JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY
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PUBLISHED BY ELSEVIER

VOL. 69, NO. 13, 2017
ISSN 0735-1097/$36.00
http://dx.doi.org/10.1016/j.jacc.2017.01.043

ORIGINAL INVESTIGATIONS

Autosomal Recessive Cardiomyopathy Presenting as Acute Myocarditis

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ABSTRACT

BACKGROUND Myocarditis is inflammation of the heart muscle that can follow various viral infections. Why children only rarely develop life-threatening acute viral myocarditis (AVM), given that the causal viral infections are common, is unknown. Genetic lesions might underlie such susceptibilities. Mouse genetic studies demonstrated that interferon (IFN)-α/β immunity defects increased susceptibility to virus-induced myocarditis. Moreover, variations in human *TLR3*, a potent inducer of IFNs, were proposed to underlie AVM.

OBJECTIVES This study sought to evaluate the hypothesis that human genetic factors may underlie AVM in previously healthy children.

METHODS We tested the role of TLR3-IFN immunity using human induced pluripotent stem cell-derived cardiomyocytes. We then performed whole-exome sequencing of 42 unrelated children with acute myocarditis (AM), some with proven viral causes.

RESULTS We found that *TLR3*- and *STAT1*-deficient cardiomyocytes were not more susceptible to Coxsackie virus B3 (CVB3) infection than control cells. Moreover, CVB3 did not induce IFN-α/β and IFN-α/β-stimulated genes in control cardiomyocytes. Finally, exogenous IFN-α did not substantially protect cardiomyocytes against CVB3. We did not observe a significant enrichment of rare variations in TLR3- or IFN-α/β-related genes. Surprisingly, we found that homozygous but not heterozygous rare variants in genes associated with inherited cardiomyopathies were significantly enriched in AM-AVM patients compared with healthy individuals ($p = 2.22E-03$) or patients with other diseases ($p = 1.08E-04$). Seven of 42 patients (16.7%) carried rare biallelic (homozygous or compound heterozygous) nonsynonymous or splice-site variations in 6 cardiomyopathy-associated genes (*BAG3*, *DSP*, *PKP2*, *RYR2*, *SCN5A*, or *TNNI3*).

CONCLUSIONS Previously silent recessive defects of the myocardium may predispose to acute heart failure presenting as AM, notably after common viral infections in children. (J Am Coll Cardiol 2017;69:1653–65)

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ABBREVIATIONS AND ACRONYMS

AM = acute myocarditis

ARVC = arrhythmogenic right ventricular cardiomyopathy

AVM = acute viral myocarditis

CVB3 = Coxsackie virus B3

DCM = dilated cardiomyopathy

iPSC = induced pluripotent stem cell

MAF = minor allele frequency

WES = whole-exome sequencing

Acute myocarditis (AM) is an inflammatory disease of the myocardium. The estimated annual incidence of myocarditis is 1 per 100,000 children in the United States, corresponding to a prevalence of ~1/10,000 (1). The actual incidence of this heterogeneous disease is probably higher because of unrecognized cases.

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Myocarditis most typically presents with sudden onset of congestive heart failure or cardiogenic shock. Some patients recover fully and spontaneously or following symptomatic treatment. Others suffer from cardiac sequelae such as dilated cardiomyopathy (DCM) (1), requiring long-term anticongestive therapy or heart transplantation. In some cases, sudden death occurs (1). Biopsy-proven myocarditis is reported in up to 46% of children with an identified cause of DCM (2). Although myocarditis has many causes, most cases apparently result from infection with viruses, including enteroviruses, adenoviruses, and parvoviruses (1). Infections from such viruses are common, particularly during childhood; it is unclear, therefore, why only a very small proportion of children develop acute viral myocarditis (AVM). Of note, clinical severity is uncorrelated with viral infectious causes, suggesting a human genetic predisposition. Studies in inbred mice have implicated the major histocompatibility complex and other loci in genetic susceptibility to Coxsackie virus-induced myocarditis (3,4). Overall, these observations suggest that human genetic variations, rendering the heart more sensitive to common viral infections, may contribute to the progression of myocarditis, at least in some children.

Severe childhood infectious diseases, including viral diseases striking healthy children, can result from single-gene inborn errors of immunity (5,6). Some of these immunodeficiencies have tissue-specific phenotypes, which could reflect restricted viral tropism or tissue specificity of the inborn error. Herpes simplex encephalitis (HSE) and severe pulmonary influenza can be due to brain- and lung-intrinsic defects in interferon (IFN) immunity, respectively (6). Interestingly, heart, brain, and lungs are typically the only organs severely affected in patients with AVM, HSE, and severe influenza, respectively, despite the ability of the causal viruses to replicate in many tissues. Moreover, inborn errors of adaptive immunity do not predispose to any of these conditions (7).

Recently, the case of an adult patient with enteroviral AVM carrying a rare, dominant-negative toll-like receptor 3 (*TLR3*) allele was reported (8). Similarly, mice lacking genes encoding key proteins in antiviral IFN immunity, such as IFN-β and TLR3, were more susceptible to Coxsackie virus B3 (CVB3) infection and virus-induced myocardial injury (9,10). Whether variations in these human genes influence the outcome of infection by cardiotropic viruses remains unclear. To test the hypothesis that inborn errors of heart-intrinsic TLR3-IFN immunity may underlie AVM in previously healthy children, we explored the impact of mutations in TLR3-IFN-related genes on antiviral immunity of human cardiomyocytes derived from induced pluripotent stem cells (iPSCs) and searched for TLR3-IFN-related gene mutations in AVM using whole-exome sequencing (WES). As our results were inconsistent with the TLR3-IFN hypothesis, we then tested the hypothesis that hitherto silent cardiomyopathies may underlie AVM.

