Evidence from Family Studies for Autoimmunity in Arrhythmogenic Right Ventricular Cardiomyopathy: Associations of Circulating Anti-Heart and Anti-Intercalated Disk Autoantibodies with Disease Severity and Family History

Alida L.P. Caforio, Federica Re, Andrea Avella, Renzo Marcolongo, Pasquale Baratta, Mara Seguso, Nicoletta Gallo, Mario Plebani, Alvaro Izquierdo-Bajo, Chun-Yan Cheng, Petros Syrris, Perry M. Elliott, Giulia d'Amati, Gaetano Thiene, Cristina Basso, Dario Gregori, Sabino Iliceto, and Elisabetta Zachara

Ahead of Print • Publication Date (Web): 02 Mar 2020

10.1161/CIRCULATIONAHA.119.043931

Evidence from Family Studies for Autoimmunity in Arrhythmogenic Right Ventricular Cardiomyopathy: Associations of Circulating Anti-Heart and Anti-Intercalated Disk Autoantibodies with Disease Severity and Family History

Running Title: *Caforio et al.; Autoimmunity in Arrhythmogenic Cardiomyopathy*

Alida L.P. Caforio, MD, PhD[1*]; Federica Re, MD[2]; Andrea Avella, MD[2]; Renzo Marcolongo, MD[3]; Pasquale Baratta, MD[2]; Mara Seguso, MSc[4]; Nicoletta Gallo, MD[4]; Mario Plebani, MD[4]; Alvaro Izquierdo-Bajo, MD[1*]; Chun-Yan Cheng, MD[1*]; Petros Syrris, PhD[5*]; Perry M. Elliott, MD[5*]; Giulia d'Amati, MD[6]; Gaetano Thiene, MD[7*]; Cristina Basso, MD, PhD[7*]; Dario Gregori, PhD[8]; Sabino Iliceto, MD[1*]; Elisabetta Zachara, MD[2]

[1*]Division of Cardiology, Department of Cardiac, Thoracic, Vascular Sciences and Public Health, University of Padova, Padova, Italy; [2]I Cardiology Division, San Camillo Hospital, Rome, Italy; [3]Department of Medicine, Hematology and Clinical Immunology, University of Padova, Padova, Italy; [4]Department of Laboratory Medicine, University of Padova, Padova, Italy; [5*]University College London and Inherited Cardiac Diseases Unit, Barts Heart Centre, St Bartholomew's Hospital, London, UK; [6] Department of Radiological, Oncological and Anatomo-pathological Sciences, Sapienza University of Rome, Rome, Italy; [7*]Cardiovascular Pathology Unit, [8]Statistics, Department of Cardiac, Thoracic, Vascular Sciences and Public Health, University of Padova, Padova, Italy

*European Reference Networks for rare, low prevalence and complex diseases of the heart (ERN GUARD-Heart)

Address for Correspondence:
Alida L.P. Caforio, MD, PhD
Division of Cardiology
Department of Cardiac, Thoracic, Vascular Sciences and Public Health
Via N Giustiniani, 2, 35100 Padova, Italy
Tel +39 0498212348
Fax +39 049 8211802
Email: alida.caforio@unipd.it
Twitter: @AlidaCaforio

Abstract

Background: Serum anti-heart autoantibodies (AHA) and anti-intercalated disk autoantibodies (AIDA) are autoimmune markers in myocarditis. In arrhythmogenic right ventricular cardiomyopathy (ARVC) myocarditis has been reported. To provide evidence for autoimmunity, we searched for AHA and AIDA in ARVC.
Methods: We studied: 42 ARVC probands, 23 male, aged 42, interquartile range (IQR) 33;49, 20 from familial and 22 non-familial pedigrees; 37 clinically affected relatives (AR), 24 male aged 35, IQR 18;46; 96 healthy relatives (HR), 49 male, aged 27, IQR 17;45. Serum AHA and AIDA were tested by indirect immunofluorescence on human myocardium and skeletal muscle in 171 of the 175 ARVC individuals and in controls with: non-inflammatory cardiac disease (NICD) (n=160), ischemic heart failure (IHF) (n=141), normal blood donors (NBD) (n=270). Screening of five desmosomal genes was performed in probands; when a sequence variant was identified, cascade family screening followed, blind to immunological results.
Results: AHA frequency was higher (36.8%) in probands, AR (37.8%) and HR (25%) than in NICD (1%), IHF (1%) or NBD (2.5%) (p=0.0001). AIDA frequency was higher in probands (8%, p=0.006), in AR (21.6%, p=0.00001) and in HR (14.6% p=0.00001) than in NICD (3.75%), IHF (2%) or NBD (0.3%). AHA positive status was associated with higher frequency of palpitation (p=0.004), ICD implantation (p=0.021), lower left ventricular ejection fraction (LVEF) (p=0.004), AIDA positive status with both lower RV and LVEF (p=0.027 and p=0.027 respectively). AHA and/or AIDA positive status in the proband and/or at least one of the respective relatives was more common in familial (17/20, 85%) than in sporadic (10/22, 45%) pedigrees (p=0.007).
Conclusions: Presence of AHA and AIDA provides evidence of autoimmunity in the majority of familial and in almost half of sporadic ARVC. In probands and in AR these antibodies were associated with disease severity features; longitudinal studies are needed to clarify whether they may predict ARVC development in HR or if they be a result of manifest ARVC.

Key Words: arrhythmogenic right ventricular cardiomyopathy; autoimmunity; autoantibodies

Nonstandard Abbreviations and Acronyms
AHA: anti-heart autoantibodies
AIDA: anti-intercalated disk autoantibodies
AR: affected relatives
ARVC: arrhythmogenic right ventricular cardiomyopathy
DSC2: desmocollin-2
DSG2: desmoglein-2
DSP: desmoplakin
EF: ejection fraction
EMB: endomyocardial biopsy
PKP2: plakophilin-2
HR: healthy relatives
JUP: plakoglobin
IF: indirect immunofluorescence
IHF: ischemic heart failure
LVEF: left ventricular ejection fraction

NBD: normal blood donors
NICD: non-inflammatory cardiac disease
RV: right ventricular

Clinical Perspective

What is new?

- This is the first family study reporting an increased frequency of serum organ-specific anti-heart autoantibodies (AHA) and anti-intercalated disk autoantibodies (AIDA) in a sizable arrhythmogenic right ventricular cardiomyopathy (ARVC) cohort of patients and relatives as compared to controls, in keeping with autoimmune involvement.
- Positive AHA status was associated with lower left ventricular ejection fraction (LVEF), higher frequency of cardiac symptoms and implantable cardioverter defibrillator implantation, positive AIDA with lower biventricular ejection fraction (EF).
- Another unique finding is that AHA and/or AIDA positive status was more common in familial than in sporadic pedigrees.

