1	NM_005633.3	c.3220G>C	p.Glu1074Gln	MYC-DCM	CMP-85-02	0	Uncertain significance (PP3, PM2)	?	yes
LMNA	ENST00000368300	c.868G>A	p.Glu290Lys	MYC-DCM	CMP-89-01	0	Likely pathogenic (PM1, PM2, PS1, PP3)	?	no
TNNI3	ENST00000344887	c.178G>A	p.Glu60Lys	MYC-DCM	CMP-89-02	0	Uncertain significance (PP3, PM2)	?	yes

Codes for pathogenicity prediction were used according to American College of Medical Genetics (ACMG) guidelines [43].

Supplemental Table V. Clinical characteristics of 20 index patients in RIKADA-DCM

	DCM
General patient parameter	
Patients	20
Female individuals	12 (60)
Age (years)	7.3 (1.9-14.2)
BSA (kg/m²)	0.9 (0.3-1.5)
Symptoms	
NYHA I	8 (40)
NYHA II	0 (0)
NYHA III	4 (20)
NYHA IV	1 (5)
NYHA n.a.	7 (35)
Heart failure signs	9 (45)
NT-proBNP (pg/ml)	5052.5 (91.2-25912.8)
Arrhythmias*	
SVT	1 (5)
nsVTs	3 (25); (n=12)
Echocardiography	
Z-score LVIDD (mm)	5.8 (3.5-10.3)
Z-Score IVSD (mm)	-0.4 (-1.3-1.3); n=20
LV-EF (%)	35.0 (17.0-50.5)
CMR	
LVEDVi (ml/m²)	124.1 (113.2-224.0); n=11
LVEF (%)	33.0 (14.0-52.0); n=11
LGE positive	2 (18); n=11
MCS & complications	
LVAD	6 (30)
BVAD	1 (5)
ECMO	1 (5)
ICD	1 (5)
HTx	9 (45)
Death	0 (0)

Values are n (%) or median (interquartile range). *Arrhythmias were recorded with Holter-ECG. BSA = body surface area; BVAD = biventricular assist device; CMR = cardiovascular magnetic resonance; DCM = dilated cardiomyopathy; ECMO = extracorporal membrane oxygenation; HTx = heart transplantation; ICD = implantable cardioverter-defibrillator; IVSD = interventricular septum thickness at end-diastole; LA = left atrial area; LGE = late gadolinium enhancement; LVAD = left ventricular assist device; LVEDVi = indexed left ventricular enddiastolic volume; LVEF = left ventricular ejection fraction; LVIDD = left ventricular internal dimension at end-diastole; n.a. = not applicable; MCS = Mechanical circulatory support; nsVT = non-sustained ventricular tachycardia; NT-proBNP = N-terminal pro brain natriuretic peptide; NYHA = New York Heart Association; SVT = supraventricular tachycardia; VO_2max = maximum oxygen consumption.

Parts of the data have been published [21,31].