METHODS

Control and mutant iPSCs carrying deleterious mutations in *STAT1* (c.1928_1929insA/c.1928_1929insA) or *TLR3* (p.P554S/p.E746*) were derived from primary dermal fibroblasts of healthy donors or patients with severe viral diseases other than AM, respectively (11,12). Using a modified version of the original protocol, iPSCs were differentiated into cardiomyocytes (13). Details of cardiomyocyte differentiation and

Institutes of Health/National Institute of Neurological Disorders and Stroke (2R01NS072381), the French National Research Agency (IEIHSEER ANR-14-CE14-0008-01), and, under the "Investments for the Future" program (ANR-10-IAHU-01), the Institut National de la Santé et de la Recherche Médicale, Paris Descartes University, the St. Giles Foundation, The Rockefeller University, and the Howard Hughes Medical Institute (to Dr. Casanova); and the Association pour la Recherche en Cardiologie du Fœtus à l'Adulte (to Dr. Bonnet). Dr. Belkaya is a recipient of a postdoctoral fellowship (16POST27260016) from the American Heart Association and an Early Career Award (12775) from the Thrasher Research Fund. Dr. Kontorovich is a recipient of the Glorney-Raisbeck fellowship in cardiovascular diseases from the Corlette Glorney Foundation and The New York Academy of Medicine. Donors had no role in the design and conduct of the study, the collection, analysis, and interpretation of the data, or in the preparation, review, or approval of the manuscript. Dr. Casanova is a consultant for ADMA, Nimbus, and Vitae Pharmaceuticals. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose. Drs. Belkaya, Kontorovich, and Byun, are joint first authors and contributed equally to this work. Drs. Abel and Zhang contributed equally to this work. Drs. Bonnet, Gelb, and Casanova are joint senior authors and contributed equally to this work. Jeffrey A. Towbin, MD, served as Guest Editor for this article.

Manuscript received October 3, 2016; revised manuscript received December 11, 2016, accepted January 19, 2017.

CVB3 infection studies are described in the Online Appendix.

PATIENT RECRUITMENT AND ETHICS. Our subject inclusion criteria were the following: between 1 month and 16 years of age at the onset of cardiac symptoms; presentation with acute onset of chest pain, dyspnea, and/or congestive heart failure; cardiac magnetic resonance (CMR) findings consistent with inflammation; recent history of febrile illness; absence of known family history of or metabolic disorder associated with cardiomyopathy; and histological evidence of inflammatory infiltrates within the myocardium on myocardial biopsy or cardiac explant when available, according to the Dallas classification (14). CMR criteria used to diagnose myocardial inflammation were modified from the Lake Louise criteria that apply in the adult population but are less sensitive in children, for whom early gadolinium enhancement findings are difficult to quantify (15), including: 1) evidence of regional or global myocardial edema with T2 hyperintensity (T2 ratio: 2; where T2 ratio = signal intensity$_{myocardium}$-to-signal intensity$_{skeletal\ muscle}$); 2) evidence of myocardial hyperemia and capillary leakage with early gadolinium enhancement on cine-steady-state free precession and/or T1-weighted images (acquired early after contrast injection) compared with the skeletal muscle; and 3) evidence of myocardial necrosis and fibrosis (visual assessment) with nonischemic regional distribution at late gadolinium enhancement imaging. Acute myocarditis was diagnosed when at least 2 of the CMR criteria were present. Overall, a total of 42 unrelated patients (23 female, 19 male) with AM, some with proven AVM, were recruited. Time from symptom onset to diagnosis varied, ranging from a few hours to 3 months. All patients were referred from the Necker Hospital for Sick Children in Paris, France, after a review of the clinical evidence for AM by a clinical expert (D.B.). Clinical history and biological specimens were obtained after obtaining consent from the patients and/or their participating family members under an approved protocol in accordance with institutional, local, and national ethical guidelines.

WES and data analysis are described in the Online Appendix. We excluded polymorphisms with a minor allele frequency (MAF) of ≥1% in public databases: Exome Variant Server; 1000 Genomes; and Exome Aggregation Consortium (ExAC), excluding more common variants in each ethnic subpopulation in ExAC. Only damaging and missense mutations were retained. We then searched for: 1) heterozygous variations with MAF <0.01% in ExAC (excluding variants with MAF ≥0.1% in any ethnic subpopulation) under an autosomal dominant (AD) model; 2) homozygous or compound heterozygous variants with MAF <1% under an autosomal recessive (AR) model; and 3) monoallelic variations with MAF <0.01% in ExAC (excluding variants with MAF ≥0.1% in any ethnic subpopulation) or biallelic variations with MAF <1% under X-linked genetic models.

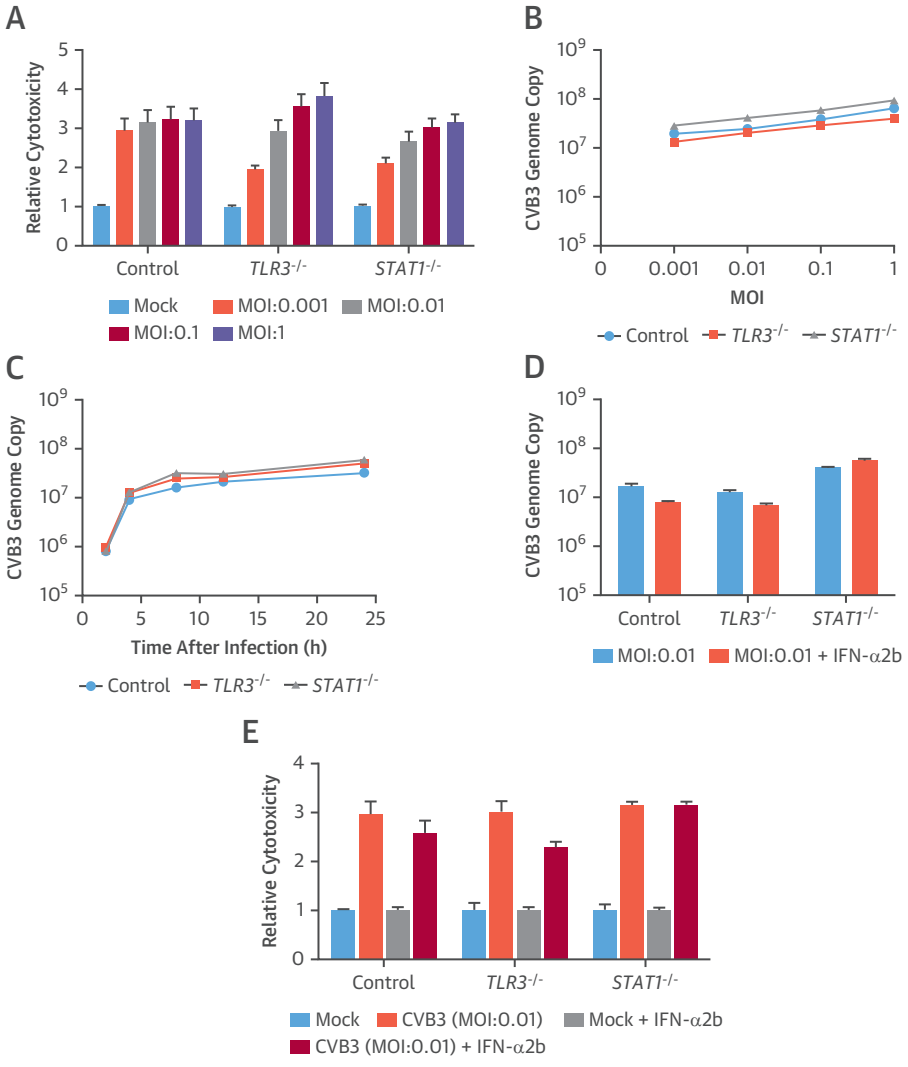
We performed an enrichment analysis of variations in 3 pathways: 1) TLR3 cascade; 2) IFN-α/β signaling; and 3) cardiomyopathy-associated genes (Online Tables 1 and 2) in our comparison of AM-AVM cohort with the 2 control cohorts of 1,164 healthy individuals from 1000 Genomes database and our in-house database of 2,324 exomes from healthy individuals and patients with severe infectious diseases of childhood other than myocarditis.