What are the clinical implications?

- Presence of organ-specific AHA and AIDA provides evidence of autoimmunity in the majority (85%) of familial and in almost half (45%) of sporadic ARVC. In probands and in affected relatives these antibodies were associated with disease severity features.

Introduction

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a significant cause of sudden cardiac death in the young. It is considered a genetically determined heart muscle disease of the desmosome, characterized by progressive fibrous or fibro-fatty replacement of the myocardium and ventricular arrhythmias,[1–9] although non-desmosome genetic causes and sporadic disease may occur.[1–11] Virus-negative myocarditis is reported in a high proportion of histologically-proven ARVC, but its pathogenic significance remains elusive.[12–14]

ARVC is a complex diagnosis, requiring fulfillment of a set of clinical, pathological and genetic criteria, first proposed in 1994[2] and revised in 2010 by an international expert Task Force[3]. In the early stages, ARVC may present with "hot phases" of chest pain, palpitations, and release of troponins, closely resembling clinically suspected myocarditis with pseudo-infarct presentation.[12,14] The myocarditis phenotype in the early stages of disease onset has been clearly documented also in transgenic animal models of ARVC.[15] Virus-negative myocarditis is often an autoimmune disease[16] in which organ-specific and disease-specific serum anti-heart autoantibodies (AHA) and anti-intercalated disk autoantibodies (AIDA) represent reliable autoimmune biomarkers in affected patients (with or without ventricular dysfunction and regardless of the clinical presentation) as well as in their apparently healthy relatives at risk of disease development. [17-21] A recent study, on 45 index ARVC patients and a limited number of normal and non ARVC cardiomyopathy sera, reported an anti-desmoglein-2 (DSG-2) antibody to be associated with ARVC; relatives were not studied. [22] To provide evidence for autoimmunity from family studies, this study aimed at assessing prevalence, clinical and genetic correlates of AHA and AIDA in a sizable single centre cohort of ARVC probands, affected (AR) and healthy relatives (HR) as compared to a large number of normal and non ARVC cardiomyopathy sera.

Methods

Study patients

The study groups included 175 individuals (42 ARVC probands, of whom 20 from familial and 22 from sporadic pedigrees, 37 AR fulfilling the 2010 revised Task force diagnostic criteria[3] and 96 HR followed at the Cardiomyopathy Unit, San Camillo Hospital, Rome, Italy) (Figure 1). Familial disease was defined as at least 1 AR besides the proband, sporadic as no AR besides the proband; 117 relatives were from familial and 16 from sporadic pedigrees. The study protocol followed the ethical guidelines of the Declaration of Helsinki, obtained Institutional Review Board approval at S. Camillo Hospital and all participants gave written informed consent. In keeping with the 2010 revised diagnostic Task force criteria,[3] individuals underwent standard 12-lead ECG, signal-averaged ECG, 24 -hour Holter monitoring, echocardiography, and cardiovascular magnetic resonance imaging (CMR). Probands and AR underwent complete cardiac catherization and endomyocardial biopsy (EMB) when clinically indicated to reach diagnosis.

Serum AHA and AIDA testing by indirect immunofluorescence (IF)

The data, analytic methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure because of lack of diagnostic sera after testing for the present study. AHA and AIDA were detected by indirect immunofluorescence (IF) at 1/10 dilution on 4 μm-thick unfixed fresh frozen cryostat sections of blood group O normal human atrium and skeletal muscle.[19-21] Two sera were used as standard positive and negative controls and titrated in every assay. All sera were read blindly against these standards using a fluorescence microscope (Zeiss Axioplan 2 imaging, Zeiss, New York). An additional positive control serum was titrated to assess reproducibility. End point titres for this

serum were reproducible within one double dilution in all assays.[17,19-21] The frequency of AHA and of AIDA in ARVC was compared with that observed in previously established control groups of non-inflammatory heart disease (NICD) (n=160, 80 male, aged 37±17 years, of whom n=55 rheumatic heart disease, n = 67 hypertrophic cardiomyopathy, and n= 38 congenital defects), ischemic heart disease (n=141, 131 male, aged 44±14 years) and normal individuals (n=270, 123 male, age 35±11). [17,19-21] These control sera were obtained with informed consent from patients admitted to hospital and tested blindly from diagnosis at the time of description and validation of the IF assay. [17,19-21]

Genetic testing

Genetic screening of five desmosomal genes associated with ARVC was performed on 38 of the 42 ARVC index cases (Figure 1). When a sequence variant was identified in an index case, cascade screening followed in the corresponding families. In all cases, genetic screening was carried out blind to the immunological test results. In total, genomic DNA from 139 of the 175 index patients and family members was extracted from whole blood with QIAamp DNA Blood mini kits (Qiagen). Primer pairs were designed to amplify the coding exons and the flanking intronic sequences of five ARVC related desmosomal genes: plakophilin-2 (PKP2), desmoplakin (DSP), desmocollin-2 (DSC2), desmoglein-2 (DSG2) and plakoglobin (JUP). PCR amplification was carried out using standard protocols (AmpliTaq Gold, Applied Biosystems) for all fragments except those with high GC content which were amplified with the GC RICH PCR system (Roche), as previously described.[5,6,23] After amplification, PCR fragments were sequenced in both directions on an ABI PRISM 3130 DNA analyzer using BigDye Terminator chemistry (version 3.1) and analyzed by Seqscape version 2.0 software (Applied Biosystems). Sequence variants detected in ARVC patients were cross referenced to the updated version of the ARVD/C

Genetic Variants Database (https://molgenis07.gcc.rug.nl/# - accessed on 25 Sep 2018).[24] The minor allele frequency of each variant on the Genome Aggregation Database (gnomAD) was determined using the gnomad browser (http://gnomad.broadinstitute.org/ - gnomAD r2.0.2 - accessed on 25 Sep 2018). Classification of identified variants was based on the American College of Medical Genetics (ACMG) guidelines for the interpretation of sequence variants [25]. In particular, missense variants were evaluated using the InterVar bioinformatics software tool (http://wintervar.wglab.org/) [26] whilst the pathogenicity of nonsense and frameshift variants was determined with the online Genetic Variant Interpretation Tool provided by the University of Maryland, School of Medicine at http://www.medschool.umaryland.edu/Genetic_Variant_Interpretation_Tool1.html/ [27].

Statistical analysis

Results for quantitative measures are given as mean±SD or as median (interquartile range) for variables deviating from normal distribution, qualitative measures are given as frequency (percentage). Quantitative variables were compared by one-way analysis of variance, Student's t-test, if normally distributed, or by Mann-Whitney test if deviating from normal distribution. Qualitative measures were compared by χ^2 test or Fisher's exact test as appropriate.

