ESC European Society of Cardiology

European Heart Journal (2020) **0**, 1–14
doi:10.1093/eurheartj/ehaa383

CLINICAL RESEARCH
Arrhythmia/electrophysiology

An autoantibody profile detects Brugada syndrome and identifies abnormally expressed myocardial proteins

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Received 13 December 2019; revised 23 March 2020; editorial decision 23 April 2020; accepted 24 April 2020

Aims Brugada syndrome (BrS) is characterized by a unique electrocardiogram (ECG) pattern and life-threatening arrhythmias. However, the Type 1 Brugada ECG pattern is often transient, and a genetic cause is only identified in <25% of patients. We sought to identify an additional biomarker for this rare condition. As myocardial inflammation may be present in BrS, we evaluated whether myocardial autoantibodies can be detected in these patients.

Methods and results For antibody (Ab) discovery, normal human ventricular myocardial proteins were solubilized and separated by isoelectric focusing (IEF) and molecular weight on two-dimensional (2D) gels and used to discover Abs by plating with sera from patients with BrS and control subjects. Target proteins were identified by mass spectrometry (MS). Brugada syndrome subjects were defined based on a consensus clinical scoring system. We assessed discovery and validation cohorts by 2D gels, western blots, and ELISA. We performed immunohistochemistry on myocardium from BrS subjects (vs. control). All (3/3) 2D gels exposed to sera from BrS patients demonstrated specific Abs to four proteins, confirmed by MS to be α-cardiac actin, α-skeletal actin, keratin, and connexin-43, vs. 0/8 control subjects. All (18/18) BrS subjects from our validation cohorts demonstrated the same Abs, confirmed by western blots, vs. 0/24 additional controls. ELISA optical densities for all Abs were elevated in all BrS subjects compared to controls. In myocardium obtained from BrS subjects, each protein, as well as SCN5A, demonstrated abnormal protein expression in aggregates.

Conclusion A biomarker profile of autoantibodies against four cardiac proteins, namely α-cardiac actin, α-skeletal actin, keratin, and connexin-43, can be identified from sera of BrS patients and is highly sensitive and specific, irrespective of genetic cause for BrS. The four involved proteins, along with the *SCN5A*-encoded Nav1.5 alpha subunit are expressed abnormally in the myocardium of patients with BrS.

Keywords Actin • Autoantibody • Biomarker • Brugada syndrome • Connexin-43 • Keratin • Perinexus

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Introduction

Brugada syndrome (BrS) is a heritable disorder associated with an increased risk for sudden cardiac death (SCD) from ventricular arrhythmias and is characterized by a unique electrocardiogram (ECG) pattern of 'coved' ST-segment elevation (≥2 mm) followed by a negative T wave in anterior chest leads (called the Type 1 Brugada ECG pattern).[1] Brugada syndrome tends to affect young individuals with an apparently structurally normal heart, particularly males (male:female ratio = 9:1) in their third to fourth decade of life, and SCDs tend to occur during sleep.

The worldwide prevalence of BrS is estimated at 1:2000 persons[2] but is higher in those of Southeast Asian ethnicity/descent.[3] Brugada syndrome is a major contributor to SCD in young adults, accounting for 4–12% of unexpected sudden deaths and up to 20% of all sudden death in individuals with apparently normal hearts.[4] In Southeast Asia, BrS is the second highest cause of death (following accidents), of men under age 40 years.[5] Events are exacerbated by fever, alcohol, large meals, high vagal tone, and certain medications.

Mutations in the *SCN5A*-encoded Nav1.5 sodium channel is a monogenic cause for 14–26% of BrS.[6–9] A complicating factor is that *SCN5A* is a large gene, resulting in almost 3% of normal individuals having rare, protein-altering variants.[10,11] Other implicated genes include *CACNA1C*, *CACNA2D1*, *CACNB2*, *GPD1L*, *HCN4*, *KCND3*, *KCNE3*, *KCNE5*, *KCNJ8*, *RANGRF*, *SCN1B*, *SCN2B*, *SCN3B*, *SLMAP*, and *TRPM4*, but these have been reclassified as either 'limited evidence' or 'disputed evidence' BrS susceptibility genes.[12] Brugada syndrome may be oligogenic rather than monogenic.[13] Brugada syndrome remains gene elusive in 75% of individuals/families.