Supplemental Table VI. Genetic variants in 20 index patients in RIKADA-DCM

Gene	Transcript	cDNA alteration	Protein alteration	Diagnosis	Variant ID	gnomAD MAF	Pathogenicity	de novo	novel	CADD analysis*	Gene-based burden analysis†
Pathogenic and likely pathogenic genetic variants											
ACTC1	NM_005159.4 ENST00000290378.4	c.328G>A	p.Ala110Thr	RIKADA-DCM, HTX	CMP-77-01	0	Likely pathogenic (PM2, PM6, PP2-3)	yes	no	no	yes
TNNT2	NM_001001431.2 ENST00000367315.2	c.620_622delAGA	p.Lys207del	RIKADA-DCM, HTX	CMP-12-02	0	Likely pathogenic (PS1, PM1-2)	?	no	no	yes
	NM_001001431.2 ENST00000367315.2	c.620-622delAGA	p.Lys207del	RIKADA-DCM, HTX	CMP-74-02	0	Pathogenic (PS1, PM1-2, PM6)	yes	no	no	yes
TTN	NM_001267550.1 ENST00000589042.1	c.85891delG	p.Ala28631Leufs*3	RIKADA-DCM, HTX	CMP-30-01	0	Likely pathogenic (PM1-2, PM4)	no	yes	yes	yes
Genetic variants of uncertain significance (VUS)											
BAG3	NM_004281.3	c.1634C>G	p.Pro545Arg	RIKADA-DCM	CMP-16-01	0.00009385	Uncertain significance (PP3, PM2)	no	no	no	yes
FHL2	NM_201555.1	c.143G>A	p.Gly48Asp	RIKADA-DCM	CMP-16-02	0	Uncertain significance (PP3, PM2)	no	yes	no	yes
MYH7	NM_000257.2	c.4501G>T	p.Glu1501*	RIKADA-DCM	CMP-32-01	0	Uncertain significance (PM4, PM2)	no	yes	yes	yes
	NM_000257.2	c.5767A>G	p.Lys1923Glu	RIKADA-DCM	CMP-18-01	0	Uncertain significance (PP3, PM2)	?	yes	no	yes
MYL2	NM_000432.3	c.421G>A	p.Ala141Thr	RIKADA-DCM, HTX	CMP-56-01	0	Uncertain significance (PP3, PM2)	?	no	no	yes
MYPN	NM_001256267.1	c.259C>G	p.Pro87Ala	RIKADA-DCM	CMP-16-03	0.0000433	Uncertain significance (PP3, PM2)	no	no	no	yes
PKP2	NM_004572.3	c.1536T>A	p.Asn512Lys	RIKADA-DCM, HTX	CMP-26-01	0.00001446	Uncertain significance (PP3, PM2)	no	yes	no	yes
TBX20	NM_001077653.2	c.208G>A	p.Gly70Ser	RIKADA-DCM	CMP-16-06	0	Uncertain significance (PM2)	no	yes	no	yes

	NM_001077653.2	c.994C>T	p.Pro332Ser	RIKADA-DCM, HTX	CMP-19-02	0.0000366	Uncertain significance (PM2)	no	yes	no	yes
TNNT2	NM_000364.2	c.808G>A	p.Val270Ile	RIKADA-DCM	CMP-20-01	0	Uncertain significance (PP3, PM1, PM2)	no	yes	no	yes
TPM1	NM_001018005.1	c.340G>C	p.Glu114Gln	RIKADA-DCM, HTX	CMP-26-02	0	Uncertain significance (PP3, PM2, PM5)	no	yes	no	yes

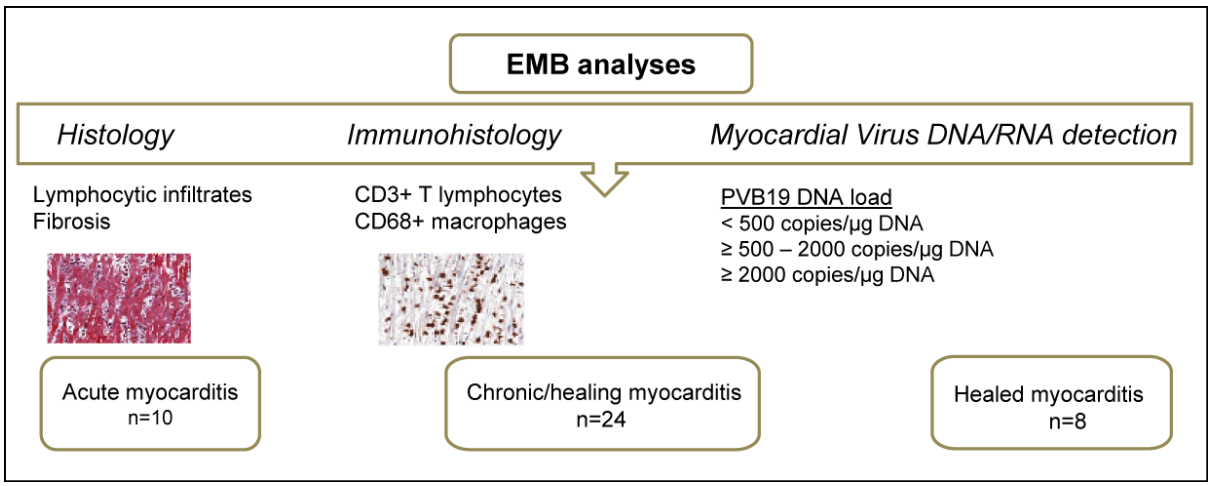
Parts of the data have been published [21,31]. Codes for pathogenicity prediction were used according to American College of Medical Genetics (ACMG) guidelines [43]. * Inclusion of the variant for CADD based rare variant enrichment analysis when CADD score >30. † Inclusion of the variant in gene-based burden testing according to Guo et al. and Mazzarotto et al. [30,50].