To account for clustering of observations in families a random effect model was implemented for each variable of interest also including family size in the model. Random effect model was in the class of the Generalised Linear Mixed Models fitted using Markov chain Monte Carlo techniques[28].

Given the high number of statistical tests performed, all p-values were adjusted for multiplicity to control the false discovery rate (i.e.: the expected proportion of false discoveries amongst the rejected hypotheses) to keep power also in presence of test dependence [29] . Adjusted

p-values less than 0.05 were considered to indicate statistical significance and explicitly reported if below 0.10, otherwise "ns" was stated. All descriptive statistical analyses were performed using the SPSS software version 25.0 (SPSS, Inc, Chicago, IL; 2017) and inferential evaluations were conducted using the R-System [30] and the MCMCglmm libraries [31].

Results

Baseline features in ARVC patients and relatives.

The clinical and diagnostic features at baseline are given in Table 1. Briefly, probands compared to AR and HR were more symptomatic, had larger right ventricular (RV) dimensions and lower biventricular function, and a higher number of fulfilled revised Task Force diagnostic criteria. Ventricular arrhythmia burden on ECG and 24-hour ECG Holter monitoring was higher in probands; an implantable cardioverter defibrillator (ICD) was implanted in 11 (26%) of probands, in 1 (2.7%) AR and in none of the HR (p=0.007). All patients with an EMB sample of sufficient quality for unequivocal pathological diagnosis showed typical ARVC, none had histological or immunohistochemical evidence of myocarditis, but focal areas of inflammation might have not been sampled. Of interest, a family history for autoimmune disease was found in a sizable proportion of probands (38%) and AR (40.5%).

AHA and AIDA: frequency and associations with clinical and diagnostic features.

Sera from 171 (38 probands, 37 AR and 96 HR) out of the 175 ARVC probands and relatives, taken at baseline evaluation with informed consent, were collected and tested at the same time of genetic study (Figure 1) blindly from clinical diagnosis. Organ-specific and cross-reactive AHA patterns were classified as described; [19-21] representative examples are shown in Figure 2. Briefly, organ-specific AHA gave diffuse cytoplasmic with or without additional fine striational

staining of atrial myocytes, but were negative on skeletal muscle; cross-reactive 1 or partially organ-specific AHA gave a fine striational staining on atrium, and were negative or only weakly stained skeletal muscle; cross-reactive 2 AHA gave a broad striational pattern on longitudinal sections of heart and skeletal muscle. [19-21]Absorption studies with relevant tissues had confirmed the organ-specificity and cross-reactivity of the AHA patterns.[21] AIDA gave a linear staining of the intercalated disks between cardiac myocytes.[20] The frequency of AHA was higher in ARVC probands (14/38, 36.8%, p=0.0001), AR (14/37, 37.8%, p=0.0001) and HR (24/96, 25%, p=0.0001) than in NICD (2/160, 1%), IHF (2/141, 1%) or NBD (7/270, 2.5%). The frequency of AIDA was higher (3/38, 8%, p=0.006) in ARVC probands, AR (8/37, 21.6%, p=0.00001) and in HR (14/96, 14.6%, p=0.00001) than in NICD (6/160, 3.75%), IHF (3/141, 2%) or NBD (1/270, 0.37%). The frequency of AHA was similar in probands (14/38, 36.8%), AR (14/37, 37.8%,) and in HR (24/96, 25%) (p=NS). Similarly, the frequency of AIDA did not differ in probands (3/38, 8%), AR (8/37, 21.6%) and in HR (14/96,14.6%) (p=NS).

Associations of AHA and AIDA status with clinical and diagnostic features are shown in Table 2. AHA positive status was associated with higher frequency of palpitation (p=0.004), ICD implantation for primary prevention of sudden cardiac death (p=0.021), greater left ventricular (LV) septal (p=0.004) and posterior wall end-diastolic thickness (p=0.004), lower LV ejection fraction (p=0.004) and tended to be associated with chest pain (p=0.08). AIDA positive status was associated with both lower RV and LV echocardiographic ejection fraction (p=0.027 and p=0.027 respectively). AHA and/or AIDA positive status in the proband and/or at least one of the respective relatives was more common in familial (17/20, 85%) than in sporadic (10/22, 45%) pedigrees (p=0.007).

Frequency of mutations in ARVC genes and associations with clinical, diagnostic features and autoantibody status.

Classification of the detected desmosome variants by ACMG criteria identified 8 pathogenic loss-of-function (nonsense and frameshift) variants in 8 probands of the 38 tested (21%), 4 variants of unknown significance and 12 benign/likely benign variants (Table 3). The degree of relatedness of relatives in gene-elusive families was similar to that of gene-positive relatives (first degree 55/71, 77% vs 25/30, 83% respectively, p=NS). Pathogenic gene mutations were present in similar proportions of probands (8/38, 21%), AR (6/25, 24%) and HR (9/76, 11.8%) (p=NS). The most common mutated gene was PKP2, with relative frequencies of 5 (13%) probands, 4 (16%) AR and 6 (8%) of HR respectively (p=NS); the second most common gene was DSP, with relative frequencies of 2 (5 %) probands, 2 (8%) AR and 2 (3%) of HR respectively (p=NS). Pathogenic DSG2 mutations had relative frequencies of 1 (2.6%) of probands, 0 (0%) AR and 2 (2.6%) of HR respectively (p=NS); no DSC2 or JUP pathogenic mutations were found.

Significant associations of pathogenic mutations with clinical, diagnostic features and with autoantibody status in probands and relatives are shown in Table 4. Overall, individuals with any pathogenic mutation as compared to those without had a larger RV end-diastolic volume (p=0.02), a higher frequency of negative t waves in leads V1-V3 (p=0.002), and tended to have higher frequency of ICD (p=0.09). PKP2 mutation positive, as compared with PKP2 negative patients, had a higher frequency of ICD implants for primary or secondary prevention (p=0.01), larger RV dimensions (p=0.004). DSP positive mutations were associated with larger RV outflow tract dimensions (p=0.004) and longer QRS duration (p=0.005). DSG-2 mutations did not show significant associations. Excluding probands, AHA and of AIDA rates were

similar in gene-positive (carriers) relatives, gene-negative (NOT carriers) relatives from gene-positive families, and gene-elusive (or not gene-tested) relatives. Conversely, each of the 3 subgroups of relatives had significantly higher frequencies of inflammatory markers compared to individuals from control groups (NBD, IHF and NICD) (Suppl. Tables 1 and 2).