STATISTICAL ANALYSIS. The proportion of individuals with mutant alleles in each cohort were compared by means of logistic regression, using the likelihood ratio test. To account for cohort ethnic heterogeneity, the 3 first principal components of the principal component analysis were systematically included in the logistic regression model (Online Appendix). Bonferroni correction was applied to set the p value threshold for statistical significance to 8.33E-03 (=0.05/6) as 3 gene lists were tested under 2 different genetic models. We also compared the proportion of individuals with variations in cardiomyopathy-associated genes in the AM cohort with each ethnic ExAC subpopulation, using Fisher exact test, as principal components could not be obtained from available ExAC data. Finally, 1-tailed unpaired Student *t* test was used to compare the combined annotation-dependent depletion (CADD) scores of rare cardiomyopathy-associated alleles in AM-AVM patients to those of all variations known to cause inherited cardiomyopathy, using $p < 0.05$ as the threshold for statistical significance.

RESULTS

Human iPSC-derived cardiomyocytes are permissive to CVB3 infection (16). Here, we generated cardiomyocytes from iPSCs and selected the SIRPα^{+}/CD90^{-} population, achieving >95% cardiomyocyte purity. We infected control human iPSC-derived cardiomyocyte lines from 3 unrelated healthy individuals with CVB3 at increasing multiplicities of infection (MOIs) to assess virus-induced cytotoxicity and viral replication at various time points upon infection. Cardiomyocyte viability was severely decreased upon CVB3 infection, with complete cell death occurring 24 h post infection at MOIs >1 (data not shown). CVB3 replication peaked at between 10^7 and 10^8 genome copies within 8 h and remained unchanged up to 72 h post infection at MOI 1

FIGURE 1 CVB3 Infection of Human iPSC-Derived Cardiomyocytes



(A) Virus-induced cytotoxicity in representative lines of control, *TLR3*-, and *STAT1*-deficient cardiomyocytes at 24 h post-infection with CVB3 is compared at indicated MOIs. Percentages of cytotoxicity were determined by lactate dehydrogenase release assay and normalized to that of mock-infected cells for each iPSC line, which is set as 1. CVB3 replication was determined by quantitative PCR in control and mutant cardiomyocyte lines at 24 h post infection with CVB3 at various MOIs **(B)** and at different time points post infection at MOI of 1 **(C)**. **(A to C)** Data are mean ± SEM from several independent duplicated experiments. Viral replication **(D)** and virus-induced cytotoxicity **(E)** were measured at 24 h post infection with CVB3 at MOI of 0.01 in control and mutant cardiomyocytes pre-treated with exogenous IFN-α2b. Data are mean ± SEM from at least 2 independent experiments. CVB3 = Coxsackie virus B3; IFN = interferon; iPSC = induced pluripotent stem cell; MOI = multiplicity of infection; SEM = standard error of the mean.

(data not shown). Moreover, we performed transcriptomic profiling of control cardiomyocytes upon infection with CVB3 or a mutant strain of vesicular stomatitis virus (mVSV), which is unable to attenuate host antiviral immunity (17), at MOI 5 for 8 h. We found that 88 and 155 genes were differentially expressed (>2-fold) in CVB3- and mVSV-infected cardiomyocytes, respectively (Online Figure 1A). Notably, CVB3 infection did not trigger *IFN* expression or IFN-stimulated genes. Genes dysregulated upon CVB3 were disproportionately in pathways associated with unfolded protein response and cellular senescence, whereas mVSV-induced genes were significantly enriched within virus clearance pathways, indicating that antiviral IFN immunity is intact in human cardiomyocytes (Online Figures 1B and 1C).

TABLE 1 No Enrichment of Variations in TLR3- or IFN-α/β-Related Genes in Patients With AM

Pathways	Acute Myocarditis (n = 42)	1000 Genomes (n = 1,164)	p Value
Heterozygous variations with MAF <0.01%*			
TLR3 cascade	13 (31.0)	427 (36.7)	8.46E-01
IFN-α/β signaling	15 (35.7)	337 (29.0)	4.56E-02
Homozygous variations with MAF <1%*			
TLR3 cascade	1 (2.4)	3 (0.3)	9.80E-01
IFN-α/β signaling	0 (0.0)	6 (0.5)	8.07E-01

*Values show the number of individuals as n (%) carrying heterozygous or homozygous variations in at least one gene.

AM = acute myocarditis; MAF = minor allele frequency.