Supplemental Table VII. Selected complex genotypes in 20 index patients in RIKADA-DCM

Gene	Transcript	cDNA Position	Protein position	Diagnosis	Variant ID	gnomAD allele frequency	Pathogenicity	de novo	novel
Truncating TTN variants									
TTN	NM_001267550.1	c.85891delG	p.Ala28631Leufs*3	RIKADA-DCM, HTX	CMP-30-01	0	Likely pathogenic (PM1-2, PM4)	no	yes
Index patients with >1 variant									
BAG3	NM_004281.3	c.1634C>G	p.Pro545Arg	RIKADA-DCM	CMP-16-01	0.00009385	Uncertain significance (PP3, PM2)	no	no
FHL2	NM_201555.1	c.143G>A	p.Gly48Asp	RIKADA-DCM	CMP-16-02	0	Uncertain significance (PP3, PM2)	no	yes
MYPN	NM_001256267.1	c.259C>G	p.Pro87Ala	RIKADA-DCM	CMP-16-03	0.0000433	Uncertain significance (PP3, PM2)	no	no
TBX20	NM_001077653.2	c.208G>A	p.Gly70Ser	RIKADA-DCM	CMP-16-06	0	Uncertain significance (PM2)	no	yes
PKP2	NM_004572.3	c.1536T>A	p.Asn512Lys	RIKADA-DCM, HTX	CMP-26-01	0.00001446	Uncertain significance (PP3, PM2)	no	yes
TPM1	NM_001018005.1	c.340G>C	p.Glu114Gln	RIKADA-DCM, HTX	CMP-26-02	0	Uncertain significance (PP3, PM2, PM5)	no	yes