Discussion

Significance and specificity of AHA and AIDA in ARVC families

In this study organ-specific AHA were found in 37% of affected ARVC patients and in 25% of HR, AIDA in 15% of affected patients and HR. In addition AHA and/or AIDA positive status in probands and/or in their relatives was present in the majority (85%) of familial and in 45% of sporadic ARVC pedigrees. Conversely, these antibodies were absent or uncommon in a large number of NICD (including 67 patients with another specific genetically determined cardiomyopathy, hypertrophic cardiomyopathy), ischemic heart disease and normal control individuals.

The IF technique used on human myocardium and skeletal muscle is standardized, validated and able to distinguish organ-specific cardiac from partially organ-specific (cross-reactive 1 pattern) or fully skeletal muscle cross-reactive AHA (cross-reactive 2 pattern) [21]. Each assay includes controls for non-specific antibody binding[21]. The organ-specific vs cross-reactive AHA patterns have previously been confirmed by absorption studies on heart, skeletal muscle, and liver as control[21], therefore IF per se on human substrate is able to distinguish cardiac-specific from skeletal muscle cross-reactive AHA. Recognized autoantigens for AHA are alpha (entirely cardiac-specific isoform) and beta myosin heavy chain (partially cross-reactive with skeletal muscle) and other unidentified autoantigens by Western blot[32]. The AIDA pattern is

organ-specific; the intercalated disks are specialized cardiac structures, no AIDA binding is present on skeletal muscle[20].

The higher frequency of AHA and AIDA in ARVC probands and relatives than in controls is in keeping with autoimmune involvement in ARVC, as previously seen in autoimmune myocarditis/dilated cardiomyopathy and in other organ-specific autoimmune diseases, such as type 1 insulin-dependent diabetes mellitus (IDDM).[16-21] The findings of this study are also in keeping with a recent report of anti-DSG-2 antibodies in 45 index ARVC patients.[22] A unique finding of the present study is that, for the first time, AHA and/or AIDA were found in HR (e.g. symptom-free, with normal ECG and echocardiogram). In autoimmune diseases apparently healthy relatives are potentially at risk of disease development, particularly if they are autoantibody positive. Autoimmune diseases result from both genetic and environmental triggers. [17,19] In autoimmune dilated cardiomyopathy AHA are found years before disease development and identify family members at risk.[17,19] Although the same may apply to ARVC, this is a cross-sectional study; longitudinal prospective studies are warranted to prove the possible role of AHA and AIDA as early predictors of disease development in antibody-positive unaffected ARVC relatives with or without a pathogenic mutation. The trend towards a lower frequency of AIDA in probands as compared to AR and HR may be related to older age and long-standing disease in probands, with reduction of antibody titres with disease progression, similar to what is seen in autoimmune dilated cardiomyopathy and in IDDM.[17,18]

AHA, AIDA status and genetic background.

In this study, AHA and/or AIDA were found in similar proportions of patients and relatives with and without pathogenic mutations. Similarly, in another study the anti-DSG2 antibodies were present regardless of the underlying mutation [22]. This may relate, in both studies, to the small

number of mutations in single genes and subsequently a reduced statistical power to detect associations. Any association between an antibody type and specific gene mutations warrants confirmation on larger numbers. The finding of genotype negative, antibody positive pedigrees may suggest that a subset of ARVC cases are non-genetic and entirely caused by autoimmune disease, or that new disease genes are yet to be discovered. Currently, the genetic cause of ARVC is still elusive in about 50% of index cases.[1,3–6,11,23,24,26] However, this is the first family study to show that autoimmunity seems to be involved in the majority of familial ARVC (85%) and in 45% of sporadic ARVC pedigrees, similar to what is found with the same autoantibody markers, AHA, in autoimmune dilated cardiomyopathy. [17]

ARVC and myocarditis: autoimmunity as a common pathogenetic link

Autoimmune involvement in ARVC is in keeping with the pathological description of virus-negative myocarditis in up to 70% of biopsy or autopsy tissue in ARVC[12–14]; in addition, the "hot phases" of ARVC are clinically indistinguishable from the pseudo-infarct presentation of myocarditis[14]. Therefore, the immunological findings shown here may provide the missing link for these observations. In other autoimmune diseases, the presence of specific autoantibody markers is associated and predicts "hot phases", or disease relapses.[16-21]

In keeping with other autoimmune diseases[18], in the present study AHA and/or of AIDA were associated with clinical findings of disease activity or severity (chest pain, palpitation, lower left and/or right ventricular ejection fraction, and ICD implantation) in ARVC probands and AR. The anti-DSG-2 antibodies reported by others were also associated with a higher frequency of ventricular ectopic beats[22].

The data provided here suggest novel insights into ARVC pathogenesis. So far, myocarditis has been considered by clinicians as a differential diagnosis from ARVC or a non-

specific phenomenon secondary to tissue injury in a genetically-determined cardiomyopathy. However, in another heart muscle disease, hypertrophic cardiomyopathy, where there is no myocardial inflammation at histopathological analysis, AHA or AIDA frequencies were not increased[21] and others did not find anti-DSG2 antibodies[22]. Conversely, in this study AHA and AIDA and, in another study, anti-DSG2 antibodies [22] were associated with ARVC, in keeping with the proposal made here and by others[22] that autoimmunity to myosin (one of the autoantigens recognised by AHA by western blotting)[32] and to intercalated disk components (recognised by AIDA and the anti-DSG2 antibodies[22]) is involved in ARVC pathogenesis.

Polyclonal humoral reactivity in ARVC

In the present IF study AIDA were identified in a subset of ARVC probands and AR, conversely others, using western blot and ELISA, found anti-DSG2 antibodies in almost all index ARVC individuals[22]. The discrepancy may relate to different sensitivity of the immunological techniques and/or heterogeneity of patients. In previous work on AHA it was showed that western blot[32] and ELISA[16] are more sensitive than IF in recognizing autoantibodies directed against specific heart autoantigens, which for AHA, include alpha and beta myosin heavy chain isoforms[32]. On the other hand, IF, the standard autoimmune serology technique, is best suited for the detection of multiple autoantibody reactions simultaneously [18,21], particularly in a newly suspected organ-specific autoimmune disease, such as ARVC, on a sizable number of patients, relatives and controls. The present IF findings show, as in other organ-specific autoimmune diseases, a polyclonal humoral autoimmune reactivity in ARVC sera including AHA (which are directed against alpha and beta myosin heavy chains and other yet unidentified autoantigens[32]) and AIDA (directed against yet unknown autoantigens). Since this study tested more ARVC patient and relative cohorts than others[22], it is likely that a greater heterogeneity of both genetic

backgrounds and autoantibody responses was present. It remains to be seen whether or not patients who are anti-DSG-2 antibody positive are also AHA and AIDA positive, or whether distinct patient subsets produce anti-DSG2 antibodies[22] or AHA and AIDA. To this end collaborative work among laboratories which are testing distinct antibodies will be of great interest.