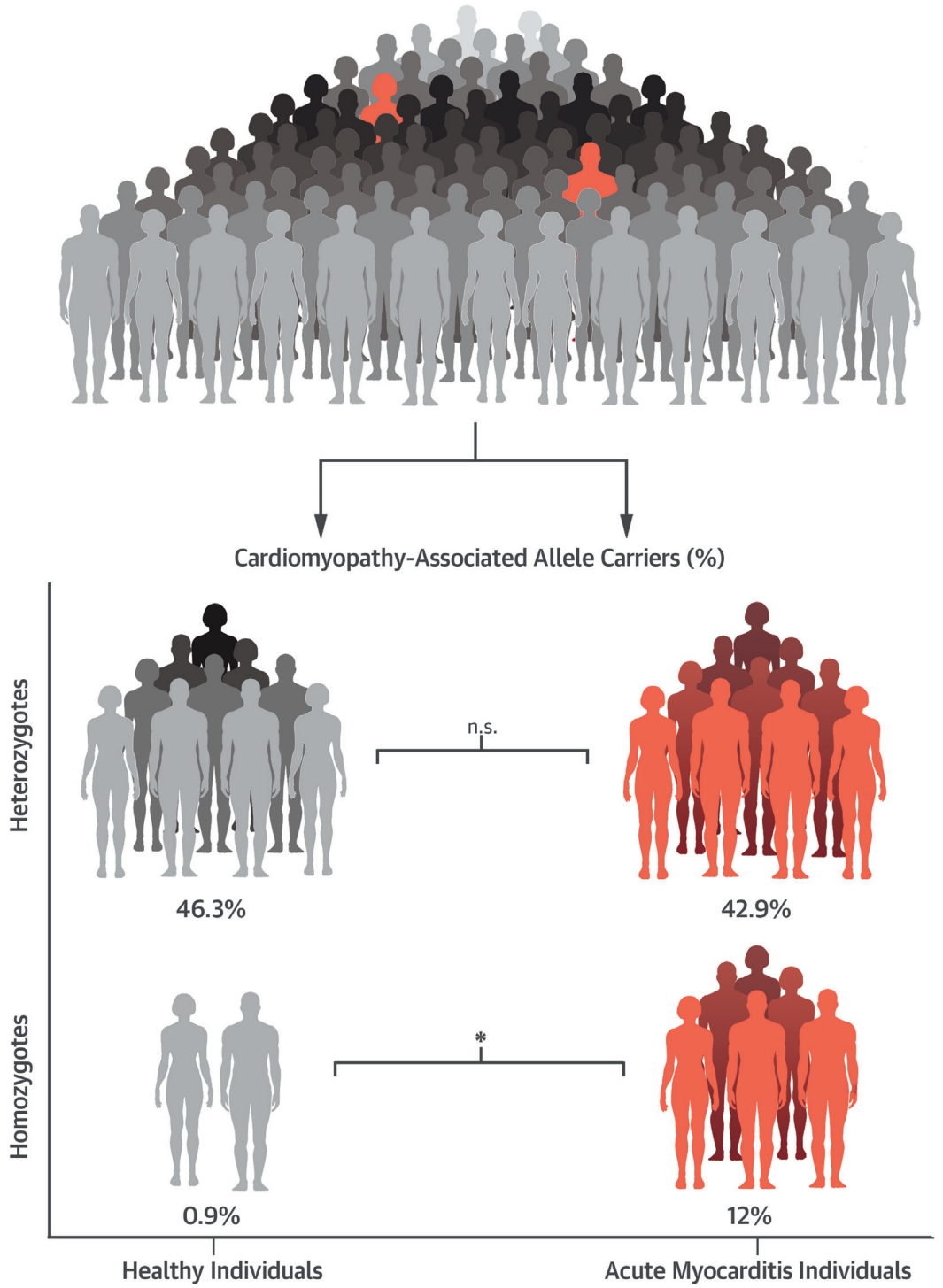
We generated cardiomyocytes from iPSC lines carrying naturally occurring mutations in *TLR3* or *STAT1*, genes relevant for IFN-mediated immunity. Of note, the *TLR3*-deficient line carries the same heterozygous dominant-negative allele (p.P554S) that was reported in a patient with enteroviral myocarditis (8). We infected *TLR3*- or *STAT1*-deficient cardiomyocyte lines with CVB3 at increasing MOIs (0.001, 0.01, 0.1, and 1.00). Although all lines were highly susceptible to CVB3, we did not observe dramatic differences in virus-induced cytotoxicity or viral replication at 24 h post infection compared to the control (**Figures 1A and 1B**).

We then assessed viral replication at different time points (2, 4, 8, 12, and 24 h) after infection at various MOIs (0.0001, 0.01, and 1.00). CVB3 genome copies were not different in *TLR3*- or *STAT1*-deficient cardiomyocytes compared to those in the control (**Figure 1C** and data not shown). We also measured CVB3 titers in supernatants but did not observe any drastic changes in virus production over time among control and mutant cardiomyocytes (data not shown). Moreover, we tested whether exogenous IFN-α confers protection against CVB3 infection in cardiomyocytes. Upon pre-treatment with IFN-α2b (1,000 IU/ml) for 18 h, cardiomyocytes were infected with CVB3 at MOI 0.01 and analyzed at 24 h. CVB3 genome copies and virus-induced cytotoxicity were slightly reduced in both control and *TLR3*-deficient cardiomyocytes but not altered in *STAT1*-deficient cardiomyocytes compared to untreated cells (**Figures 1D and 1E**). However, we did not detect any IFN-induced protection against CVB3 infection at MOI 1 in control or mutant cardiomyocytes (data not shown). Overall, these findings suggested no obvious impact of TLR3-IFN-mediated intrinsic immunity on cardiomyocytes during the course of CVB3 infection at early stages of AVM pathogenesis.

We performed WES of 42 unrelated children (21 European, 10 African, 9 North African, and 2 Middle Eastern). Principal component analysis performed in the WES data confirmed these patients' ethnic origin (Online Appendix, Online Figure 2). We first searched for variations in the TLR3 cascade (91 genes) and IFN-α/β signaling pathways (59 genes) (Online Table 1) in the AM cohort under different autosomal genetic models. For comparison and with respect to our patients' ethnic origin, we applied the same analysis to the sequencing data of 1,164 healthy individuals from the 1000 Genomes database who were of African (n = 661) or European (n = 503) origin. Using a Bonferroni-corrected p value threshold of 8.33E-03, we did not find any statistically significant enrichment of heterozygous carriers with MAF <0.01% in TLR3 cascade (p = 0.846) or IFN-α/β signaling genes (p = 0.046) in the AM cohort (**Table 1**). Similarly, there was no statistically significant enrichment of homozygous variants with MAF <1% in the TLR3 cascade (p = 0.980) or IFN-α/β signaling genes (p = 0.807) in patients with AM-AVM (**Table 1**). Notably, we did not find any AM-AVM patients bearing compound heterozygous variations with MAF <1% in the TLR3 cascade or IFN-α/β signaling genes. Finally, there were no monoallelic (MAF <0.01%) or biallelic (MAF <1%) variations found in TLR3-IFN-α/β-related genes located on chromosome X in the AM cohort. Collectively, these data were inconsistent with the hypothesis that inborn errors of heart-intrinsic TLR3-IFN immunity may underlie AM-AVM.