Reliance on ECG Type 1 pattern to identify BrS may result in under-diagnosis in 65% due to the fluctuant nature of this ECG finding,[14] in 28–42% due to the need for modified high leads,[15,16] and in 40% due to the need for drug provocation to identify a Type 1 ECG pattern.[17] A simpler, less fluctuant and accurate test for BrS would be beneficial. Lack of a simple, accurate test that also can identify preclinical disease makes cascade screening (a major tool in other inherited arrhythmia conditions) difficult. Brugada syndrome has been diagnosed based on evolving expert consensus conferences focusing on diagnostic criteria (in 2000[18]), risk stratification and therapy (in 2004[19]), and (along with early repolarization syndrome) the emerging concept of J-wave syndromes (in 2015[20]). For this study, we have used the point scoring system for BrS diagnosis from this most recent consensus conference, also known as the Shanghai score (ShS). Subjects with ShS ≥3.5 are considered to have probable or definite BrS.

The pathophysiology of BrS may involve depolarization abnormalities, changes in transmural dispersion of repolarization, or both.[21] Histological changes in the right ventricular (RV) myocardium similar to that seen in arrhythmogenic RV cardiomyopathy (ARVC) have been described in a series of patients with a Type 1 Brugada ECG pattern.[22,23] Imaging studies demonstrate mild structural abnormalities within the right ventricle of some BrS patients.[24] Inflammatory infiltrates have been identified, and fibrosis has also been described.[25] Recently, Pieroni *et al.*[26] identified pathological myocardial inflammation in 80% of RV outflow tract biopsies from BrS patients. Thus, it seems likely that inflammation plays a role in BrS. Given a recent discovery of an autoantibody characterizing ARVC,[27] autoimmunity might also explain the inflammation now being recognized in BrS.

The cardiac intercalated (IC) disk is implicated in BrS, and our understanding of IC disk structure has evolved from independent adherens junctions, desmosomes, and gap junctions, to an area composita (merging desmosomal and adherens junction components), and to a connexome (where desmosomal, gap junction, sodium channel complex molecules interact with each other).[28,29] Actin filaments insert into the adherens junction components of the connexome but also into gap junction plaques and perinexus connexon complexes with ZO-1, as well as into potassium channel complexes of Kv1.5 and Kv4.2.[29] Actin forms an extensive submembrane cytoskeleton that anchors ion channels via linker proteins SAP97, filamin, and α-actinin-2.[30] Within the heart, desmin is the main intermediate filament (IF) protein, connecting into the desmosome via desmoplakin. However, an alternative IF network using keratin is induced by cardiac stress such as heart failure via tumour necrosis factor-α as a compensatory mechanism.[31,32] Micrographs from Human Protein Atlas (www.proteinatlas.org, 6 May 2020, date last accessed) demonstrate that keratin-24 is present in the cardiac IC disk based on antibody (Ab) staining, although its function there is unclear.

In this study, we report the presence of serum autoantibodies against cardiac α-actin, skeletal α-actin, keratin, and connexin-43 as highly sensitive and specific biomarkers identifying ShS-positive BrS human subjects, and demonstrate that these proteins are expressed abnormally in the myocardium of BrS patients.