Regarding the AHA, identified autoantigens include alpha and beta myosin heavy chain[32], which represents the most abundant heart proteins. Although in ARVC the genetically-defective cardiomyocyte structures are thought to be predominantly the intercalated disks, it is quite conceivable that, following myocyte cell damage related to these specialised structures, the whole cell becomes dysfunctional or dies, leading to release of myosin, as well as other autoantigens, and stimulating the immune system to AHA production[33], as well as AIDA and anti-DSG2 antibodies[22].

Future clinical perspectives

AHA and AIDA detected by indirect IF represent recognized organ-specific and disease-specific markers in ARVC, in keeping with Rose-Witebsky criteria[18], thus in ARVC one major criteria for organ-specific autoimmunity is met. However, at least two major Rose-Witebsky criteria should be fulfilled to classify a new disease entity as autoimmune[18]. More work is needed, particularly in early genetically confirmed ARVC and in clinically "hot phases", to detect potential involvement of cell-mediated autoimmunity, and to clarify immune features in situ, such as quantity and phenotype of inflammatory myocardial infiltrates and potential expression of Human Leucocyte Antigens (HLA) on EMB[16,18,33]. Another clinical research direction is the potential use of immunosuppression in biopsy-proven virus-negative autoantibody-positive inflammatory ARVC. Response to immunosuppression is a major criterion for an autoimmune

disease and is therefore the standard therapy in organ-specific and systemic autoimmune disorders.[16,18] Similarly, it is becoming of standard use in biopsy-proven virus-negative myocarditis/ inflammatory dilated cardiomyopathy.[16,17,19] In ARVC there is no aetiology-directed therapy to stop or slow down disease progression. Therefore immunosuppression/immunomodulation, with its current wide range of drugs and interventions, may be a promising new clinical perspective. Response to immunosuppression, to be tested by an appropriate controlled trial design, would also provide a second fulfilled major Rose-Witebsky criteria to classify ARVC as autoimmune.[18]

Study limitations

A first limitation of this study is deriving from being a cross-sectional study, and caution should be taken in inferring causality; this study is unable to determine whether auto-immunity is primary/causative or is secondary to the primary ARVD process. A second limitation is that only a small subset of patients, 26 out of a total of 78 (including probands and affected relatives) underwent EMB, thus it is not feasible to relate the presence of AHA or AIDA with the histological confirmation of virus-negative myocardial inflammation. In the present study myocarditis was not found in biopsy-proven patients; this may reflect EMB sampling error, and/or the fact that EMB, an invasive procedure, was performed late in the disease stage, and in those patients who did not reach Task Force criteria at the end of non-invasive diagnostic work-up. Nonetheless, since all study patients fulfilled 2010 revised Task force criteria, including genetic characterisation[3], we think that they are truly representative of typical ARVC.

Work is in progress to identify the specific autoantigenic targets in AIDA, although this was beyond the study aims. By passive transfer experiments, it has been previously reported that AHA purified from dilated cardiomyopathy and myocarditis sera may be directly pathogenic.[34]

However, this has not yet been shown for AHA or AIDA purified from ARVC patients, therefore, although in this study AHA and AIDA were associated with clinical features of disease activity or severity in ARVC, this does not imply that they are directly pathogenic[22]. Future work is needed to clarify this issue.

The present study focused on the main cause of ARVC, the desmosomal genes; the majority of patients with an identified pathogenic variant have a mutation in one of these five genes. Desmosomal gene mutations are typically associated with a classic form of ARVC as it is extensively described in the literature[23-24,35]. Mutations in other genes associated with ARVC account for a very small percentage of cases[23-24]. Other, non-desmosomal, genes have been reported to be responsible for other forms of the disease (recently collectively termed arrhythmogenic cardiomyopathy) which are phenotypically distinct[35]. For example, the phenotypes associated with mutations in phospholamban, desmin and lamin are characterised by increased risk of life-threatening arrhythmia, myocardial structural abnormalities, usually predominantly of the left ventricle and differ from the classic ARVC phenotype[35].

In addition, to date there have been no reports linking non-desmosomal genes with autoimmunity and ARVC. The only report of autoimmunity in ARVC concerned antibodies against desmoglein-2 which is a desmosomal protein[22].

Conclusions

Presence of AHA and AIDA provides evidence of autoimmunity in the majority (85%) of familial and in 45% of sporadic ARVC. Although in probands and in affected relatives these antibodies were associated with disease severity features, longitudinal studies are needed to clarify whether they may predict ARVC development in healthy relatives.

Sources of Funding

None

Disclosures

None.

References

1. Basso C, Corrado D, Marcus FI, Nava A, Thiene G. Arrhythmogenic right ventricular cardiomyopathy. *Lancet*. 2009;373:1289–1300.
2. McKenna WJ, Thiene G, Nava A, Fontaliran F, Blomstrom-Lundqvist C, Fontaine G, Camerini F. Diagnosis of arrhythmogenic right ventricular dysplasia/cardiomyopathy. Task Force of the Working Group Myocardial and Pericardial Disease of the European Society of Cardiology and of the Scientific Council on Cardiomyopathies of the International Society and Federation of Cardiology. *Br Heart J*. 1994;71:215-218.
3. Marcus FI, McKenna WJ, Sherrill D, Basso C, Bauce B, Bluemke DA, Calkins H, Corrado D, Cox MG, Daubert JP. Diagnosis of arrhythmogenic right ventricular cardiomyopathy/dysplasia: proposed modification of the task force criteria. *Circulation*. 2010;121:1533–1541.
4. Pilichou K, Nava A, Basso C, Beffagna G, Bauce B, Lorenzon A, Frigo G, Vettori A, Valente M, Towbin J. Mutations in desmoglein-2 gene are associated with arrhythmogenic right ventricular cardiomyopathy. *Circulation*. 2006;113:1171–1179.
5. Syrris P, Ward D, Evans A, Asimaki A, Gandjbakhch E, Sen-Chowdhry S, McKenna WJ. Arrhythmogenic right ventricular dysplasia/cardiomyopathy associated with mutations in the desmosomal gene desmocollin-2. *Am J Hum Genet*. 2006;79:978–984.
6. Syrris P, Ward D, Asimaki A, Sen-Chowdhry S, Ebrahim HY, Evans A, Hitomi N, Norman M, Pantazis A, Shaw AL, Elliott PM, McKenna WJ. Clinical expression of plakophilin-2 mutations in familial arrhythmogenic right ventricular cardiomyopathy. *Circulation*. 2006;113:356–364.
7. Rampazzo A, Nava A, Malacrida S, Beffagna G, Bauce B, Rossi V, Zimbello R, Simionati B, Basso C, Thiene G. Mutation in human desmoplakin domain binding to plakoglobin causes a dominant form of arrhythmogenic right ventricular cardiomyopathy. *Am J Hum Genet*. 2002;71:1200–1206.
8. Thiene G, Nava A, Corrado D, Rossi L, Pennelli N. Right ventricular cardiomyopathy and sudden death in young people. *N Engl J Med*. 1988;318:129–133.
9. Marcus FI, Fontaine GH, Guiraudon G, Frank R, Laurenceau JL, Malergue C, Grosgogeat Y. Right ventricular dysplasia: a report of 24 adult cases. *Circulation*. 1982;65:384–398.
10. Garcia-Gras E, Lombardi R, Giocondo MJ, Willerson JT, Schneider MD, Khoury DS, Marian AJ. Suppression of canonical Wnt/β-catenin signaling by nuclear plakoglobin