The occurrence of AM has been associated with the progression of myocardial dysfunction in certain inherited cardiomyopathies, such as arrhythmogenic right ventricular cardiomyopathy (ARVC), left ventricular noncompaction, and Duchenne muscular dystrophy (DMD)-associated DCM (18–20). Therefore, we searched for alterations in 47 genes associated with ARVC, DCM, or left ventricular noncompaction (Online Table 2) in the AM and 1000 Genomes cohorts. We did not find a statistically significant enrichment of heterozygous variations with MAF <0.01% in cardiomyopathy-associated genes in patients with AM-AVM compared to those in the 1000 Genomes cohort (p = 0.223). However, homozygous carriers (MAF <1%) were enriched significantly in the AM cohort compared to those in the 1000 Genomes, taking into account ethnic heterogeneity (p = 2.22E-03) (**Central Illustration**, **Table 2**). Next, we performed the same analyses of our in-house database of 2,324 exomes from healthy individuals and patients with diseases other than myocarditis. Like the analysis using the 1000 Genomes cohort, we found a statistically significant enrichment of homozygous

CENTRAL ILLUSTRATION **Genetics of Acute Myocarditis**



Belkaya, S. et al. J Am Coll Cardiol. 2017;69(13):1653–65.

Acute myocarditis (AM), a rare, life-threatening disease that often results from common viral infections, occurs annually in ~1 in every 100,000 children. AM has been associated with the progression of myocardial dysfunction in certain inherited cardiomyopathies. Results of whole-exome sequencing of 42 children with AM **(orange)** were compared with those of healthy individuals **(gray)** from 1000 Genomes database. Although there were no significant (n.s.) differences between the groups with heterozygous rare genetic variants, homozygous damaging mutations were significantly enriched in the AM patients. *p < 8.33E-03. AM = acute myocarditis.

(p = 1.08E-04) but not heterozygous (p = 0.947) individuals with cardiomyopathy-associated rare alleles in the AM cohort (**Table 2**). Enrichment of homozygous rare variants in cardiomyopathy-associated genes in patients with AM-AVM was further validated by Fisher exact test, using the ExAC database of a total of 60,706 individuals and comparing with each ethnic subpopulation (African: p = 1.22E-06; Finnish: p = 5.85E-08; non-Finnish European: p = 7.04E-08; South Asian: p = 1.45E-03; East Asian: p = 6.77E-07; and Latino: p = 3.65E-07) (Online Table 3). Strikingly, we identified 2 AM-AVM patients carrying compound heterozygous variations in cardiomyopathy-associated genes in addition to 5 homozygous carriers. Finally, we searched for X-linked alleles in cardiomyopathy-associated genes such as dystrophin (*DMD*) and tafazzin (*TAZ*) in males and females, separately, but found no enrichment of monoallelic (MAF <0.01%) or biallelic (MAF <1%) carriers, respectively, in the AM cohort (data not shown). Overall, these findings demonstrated that biallelic but not monoallelic rare variations in cardiomyopathy-associated genes are dramatically enriched in patients with AM-AVM.

We identified 7 of 42 patients (16.7%) (**Table 3**, Patient #1 to #7) who were carrying biallelic rare nonsynonymous or splice-site variants in 6 cardiomyopathy-associated genes, including: P1 with compound heterozygous variations, p.P115S and p.R123Q, in BCL2-associated athanogene 3 (*BAG3*); P2 with homozygous p.R1458* alleles in desmoplakin (*DSP*); P3 with homozygous p.A840V substitutions in plakophilin-2 (*PKP2*); P4 with compound heterozygous variations, p.S688P and p.D829N, in *PKP2*; P5 with homozygous p.H464Q variants in ryanodine receptor 2, cardiac (*RYR2*); P6 with homozygous p.F1986L alleles in sodium channel, voltage gated, type V alpha subunit (*SCN5A*); and P7 with a homozygous, synonymous change, c.G150A, in troponin I type 3, cardiac (*TNNI3*), which was annotated as a splice-site variant. We validated these sequence alterations and their familial segregation by Sanger sequencing when parental samples were available (**Figure 2**, Online Figure 3). The biallelic state of the compound heterozygous variants in *BAG3* in P1 was also confirmed on the Integrative Genomics Viewer (Online Figure 3A). The *PKP2* A840V substitution in P3 was absent from ExAC, and the remainder of these variants were listed only in heterozygous states with very low allele frequencies in that database (**Table 3**). Most alleles were not previously reported in any individual with inherited cardiomyopathy, with the exception of the p.S688P (dbSNP accession number: rs144601090) allele in P4, for which heterozygous variants had been found in some patients with ARVC (21). Although hereditary cardiomyopathies in humans have generally been associated with AD inheritance, AR traits have also been reported in patients with cardiomyopathies for *DSP*, *PKP2*, and *TNNI3* (22,23). None of the 7 subjects whom we studied had any manifestation of cardiomyopathy before their AM presentations, which was unequivocal based on elevated troponin levels, CMR, and/or biopsy findings (**Table 3**). First-degree relatives (including heterozygous siblings) of these 7 individuals underwent electrocardiography and echocardiography when alive, and none had evidence of myocardial disease. The 2 patients (P2 and P7) homozygous for the most severe mutations (nonsense and splicing variants in *DSP* and *TNNI3*, respectively) died shortly after disease onset. The other 5 patients, who carried missense variants that may be less deleterious either underwent heart transplantation (P3) or completely recovered with treatment (P1, P4, P5, and P6). No survivor has developed other cardiac problems as of the writing of this article (**Table 3**).