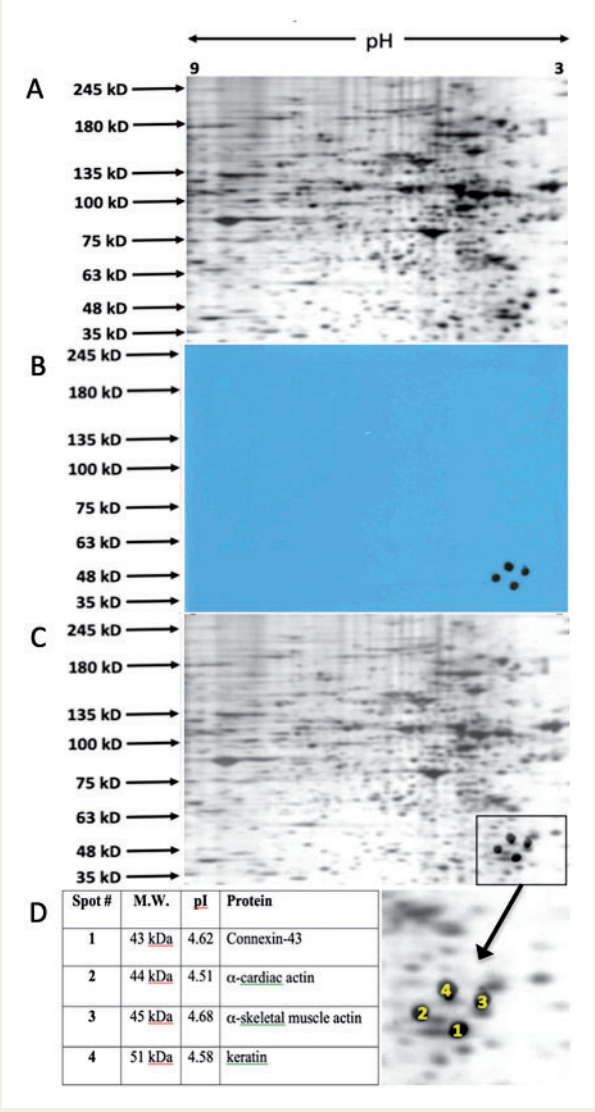
Methods

Subjects

The study protocol followed the ethical guidelines of the Declaration of Helsinki, was approved by the Research Ethics Board of each institution and all human participants gave written informed consent (see Supplementary material online, *Methods*). Patients referred to these clinics for assessment of BrS were evaluated using the 2015 J-wave syndromes expert consensus conference ShS[20] (ShS which included ECG findings, clinical history, family history, and genetic test result) and only those meeting definite/probable BrS (ShS ≥ 3.5 points, including a Type 1 Brugada ECG, spontaneous or drug-induced) were included for both the discovery cohort (Toronto) and validation cohorts (Zürich, Münster, and Mayo Clinic). Control sera for the discovery cohort were collected from eight normal control sera purchased from a commercial source: Innovative Research Inc. (Novi, MI, USA). Additional controls, including from BBI Solutions (Cardiff, UK) were used for the validation.

Development of 2D gel antibody (Ab) discovery platform

Samples of human ventricular myocardium were homogenized, solubilized, and separated using an IEF strip (pH range 3–10; BioRad, Canada), such that proteins settled at their isoelectric point and were then further separated in the second dimension by molecular weight using standard electrophoresis. The proteins in the gel were transferred to a polyvinylidene difluoride membrane and exposed to human sera in 1:100 dilution, following which they were then



Spot #	M.W.	pI	Protein
1	43 kDa	4.62	Connexin-43
2	44 kDa	4.51	α-cardiac actin
3	45 kDa	4.68	α-skeletal muscle actin
4	51 kDa	4.58	keratin

Figure 1 Two-dimensional gel of normal myocardial proteins separated by their isoelectric point and molecular weight. (*A*) Silver nitrate staining identified myocardial proteins distributed over the gel. (*B*) After exposure to sera from a patient with BrS, washing, and staining with anti-human IgG, human BrS autoantibodies were bound to four specific proteins. (*C*) Overlap of proteins and antibodies. (*D*) Protein spots 1–4 were assessed by mass spectrometry, and four protein targets: α-cardiac actin, α-skeletal muscle actin, keratin-24, and connexin-43 were identified.

developed with a horseradish peroxidase-linked anti-human IgG antibody (Ab) as described previously for one-dimensional gels[27] (see Supplementary material online, *Methods*).

Mass spectrometry

Removing the corresponding spot from a second protein gel and performing mass spectrometry identified the autoantibody-binding proteins. Raw files acquired from the mass spectrometer were processed using PEAKS software and viewed as a Scaffold file (Scaffold version 4.8.6) (see Supplementary material online, *Methods*).