recapitulates phenotype of arrhythmogenic right ventricular cardiomyopathy. *J Clin Invest*. 2006;116:2012–2021.
11. Fidler LM, Wilson GJ, Liu F, Cui X, Scherer SW, Taylor GP, Hamilton RM. Abnormal connexin43 in arrhythmogenic right ventricular cardiomyopathy caused by plakophilin-2 mutations. *J Cell Mol Med*. 2009;13:4219–4228.
12. Basso C, Thiene G, Corrado D, Angelini A, Nava A, Valente M. Arrhythmogenic right ventricular cardiomyopathy: dysplasia, dystrophy, or myocarditis? *Circulation*. 1996;94:983–991.
13. Calabrese F, Angelini A, Thiene G, Basso C, Nava A, Valente M. No detection of enteroviral genome in the myocardium of patients with arrhythmogenic right ventricular cardiomyopathy. *J Clin Pathol*. 2000;53:382–387.
14. Thiene G, Corrado D, Nava A, Rossi L, Poletti A, Boffa GM, Daliento L, Pennelli N. Right ventricular cardiomyopathy: is there evidence of an inflammatory aetiology? *Eur Heart J*. 1991;12:22–25.
15. Pilichou K, Remme CA, Basso C, Campian ME, Rizzo S, Barnett P, Scicluna BP, Bauce B, van den Hoff MJ, de Bakker JM. Myocyte necrosis underlies progressive myocardial dystrophy in mouse dsg2-related arrhythmogenic right ventricular cardiomyopathy. *J Exp Med*. 2009;206:1787–1802.
16. Caforio AL, Pankuweit S, Arbustini E, Basso C, Gimeno-Blanes J, Felix SB, Fu M, Heliö T, Heymans S, Jahns R. European Society of Cardiology Working Group on Myocardial and Pericardial Diseases. Current state of knowledge on aetiology, diagnosis, management, and therapy of myocarditis: a position statement of the European Society of Cardiology Working Group on Myocardial and Pericardial Diseases. *Eur Heart J*. 2013;34:2636–2648.
17. Caforio AL, Keeling PJ, McKenna WJ, Mann JM, Bottazzo GF, Zachara E, Mestroni L, Camerini F. Evidence from family studies for autoimmunity in dilated cardiomyopathy. *Lancet*. 1994;344:773–777.
18. Rose NR, Bona C. Defining criteria for autoimmune diseases (Witebsky's postulates revisited). *Immunol today*. 1993;14:426–430.
19. Caforio Alida L.P., Mahon Niall G., Baig M. Kamran, Tona Francesco, Murphy Ross T., Elliott Perry M., McKenna William J. Prospective Familial Assessment in Dilated Cardiomyopathy. *Circulation*. 2007;115:76–83.
20. Caforio ALP, Brucato A, Doria A, Brambilla G, Angelini A, Ghirardello A, Bottaro S, Tona F, Betterle C, Daliento L, Thiene G, Iliceto S. Anti-heart and anti-intercalated disk autoantibodies: evidence for autoimmunity in idiopathic recurrent acute pericarditis. *Heart*. 2010;96:779–784.
21. Caforio AL, Bonifacio E, Stewart JT, Neglia D, Parodi O, Bottazzo GF, Mckenna WJ. Novel organ-specific circulating cardiac autoantibodies in dilated cardiomyopathy. *J Am Coll Cardiol*. 1990;15:1527–1534.
22. Chatterjee D, Fatah M, Akdis D, Spears DA, Koopmann TT, Mittal K, Rafiq MA, Cattanach BM, Zhao Q, Healey JS. An autoantibody identifies arrhythmogenic right ventricular cardiomyopathy and participates in its pathogenesis. *Eur Heart J*. 2018;39:3932–3944.
23. Syrris P, Ward D, Asimaki A, Evans A, Sen-Chowdhry S, Hughes SE, McKenna WJ. Desmoglein-2 mutations in arrhythmogenic right ventricular cardiomyopathy: a genotype–phenotype characterization of familial disease. *Eur Heart J*. 2006;28:581–588.

24. van der Zwaag PA, Jongbloed JD, van den Berg MP, van der Smagt JJ, Jongbloed R, Bikker H, Hofstra RM, van Tintelen JP. A genetic variants database for arrhythmogenic right ventricular dysplasia/cardiomyopathy. *Hum Mutat*. 2009;30:1278–1283.
25. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17:405–423.
26. Li Q, Wang K. InterVar: clinical interpretation of genetic variants by the 2015 ACMG-AMP guidelines. *Am J Hum Genet*. 2017;100:267–280.
27. Kleinberger J, Maloney KA, Pollin TI, Jeng LJB. An openly available online tool for implementing the ACMG/AMP standards and guidelines for the interpretation of sequence variants. *Genet Med*. 2016;18:1165–1165.
28. Sorensen D, Gianola D. Likelihood, Bayesian, and MCMC methods in quantitative genetics. Springer Science & Business Media; 2007.
29. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. Royal Statist. Soc. Series B (Methodological)*. 1995;57:289–300.
30. R Core Team. R: A Language and Environment for Statistical Computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2018. Available from: https://www.R-project.org/
31. Hadfield JD. MCMC Methods for Multi-Response Generalized Linear Mixed Models: The MCMCglmm R Package. *JSS*. 2010;33:1–22.
32. Caforio ALP, Grazzini M, Mann JM, Keeling PJ, Bottazzo GF, McKenna WJ, Schiaffino S. Identification of alpha- and beta-cardiac myosin heavy chain isoforms as major autoantigens in dilated cardiomyopathy. *Circulation*. 1992;85:1734–1742.
33. Myers JM, Cooper LT, Kem DC, Stavrakis S, Kosanke SD, Shevach EM, Fairweather D, Stoner JA, Cox CJ, Cunningham MW. Cardiac myosin-Th17 responses promote heart failure in human myocarditis. JCI Insight. 2016;1.pii: 85851. doi: 10.1172/jci.insight.85851.
34. Caforio ALP, Angelini A, Blank M, Shani A, Kivity S, Goddard G, Doria A, Schiavo A, Testolina M, Bottaro S, Marcolongo R, Thiene G, Iliceto S, Shoenfeld Y. Passive transfer of affinity-purified anti-heart autoantibodies (AHA) from sera of patients with myocarditis induces experimental myocarditis in mice. *Int J Cardiol*. 2015;179:166–177.
35. Corrado D, Basso C, Judge DP. Arrhythmogenic cardiomyopathy. Circ Res. 2017;121:784-802.