We investigated the functional impact of the c.G150A allele in *TNNI3*, which is a synonymous variation (p.K50K) located in exon 4, 1 base pair (bp) from the exon 4-intron 4 junction (Online Figures 3G and 4A). In vitro exon-trapping experiments showed that the consensus splice donor site of intron 4 was used in most of the wild-type splice variants (79.1%), whereas this was rare for the mutant (0.6%) (Online Figure 4B). This suggested that the c.G150A substitution causes aberrant splicing of *TNNI3* messenger ribonucleic acid, consistent with its annotation. We then conducted an analysis of the impact of all AM-AVM–associated sequence variants (i.e., damaging vs. benign), using the in silico prediction algorithm CADD together with the mutation significance cutoff (MSC) score, which provides a clinically and biologically relevant CADD cutoff value for genes (24,25). For

TABLE 2 Enrichment of Homozygous Rare Variations in Cardiomyopathy-Associated Genes in Patients With AM

Cardiomyopathy-Associated Genes	Acute Myocarditis (n = 42)	1000 Genomes (n = 1,164)	In-House (n = 2,324)
Heterozygous variations with MAF <0.01%			
Number of carriers	18 (42.9)	539 (46.3)	996 (42.9)
Myocarditis vs. other cohorts p Value		2.23E-01	9.47E-01
Homozygous variations with MAF <1%			
Number of carriers	5 (12.0)	10 (0.9)	29 (1.2)
Myocarditis vs. other cohorts p Value		2.22E-03	1.08E-04

Values are n (%).
Abbreviations as in **Table 1**.

TABLE 3 Characteristics of Patients With Biallelic Rare Variations in Cardiomyopathy-Associated Genes

General Information				Genotype					Phenotype		
Patient	Sex	DOB	Origin	Gene	Mutation	Zygosity	MAF (ExAC)*	CADD/ MSC†	Age of Onset	Troponin Levels	ECG
#1	F	2009	Morocco	*BAG3*	c.C343T p.P115S	Het	8.25E-06	1.36/0.001	52 months	2 N	Atrioventricular block
					c.G368A p.R123Q	Het	2.48E-05	22.7/0.001			
#2	M	2010	Mayotte	*DSP*	c.C4372T p.R1458*	Hom	8.3E-06	38/0.001	32 months	4 N	Normal
#3	F	1997	Maghreb/ Central Europe	*PKP2*	c.C2519T p.A840V	Hom	0	33/0.001	11 yrs	Not done	Normal
#4	M	1995	Congo	*PKP2*	c.T2062C p.S688P	Het	3.30E-05	29/0.001	16 yrs	6 N	Premature ventricular beats
					c.G2485A p.D829N	Het	4.53E-04	24.1/0.001			Diffuse negative T waves
#5	M	2009	France	*RYR2*	c.C1392A p.H464Q	Hom	4.99E-05	1.51/13.58	1 month	8 N	Supraventricular tachycardia
#6	F	2009	La Reunion/ France	*SCN5A*	c.T5956C p.F1986L	Hom	2.02E-03	1.35/0.003	9 months	8 N	Normal
#7	F	2009	France	*TNNI3*	c.G150A p.K50K	Hom	4.21E-05	22.1/5.85	3 yrs	Not done	Negative T waves in V_4, V_5, V_6

*Minor allele frequency (MAF) in Exome Aggregation Consortium (ExAC) database with a total of 60,706 individuals. †Any variant with a combined annotation-dependent depletion (CADD) score equal to or above the mutation significance cutoff (MSC) value for that specific gene is considered potentially damaging.

CMR = cardiac magnetic resonance; CMV = cytomegalovirus; DCM = dilated cardiomyopathy; DOB = date of birth; ECG = electrocardiogram; EGE = early gadolinium enhancement; Het = heterozygous; Hom = homozygous; IV = intravenous; LGE = late gadolinium enhancement; LV = left ventricular; LVEF = left ventricular ejection fraction; NA = not applicable; RV = right ventricular.

each gene, any variant with a CADD score equal to or above the MSC score is considered potentially damaging. Of the 9 cardiomyopathy-associated variations found in the 7 AM-AVM patients studied, 8 had CADD scores above the MSCs of their respective genes; only the *RYR2* p.H464Q allele was below its threshold (Table 3). The CADD scores of rare cardiomyopathy-associated alleles in AM-AVM patients were mostly among lower percentiles (<50th) of ARVC- and/or DCM-causing mutations obtained from the Human Gene Mutation Database (Figure 3) (26) and significantly lower than those of all cardiomyopathy-causing variations ($p = 2.23E-03$). Among the 6 genes, *BAG3, RYR2*, and *SCN5A* were associated with only AD traits, whereas *DSP*, *PKP2*, and *TNNI3* were associated with both AD and AR traits (22,23).

Interestingly, the mutations found in *DSP*, *PKP2*, and *TNNI3* in the AM-AVM patients had CADD scores that were not significantly different from those of cardiomyopathy-causing variations ($p = 0.44$) (Figure 3A). The same result was observed when the comparison was restricted to biallelic variations of these genes known to cause AR cardiomyopathy ($p = 0.48$) and for which heterozygous carriers were known to be healthy. Conversely, for *BAG3, RYR2*, and *SCN5A*, the CADD scores of rare cardiomyopathy-associated alleles in the AM-AVM patients were markedly lower than those of all cardiomyopathy-causing variations ($p = 4.06E-05$) (Figure 3B). This suggested that AM-AVM-associated variants in these genes are probably milder than those causing AD cardiomyopathy, being asymptomatic in heterozygotes, and only manifesting clinically in biallelic carriers who developed AM or AVM.

DISCUSSION

Separating AVM from DCM has long posed a significant clinical challenge (27,28). When AVM presents in childhood, as it often does, the meaning of a history of recent viral infections is dubious given their prevalence in that age group. Obtaining histologic proof of myocarditis is also difficult; there is significant risk associated with procuring the tissue in critically ill young patients (29) and, even when the biopsy is performed, the disease's patchy nature reduces the diagnostic yield (30). Early attempts to document recent viral infections with acute and convalescing antibody titers or to culture the agents from the myocardium or blood were minimally successful. More recent studies amplifying portions of viral genomes from myocardial biopsies have been somewhat more robust and have highlighted the shifting viral causes across time (28,31). Finally, AVM can present in post-acute phases when virus is no longer detectable and active inflammation has resolved, leaving scarring that is not readily differentiated from genetically mediated DCM. For these