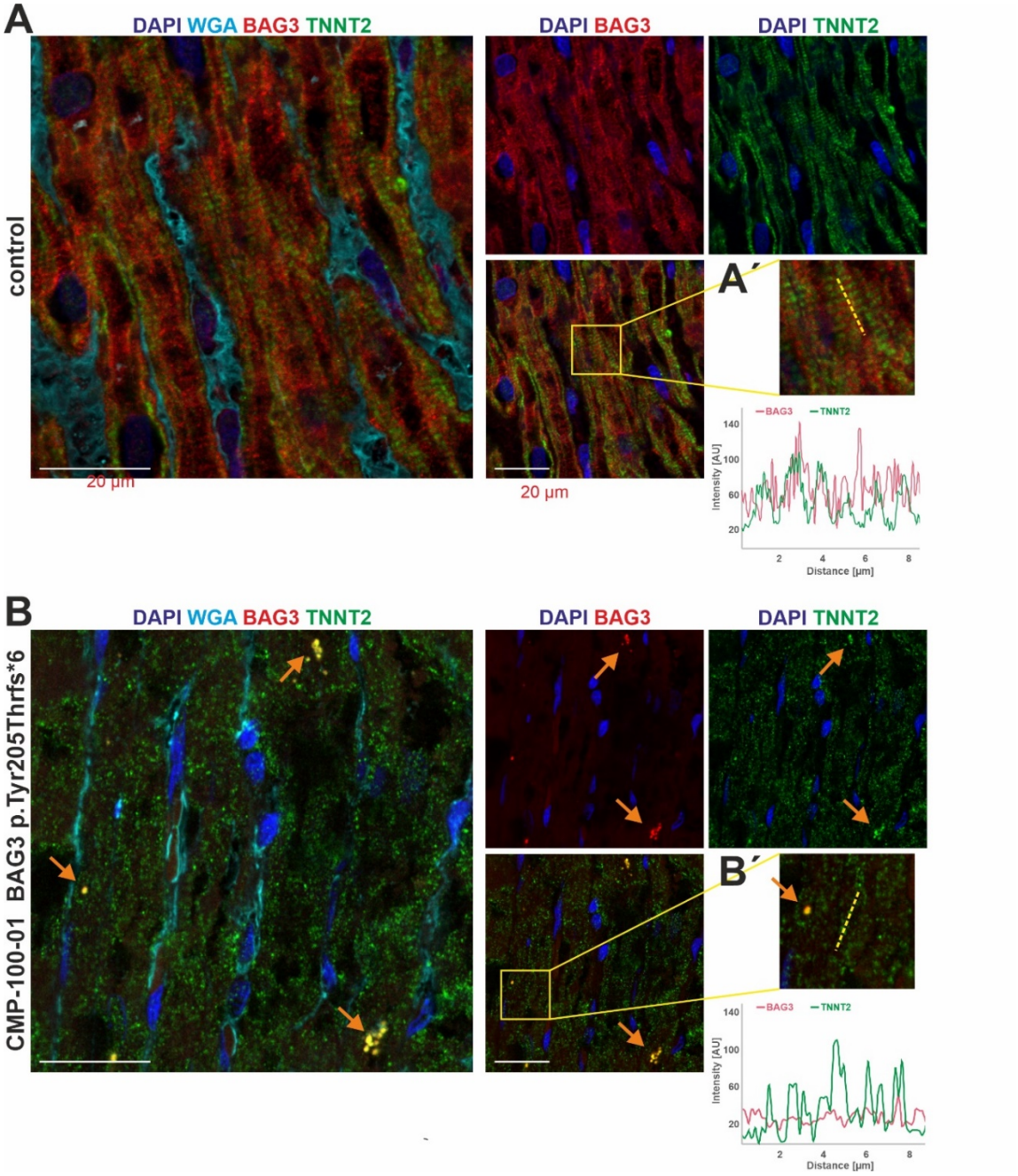
Parts of the data have been published [21,31]. Codes for pathogenicity prediction were used according to American College of Medical Genetics (ACMG) guidelines [43].

SUPPLEMENTAL FIGURES



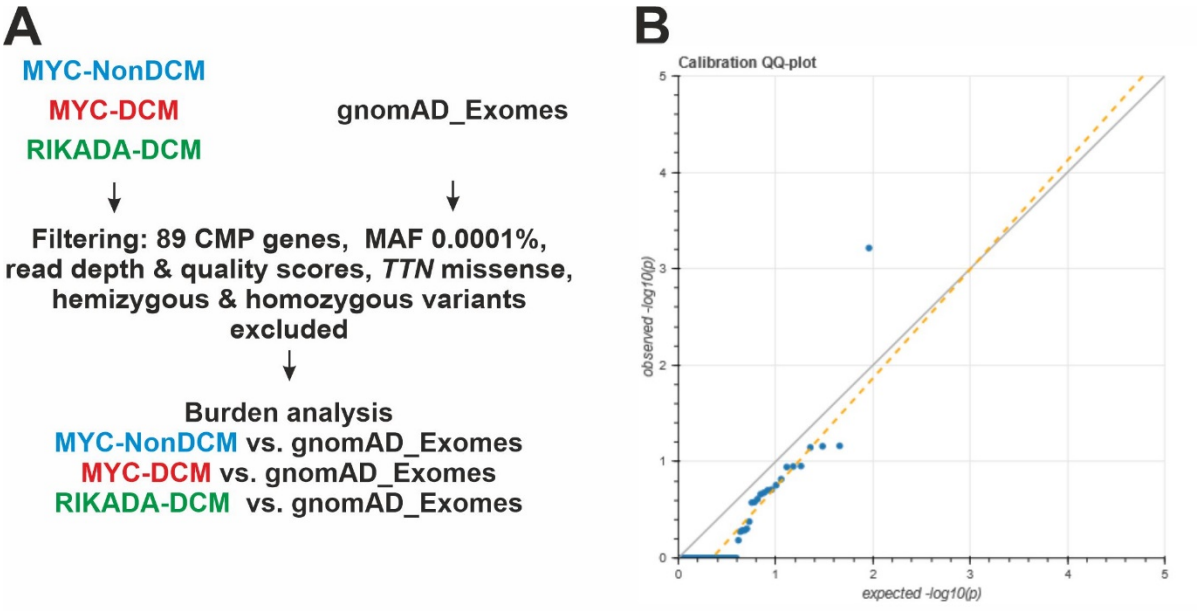
Supplemental Figure I. Schematic overview of endomyocardial biopsy (EMB) analyses