Table 1. Clinical and diagnostic baseline features in ARVC

	Probands (n=42)	**AR (n=37)**	**HR (n=96)**	**P**
Age, median (IQR)	41 (33;49)	35 (18;46)	27 (17;45)	ns
Female sex	18 (43%)	14 (38%)	47 (49%)	Ns
NYHA class: I II III or IV	 26 (62%) 13 (31%) 3 (7%)	 34 (92%) 3 (8%) 0 (0%)	 87 (91%) 8 (8%) 1 (1%)	0.007
Chest Pain	8 (19%)	6 (16%)	6 (6%)	ns
Palpitations	32 (76%)	7 (19%)	16 (19%)	0.007
Syncope	10 (24%)	4 (11%)	1 (1%)	0.007
Symptoms at follow-up	29 (69%)	5 (13%)	3 (3%)	0.007
ICD	11 (26%)	1 (2.7%)	0 (0%)	0.007
Family history of AID	16 (38%)	15 (40.5%)	20 (21%)	ns
EMB: Not done Diagnostic Non diagnostic	 19 (45%) 18 (43%) 5 (12%)	 34 (92%) 2 (5%) 1 (3%)	 96 (100%) 0 (0%) 0 (0%)	0.007
Rhythm: Sinus rhythm Atrial Fibrillation Pacemaker	 39 (93%) 2 (5%) 1 (2)	 37 (100%) 0 (0%) 0 (0%)	 96 (100%) 0 (0%) 0 (0%)	ns
VEs/24h , median (IQR)	5724 (800;165000)	37 (2,75;582)	1 (0;172)	ns
Couplets/24h, median (IQR)	149 (5,5;290)	1,5 (1;17,5)	0	0.013
NSVT, n (%)	11 (26%)	1 (3%)	0 (0%)	0.064
RV enddiastolic area, median (IQR)	20 (14;32)	16 (14;21)	15 (12;19)	0.007
RV FAC, median (IQR)	45 (32;52)	47 (35;57)	55 (47;60)	0.007
RV EDV/BSA, median (IQR)	27 (16;38)	18 (14;25)	16 (13;22)	0.007
%RVEF, median (IQR)	50 (44;60)	56 (48;64)	61 (56;68)	0.007
LV enddiastolic diameter, median (IQR)	50 (46;55)	48 (42;51)	45 (41;49)	0.007
%FS, median (IQR)	33 (29;37)	38 (33;45)	38 (32;43)	ns
2-D echo LV EDV/BSA, median (IQR)	45 (41;55)	44 (35;49)	42 (36;50)	0.007
2-D echo LVEF, median (IQR)	58 (49;63)	64 (56;68)	63 (60;69)	0.007
Revised major (>) and minor (<) 2010 Task force criteria:				
I. Global or regional dysfunction and structural alterations by 2-D echo, n (%):				
>	18 (43%)	13 (35%)	4 (4%)	0.007
<	8(19%)	7 (19%)	1 (1%)	0.007
III. repolarization abnormalities, n (%):				
>negative T wave in V1-V3	20 (48%)	9 (24%)	4 (4%)	0.007
<negative T wave in V1-V2, no RBBB, aged above 14 yrs	31 (74%)	17 (46%)	8 (8%)	0.007
<negative T wave in V4	15 (36%)	7 (19%)	1 (1%)	0.007
<negative T wave in V5	11 (26%)	0 (0%)	1 (1%)	0.007
<negative T wave in V6	9 (21%)	0 (0%)	1 (1%)	0.007
IV. depolarization/conduction abnormalities, n (%):				
> Epsilon wave in V1-V3	6 (14%)	2 (5%)	0 (0%)	0.007
V. Arrhythmias, n (%)				
< VT	11 (26%)	1 (3%)	0 (0%)	0.064

< VEs	24 (57%)	3 (8%)	1 (1%)	0.007
VI. Family history, n (%):				
<sudden death (age less than 35 yrs) due to suspected ARVC	6 (14%)	11 (30%)	7 (7%)	0.013

P-values are for overall comparison among probands, AR and HR, each row refers to a different variable (see statistics section for details on type of test used). Abbreviations: AID, autoimmune disease; AR, affected relatives; ARVC, arrhythmogenic right ventricular cardiomyopathy; BSA, body surface area; 2-D echo, 2 dimensional echocardiography; EF, ejection fraction; EDV, endiastolic volume; EMB, endomyocardial biopsy; FAC, fractional area change; %FS, %fractional shortening; HR, healthy relatives; ICD, implantable cardioverter defibrillator; IQR, interquartile range, 25% and 75% ; LV, left ventricular; NYHA, New York Heart Association; VEs, ventricular ectopic beats; NSVT, nonsustained ventricular tachycardia; VT, ventricular tachycardia; RV, right ventricular.

Table 2. AHA and AIDA Status: Association with Clinical and Diagnostic Features

	AHA positive (n=52)	**AHA negative (n=119)**	**P**
Chest Pain	10 (19.2%)	10 (8.4%)	0.080
Palpitations	22 (42.3%)	30 (25.2%)	0.004
Persistent symptoms	16 (31%)	19 (16%)	ns
ICD	7 (13.5%)	4 (3.4%)	0.021
LV enddiastolic diameter, median, median (IQR)	48 (44;52)	46 (41:51)	0.004
LV interventricular enddiastolic septum thickness, median (IQR)	10 (9;11)	9 (8;10)	0.004
LV posterior wall enddiastolic thickness, median (IQR)	9 (7;10)	8 (7;9)	0.004
2-D echo LVEF, median (IQR)	60 (53;67)	63 (57;69)	0.004
	AIDA positive (n=25)	**AIDA negative (n=146)**	**P**
Dyspnea	7 (28%)	9 (6.2%)	ns
2-D echo RVEF , median (IQR)	54 (44; 60)	60 (54;67)	0.027
2-D echo LVEF, median (IQR)	60 (53;65)	63 (57;68)	0.027

Abbreviations: AHA= Anti-heart autoantibodies; AIDA= anti-intercalated disk autoantibodies; 2-D echo= 2 dimensional echocardiography; EF, ejection fraction; ICD=implantable cardioverter defibrillator; IQR= interquartile range, 25% and 75% ; LV= left ventricular; RV= right ventricular.