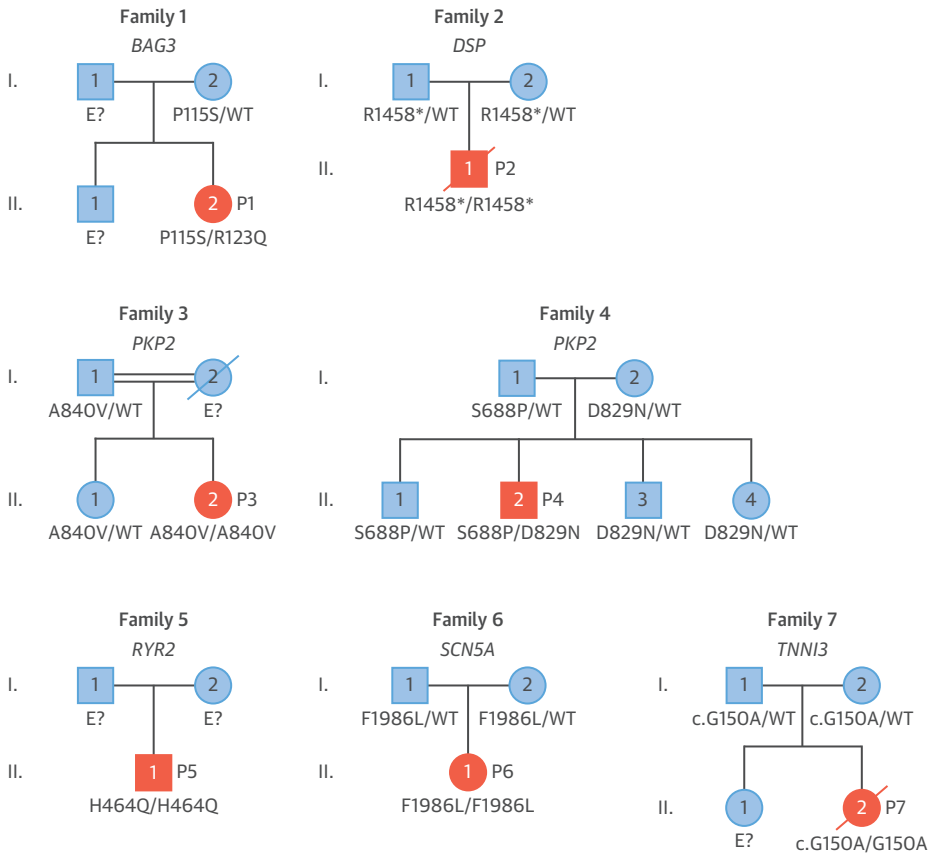
TABLE 3 Continued

Phenotype (Continued)								
Echocardiogram	CMR	Myocardial Biopsy	Viral Etiology	Clinical Diagnosis	Treatment at Diagnosis	Transplant	Family History	Follow-Up
LVEF 37% LV dilation: normal dimensions	Acute myocarditis (3 criteria - diffuse T2 signal hyperintensity)	Not done	None	Myocarditis	Immunoglobulins + steroids IV inotropes	No	No	LVEF 55% at 3 months, normal at 1 yr Normal CMR
LVEF 25% LV dilation: severely dilated (Z-score: +6.1) Global hypokinesia, no dyskinesia	Acute myocarditis (3 criteria - diffuse T2 signal hyperintensity)	Not done	None	Myocarditis	Immunoglobulins + steroids IV inotropes	No	No	Death 3 months after diagnosis after intra LV thrombosis No LV function recovery
LVEF 20% LV dilation: severely dilated (Z-score: +4.2) Global hypokinesia, no dyskinesia Intra LV thrombosis	Acute myocarditis (2 criteria - T2 signal hyperintensity + LGE)	Yes, myocarditis	CMV (blood)	DCM/ Myocarditis	No specific treatment IV inotropes	Yes	No	NA
LVEF 55% LV dilation: mildly dilated (Z-score: +2.3) Hypokinesia apex and lateral wall	Acute myocarditis (3 criteria - diffuse T2 signal hyperintensity)	Not done	None	Myocarditis	Immunoglobulins + steroids IV inotropes	No	No	Normal LVEF Control CMR (10 months): LGE limited to 1 segment of lateral wall, normal LVEF, and normal RV
LVEF 10% LV dilation: mildly dilated (Z-score +2.3) Global hypokinesia-hyperechogenicity of mitral papillary muscles	Acute myocarditis (2 criteria - diffuse EGE, diffuse LGE)	Not done	Enterovirus (blood)	Myocarditis	Immunoglobulins + steroids IV inotropes	No	No	Normal LV function after 6 months No control CMR
LVEF 10% LV dilation: mildly dilated (Z-score +5) Moderate mitral regurgitation	No CMR	Not done	None	Myocarditis	No specific treatment IV inotropes	No	No	Normal LVEF at last follow-up: 60 months
LVEF 13% LV dilation: moderately dilated (Z-score +3.1) Global hypokinesia	No CMR	Yes, myocarditis	Parvovirus B19 (LV, thymus, lymph nodes)	Fulminant myocarditis	Immunoglobulins IV Inotropes Ventricular assist device	No	No	Death 6 months after diagnosis

reasons, no virus has been unambiguously proven to cause AVM in humans, in the sense that herpes simplex virus can cause encephalitis. Accordingly, the search for viruses in children with AVM is not a standard diagnostic procedure, and its result has unclear therapeutic implications. The data presented in our study provided further reason to believe that AVM and DCM are inextricably linked.

An extensive body of work supports the notion that viruses such as enteroviruses and adenovirus infect the myocardium, activating intrinsic (non-hematopoietic), innate (hematopoietic), and acquired immunity (27). Some patients who clear the virus from the myocardium completely recover without significant sequelae. For others, an inappropriate immune reaction ensues, leading to a chronic and irreversible cardiomyopathy. Given these drastically disparate outcomes and seeming dependence on immune responses, we considered whether variations in antiviral immunity genes may underlie the difference. The ongoing discovery of inborn errors of cell-intrinsic immunity underlying susceptibility to rare infections or rare complications of common infections, often in a tissue-specific fashion, provided a strong rationale for this possibility in AVM, as did a recent report of a patient with enteroviral myocarditis who harbored a rare dominant-negative *TLR3* allele (8). Despite that, the human genetic data we produced disclosed no significant burden of mutations altering innate and intrinsic immunity in patients with AM that was presumptively viral in cause. Moreover, our in vitro models, in which normal human cardiomyocytes and those deficient in *TLR3* or *STAT1* were infected with CVB3, showed no induction of *IFN*s and IFN-stimulated genes in control cardiomyocytes and no enhanced susceptibility to infection in mutant cells compared with control cells. Therefore, the totality of our data pointed away from AM due to inborn errors of cardiomyocyte-intrinsic immunity in most affected individuals. Our previous