Confirmatory Western blot analysis

Western blot were performed with the sera at 1:100 dilutions using commercial recombinant α-cardiac actin, α-skeletal actin, keratin, and connexin-43 proteins. All recombinant proteins were purchased from Creative Biomart, USA (see Supplementary material online, *Methods*). Initially, on the basis of GC/MS data, we used recombinant keratin-14 protein to assess the reactivity of sera from BrS patients. Recognizing that BrS serum autoantibodies would be polyclonal, that keratins have substantial protein sequence homology, and that the heart expresses keratins 8, 18, and 24 (based on Human Protein Atlas), we used specific monoclonal antibodies against keratin-14 (abcam, cat # ab7800), keratin-8 (abcam; cat # ab32579), keratin-18 (Millipore Sigma; cat # SAB4501665), and keratin-24 (abcam; cat # ab150077) to assess the protein identity of spot 4 (see *Figure 1*).

Antibody ELISA protocols

A direct Enzyme-Linked Immuno-Sorbent Assay (ELISA) was performed by first coating a micro-titre plate with α-cardiac actin, α-skeletal actin, keratin, and connexin-43 proteins according to abcam protocol (www.abcam.com/protocols/indirect-elisa-protocol, 6 May 2020, date last accessed). Details of the solutions, dilutions, incubations and washing are provided in the Supplementary material online, *Methods*.

Immunofluorescence staining of myocardial tissues

Charged unstained slides were assessed from sections of formaldehyde-fixed paraffin-embedded myocardial tissue (right and left ventricular free walls) from a young adult male victim of SCD identified to have a heterozygous *SCN5A* (c.1936del p.Gln646fs) variant that was also present in his brother with a BrS Type I phenotype and has been previously reported as pathogenic and associated with BrS in ClinVar in all four submissions where a phenotype was provided. These slides were obtained from the Ontario Coroner office, along with slides from control myocardial tissue from a mixed drug overdose victim of similar age. Similarly, charged unstained slides from endomyocardial biopsies from the right ventricular outflow tract of nine BrS subjects were obtained, as well as one 'control' subject whose ShS was only two. Slides were stained for each of α-cardiac actin, keratin-24, and connexin-43 proteins, as well as SCN5A, using standard immunofluorescence (see Supplementary material online, *Methods*).

Image and statistical analysis

Image analyses were performed with ImageJ 1.52Q (National Institutes of Health). Calculations and graphs were completed with Prism 5.0 for Mac OS X Version 5.0f, except for Hodges–Lehman Difference analysis, which was performed on Prism 8 for macOS version 8.4.0 (455) (GraphPad Software, Inc.). The summarizing illustration was created under a paid academic subscription to Biorender.com.

Table 1 Clinical data and Shanghai Scores of BrS patients with serum biomarker profiling