Table 3. List of genetic variants.

ARVC index case	Gene	Variant	MAF in gnomAD	ARVD/C Genetic Variants Database classification	ACMG classification
IT2	DSC2	c.1914G>C; p.Gln638His	0.0005	VUS	Likely benign
	PKP2	c.1592T>G; p.Ile531Ser	0.0048	No known pathogenicity	Likely benign
IT5	PKP2	c.2443_2448delAACACCinsGAAA; p.Asn815GlufsX11	Not present	Not present	Pathogenic (Ia)
IT7	PKP2	c.1216delG; p.Val406PhefsX14	Not present	Not present	Pathogenic (Ia)
IT10	DSC2	c.2686_2687dupGA; p.Ala897LysfsX4	0.0086	No known pathogenicity	VUS
	DSG2	c.1038_1040delGAA; p.Lys346del	8.137e-6	Pathogenic	Pathogenic (Ia)
IT11	PKP2	c.76G>A; p.Asp26Asn	0.0079	No known pathogenicity	Likely benign
	PKP2	c.1799delA; p.Asp600ValfsX56	Not present	Pathogenic	Pathogenic (Ia)
IT12	DSP	c.2684A>G; p.Tyr895Cys	0.00014	Not present	VUS
IT13	PKP2	c.2009delC; p.Asn670ThrfsX14	Not present	Pathogenic	Pathogenic (Ia)
IT17	JUP	c.1942G>A; p.Val648Ile	0.0074	VUS	Benign
IT18	DSG2	c.2759T>G; p.Val920Gly	0.0035	VUS	Benign
	PKP2	c.1045A>G; p.Met349Val	6.61e-5	Not present	Likely benign
IT19	DSG2	c.2434G>A; p.Gly812Ser	3.60e-5	Pathogenic	VUS
IT20	JUP	c.1807G>A; p.V603M	1.24e-5	Not present	VUS
IT21	DSP	c.5851C>T; p.Arg1951X	Not present	Not present	Pathogenic (Ia)
IT23	PKP2	c.76G>A; p.Asp26Asn	0.0079	No known pathogenicity	Likely benign
IT27	PKP2	c.1759G>A; p.Val587Ile	0.0024	VUS	Likely benign
IT33	PKP2	c.209G>T; p.Ser70Ile	0.2081	No known pathogenicity	Likely benign
IT34	PKP2	c.1216delG; p.Val406PhefsX14	Novel	N/A	Pathogenic (Ia)
	DSP	c.3923G>C; p.Arg1308Pro	8.147e-5	Not present	Likely benign
IT35	DSP	c.5498A>T; p.Glu1833Val	0.0096	VUS	Benign
IT36	DSP	c.6208G>A; p.Asp2070Asn	0.0039	No known pathogenicity	Benign
IT37	DSP	c.1465G>T; p.Glu489X	Not present	Not present	Pathogenic (Ia)

Abbreviations: ACMG= American College of Medical Genetics; ARVC= Arrhythmogenic right ventricular cardiomyopathy; ARVD= Arrhythmogenic right ventricular dysplasia; Genome Aggregation Database=gnomAD; MAF=minor allele frequency; N/A= not available; desmocollin-2 (DSC2), desmoglein (DSG2); desmoplakin (DSP) and plakoglobin (JUP); plakophilin-2 (PKP2); VUS=variant of unknown significance.

Table 4. Pathogenic Mutations: Associations with Clinical, Diagnostic and Immune Features

	Any mutation pos (n=23)	Any mutation neg (n=116)	p
ICD	6 (26%)	6 (5%)	0.095
Revised major III (neg T in V1-V3)	11 (48%)	19 (16%)	0.002
RV end-diastolic area, cm^2, mean (SD)	23 (10)	18 (6)	0.02
RV EDV/BSA, ml/m^2 mean (SD)	29 (15)	21 (10)	0.02
AHA*	10 (45%)	30 (26%)	ns
AIDA*	3 (14%)	22 (19%)	0.099
AHA and/or AIDA*	11 (50%)	40 (35%)	ns
	DSP pos (n=6)	DSP neg (n=133)	p
QRS duration, msec,mean (SD)	100 (16)	88 (12)	0.005
Rev <criteria (negative T V1-V4)	1 (17%)	1 (0.7%)	ns
RV outflow tract 1/BSA, mm, mean (SD)	20 (5)	15(3)	0.004
RV outflow tract 4/BSA, mm, mean (SD)	22 (5)	16(3)	0.004
AHA*	2 (33%)	38 (29%)	ns
AIDA*	1 (17%)	24 (18%)	ns
AHA and/or AIDA*	2 (33%)	49 (36%)	ns
	PKP-2 pos (n=15)	PKP-2neg (n=124)	p
ICD	5 (26%)	9 (8%)	0.011
Syncope	6 (31%)	6 (5%)	ns
RV end-diastolic area, cm^2, mean (SD)	24 (9)	18 (7)	0.004
RV EDV/BSA, ml/m^2, mean (SD)	31 (16)	21 (10)	0.004
AHA*	0 (0%)	40 (30%)	ns
AIDA*	2 (14%)	23 (19%)	ns
AHA and/or AIDA*	9 (64%)	42 (34%)	ns
	DSG-2 pos (n=3)	DSG-2 neg (n=136)	p
AHA*	0 (0%)	40 (30%)	0.060
AIDA*	0 (0%)	25 (19%)	ns
AHA and/or AIDA*	0 (0%)	51 (39%)	0.060

Abbreviations: BSA, body surface area; neg, negative; pos, positive; see Table 1-3 for remaining abbreviations.

*Total for antibody tests: n=136; †No other significant associations were found for DSC2 mutations.

Figure Legends

Figure 1. Study flow-chart

Abbreviations: AHA= Anti-heart auto-antibodies; AIDA= anti-intercalated disk autoantibodies.

Figure 2. Anti-heart auto-antibodies (AHA) and anti-intercalated disk autoantibodies (AIDA) patterns by indirect immunofluorescence test

Negative AHA control serum pattern: panel A on human heart tissue: negative (x200), and *panel B* on human skeletal muscle: negative (x400).

Organ-specific AHA pattern: panel C on human heart tissue: strong cytoplasmic and striational staining of cardiac myocytes (organ-specific AHA pattern); *panel D* (x400) on human skeletal muscle tissue: negative.

Organ-specific AHA and AIDA pattern: *panel E* strong linear staining of the intercalated disks (AIDA pattern) and associated weak diffuse cytoplasmic organ-specific AHA pattern (x400); *panel F* (x400) on human skeletal muscle tissue: negative.