FIGURE 2 Familial Pedigrees



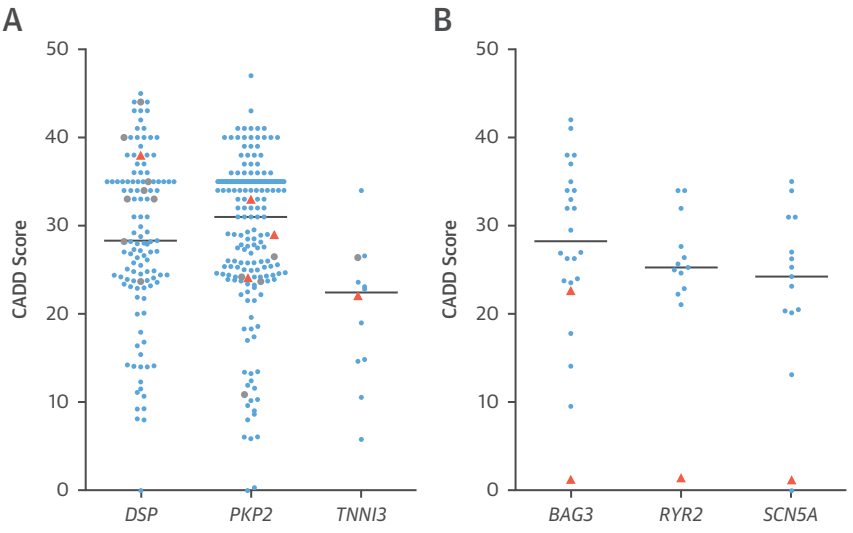
Pedigrees of 7 families affected by myocarditis and variations in 6 cardiomyopathy-associated genes are shown. **Orange** = patients; and **blue** = healthy family members. Where available, a gene's mutation status is provided. WT = wild type.

findings with HSE and severe pulmonary influenza do not apparently apply to AVM (6).

In contrast, we discovered that rare alleles altering cardiac-specific genes previously associated with typically dominant genetic cardiomyopathies underlie AM but are mediated through an autosomal recessive mechanism. Although unexpected, our findings have precedent. First, the severe disease of childhood acute necrotizing encephalopathy develops following common viral infections but has no obvious connection to antiviral immunity. Rather, familial acute necrotizing encephalopathy results from mutations in *RANBP2*, which encodes a component of the nuclear pore Ran binding protein 2 (32). Second, AM has been shown to play a role in the progression of myocardial dysfunction in several inherited cardiomyopathies. A viral cause has been directly implicated in acute deteriorations in hypertrophic cardiomyopathy (33) and DMD-associated DCM (34), through the use of polymerase chain reaction identification of viral genomes in myocardial samples. For ARVC, myocarditis has been documented histologically in association with clinical deterioration (19), and viral genomes have been detected in ARVC myocardial samples at higher rates than in controls (35).

A critical question that arises from this work is whether AVM is merely a second hit revealing inherent myocardial abnormalities of a genetic nature or whether the genetic defects render the myocardium more susceptible to viral infections (36,37), perhaps through infectivity enhanced through a loss of membrane integrity. It has been shown that enteroviral protease 2A cleaves dystrophin, the protein altered in DMD, in a specific fashion and that rendering dystrophin cleavage resistant through mutagenesis produces AVM resistance (38,39). These elegant mouse studies implied that disruption of a

FIGURE 3 **In Silico Prediction of Damaging Impact of Disease-Causing Mutations**



Human gene mutation database mutations associated with dominant **(blue)** and recessive **(gray)** cardiomyopathies are shown for 6 genes: *DSP*, *PKP2*, and *TNNI3* **(A)**; and *BAG3*, *RYR2*, and *SCN5A* **(B)**. **Orange** = variations found in patients with acute or acute viral myocarditis. The **line** indicates the median (50th percentile) of CADD scores of all mutations presented in each graph. CADD = combined annotation-dependent depletion.

cardiac structural protein is critical for enterovirus-host myocardial interaction, whether by facilitating initial infection, viral replication, or viral exit from the cardiomyocytes. By extrapolation, boys with DMD are more susceptible to AVM because their dystrophin is already disrupted. Whether this mechanism is relevant for other viruses and other cardiac proteins remains to be determined.

STUDY LIMITATIONS. We were not able to document a viral cause in all the AM cases for which mutations of cardiomyopathy genes were found due to a lack of available tissue. However, the patients in our AM cohort had clear evidence of myocardial inflammation (e.g., troponin leakage, CMR findings), and our positive histological findings in those from whom we obtained biopsy samples established the concept. We cannot exclude the possibility that some of the AM cases we studied were unrelated to a viral infection, perhaps attributable solely to the genetic defects. Another limitation in this study is the sample size for genetic analyses.

CONCLUSIONS

Our data suggest that AM is not primarily caused by inborn errors of TLR3-IFN immunity but appears to be more commonly related to defects in cardiac structural proteins. Until now, AM has been classified as a nonfamilial, acquired disorder, except when observed rarely in certain inherited cardiomyopathies, particularly ARVC; according to the present study, this nosology may need to be revisited. Future work on AM is needed to determine if the myocardium of such patients is more susceptible to viral infections per se, in which case these defects would define novel mechanism of cell-intrinsic immunity operating in the heart, or whether common viral infections merely and indirectly destabilize inherently vulnerable hearts.

ACKNOWLEDGMENTS The authors thank the patients and their family members for their contributions to this project. They also thank the Flow Cytometry Center of Research Excellence at the Icahn School of Medicine at Mount Sinai and the Genomics Resource Center at The Rockefeller University. And they thank Lei Shang, Bertrand Boisson, and Vimel Rattina for their assistance with bioinformatics.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Certain autosomal recessive defects in genes encoding various components of cardiac structure can predispose to acute myocarditis in young individuals.

TRANSLATIONAL OUTLOOK: Further studies are needed to elucidate the mechanisms by which mutations altering structural cardiac proteins are associated with acute myocarditis, often in response to common viral infections.

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KEY WORDS cardiomyocytes, children, genetics, immunity, sequencing, virus

APPENDIX For expanded Methods section as well as supplemental tables, figures, and references, please see the online version of this article.