Myocarditis was diagnosed by histological and immunhistological results. In addition, myocardial virus deoxyribonucleic and ribonucleic acid (DNA/RNA) detection was performed. Parvovirus B19 (PVB19) was assessed quantitatively.



Supplemental Figure II. Protein Expression of the BAG3 p.Tyr205Thrfs*6 Variant in a Human Heart Biopsy

Immunostaining and confocal imaging of heart biopsies visualizes nuclei (DAPI; dark blue), cell membranes (wheat germ agglutinin, WGA; turquoise), BCL2 associated athanogene 3 (BAG3; red), and troponin T2, cardiac type (TNNT2; green). (A) Control sample without variant immunostaining reveals typical sarcomere pattern as illustrated by TNNT2 and abundant BAG3 protein level. (A´) Intensity plot of TNNT2 and BAG3 staining confirmed periodic TNNT2 sarcomere staining (inset image, yellow dashed line). (B) Heart biopsy of patient CMP-100-01 shows reduced BAG3 intensity, disturbed sarcomere organization (green dots), and BAG3/TNNT2 positive accumulations (orange arrows). (B´) TNNT2 and BAG3 intensity plot validates reduced BAG3 level and disturbed sarcomere organization. Scale bars indicate 20µm.



C

	gene	case (positive)	case (negative)	gnomAD control (positive)	gnomAD control (negative)	p-value (observed)	p-value (corrected)
truncating variants							
MYC-NonDCM	*DSP*	2	20	52	56833	0.000202	**0.01798**
MYC-DCM	*BAG3*	2	18	10	56872	0.000008	**0.00071**
	TNNI3	1	19	21	56621	0.007738	0.68868
	TTN	1	19	597	56259	0.190575	16.9611
RIKADA-DCM	*MYH7*	1	18	57	56828	0.019192	1.70809
	TTN	1	18	597	56259	0.181974	16.1957
missense variants							
MYC-NonDCM	*TNNT2*	1	21	112	56773	0.042794	3.80867
	NEXN	1	21	373	56255	0.135627	12.0708
	LDB3	1	21	586	56299	0.203997	18.1557
MYC-DCM	*TNNI3*	1	19	76	56569	0.026834	2.38823
	TNNT2	1	19	112	56773	0.038981	3.46931
	EYA4	1	19	252	56620	0.085295	7.59126
RIKADA-DCM	*TNNT2*	2	17	112	56773	0.000665	0.05919
	TBX20	2	17	147	56738	0.001131	0.10066
	ACTC1	1	18	37	56847	0.012614	1.12265

Supplemental Figure III. Gene-based Burden Testing

(A) Outline of gene-based burden analysis includes data sets from MYC-NonDCM, MYC-DCM, RIKADA-DCM, and gnomAD exomes that underwent variant filtering and subsequent burden analysis [30,50]. (B) For calibration of burden analysis, a QQ-plot was generated. (C) Results of burden analysis are shown for truncating and missense variants in each subgroup. The observed p-values were corrected for the 89 analyzed genes. Significant variant enrichments are shown in bold. Correction of p-values according to the 12 validated DCM genes from Mazzarotto et al.[30] did not identify enrichment of additional significant variants for MYC-DCM and MYC-NonDCM (data not shown).