Cohort[a]	Research ID	Sex	Age[b]	Shanghai score (ShS)						
				ECG[c]	Clinical history[d]	Family history[e]	Genetics[f]	Mutation[g]	Shanghai score total[h]	Biomarker[i]
SickKids	HRC095	M	35–55	3.5	2	0	0	None	5.5	Positive
(Discovery)	HRC289	M	35–55	3.5	0	0	0.5	VUS: *CACNA1C*, *KCNE3*	3.5	Positive
	HRC0409	M	35–55	2	3	0	0	None	5	Positive
Zurich	ZH-BrS150	M	35–55	3.5	0	0	0.5	*SCN5A* c.1007C>T (p. Pro336Leu)	4	Positive
	ZH-BrS169	M	35–55	3	1	0	0	None	4	Positive
	ZH-BrS173	M	M	2	0	2	0.5	VUS: *RANGRF*	4.5	Positive
	ZH-BrS196	F	35–55	3.5	0	2	0.5	*SCN5A* c.3352C>T (p.Gln1118Ter)	6	Positive
	ZH-BrS197	M	15–35	3.5	0	0	0.5	*SCN5A* c.844C>G (p.Arg282Gly)	4	Positive
	ZH-BrS200	M	15–35	2	0	2	0.5	*SCN5A* c.3508 + 1G>A	4.5	Positive
	ZH-BrS240	F	55+	3.5	0	0	0.5	*SCN5A* c.3508 + 1G>A	4	Positive
	ZH-BrS250	F	55+	2	0	2	0		4	Positive
	ZH-BrS253	M	35–55	3.5	3	0	0.5		7	Positive
	ZH-BrS256	M	55+	3.5	1	0	0.5	*SCN5A* c.4501C>G (p.Leu1501Val)	5	Positive
	ZH-BrS263	M	15–35	3.5	3	0	0	None	6.5	Positive
	ZH-BrS258	M	35–55	3.5	2	0	0	None	5.5	Positive
Münster	12819-4	M	35–55	2	0	2	0.5	*SCN5A* c.4387A>T, (p.Asn1463Tyr)	4.5	Positive
	12924-1	M	55+	3.5	3	0	0		6.5	Positive
	10021-49	M	35–55	2	0	2	0.5	*SCN5A* c.4477_4479del (p.Lys1493del)	4.5	Positive
Mayo Clinic	BrSM-01	M	35–55	2	0	2	0.5	*SCN5A* c.1127 G>A (p.Arg376Cys)	4.5	Positive
	BrSM-02	F	35–55	3.5	0	2	0.5	*SCN5A* c.5027 T>C (p.Met1676Thr)	6	Positive
	BrSM-04	M	35–55	3.5	0	0	0.5	*SCN5A* c.3695 G>A (p.Arg1232Trp)	4	Positive

[a]Source of samples defined as a discovery cohort (SickKids) and validation cohorts (Zurich, Münster, and Mayo Clinic).
[b]Age at time of blood draw.
[c]ECG (12-lead/ambulatory) as defined by the Shanghai criteria where 3.5 points = spontaneous type 1 Brugada ECG pattern at nominal or high leads, 3 points = fever-induced type 1 Brugada ECG pattern at nominal or high leads, and 2 points = type 2 or three Brugada ECG pattern that converts with provocative dug challenge.
[d]Clinical history as defined by the Shanghai criteria where 3 points = unexplained cardiac arrest or documented ventricular fibrillation/polymorphic ventricular tachycardia, 2 points = either nocturnal agonal respirations or suspected arrhythmic syncope, 1 point = syncope of unclear mechanism/unclear aetiology, 0.5 points = atrial flutter/fibrillation in patients under 30 years without alternative aetiology.
[e]Family history as defined by the Shanghai criteria where 2 points = first or second degree relative with definite BrS, 1 point = suspicious SCD (fever, nocturnal, Brugada aggravating drugs) in a first or second degree relative, 0.5 points = unexplained SCD less than 45 years in first- or second-degree relative with negative autopsy.
[f]Genetics as defined by the Shanghai criteria where 0.5 points = probable pathogenic mutation in Brugada Syndrome susceptibility gene.
[g]Mutation found in individual; no genetic data available.
[h]Total Shanghai score (ShS) as defined by the Shanghai criteria where a score of ≥3.5 is a probable/definite diagnosis of BrS, 2–3 points is a possible diagnosis of BrS, and < 2 is non-diagnostic of Brugada Syndrome.
[i]BrS biomarker result.

Results

Study population

Three cases of probable/definite BrS based on 2015 consensus conference criteria (ShS ≥ 3.5) from families followed at the Hospital for Sick Children formed a discovery cohort for serum analysis (*Table 1*). These include cases referred for ventricular arrhythmias or resuscitated sudden cardiac arrest. Subjects had undergone a 12-lead ECG, Brugada high-lead ECG, signal-averaged ECG, ambulatory ECG, and clinical panel testing for genetic causes of BrS. None had known BrS pathogenic variants in the *SCN5A* gene. Eight commercially sourced normal controls provided control sera for this discovery cohort.

A validation cohort of 12 adults with probable/definite BrS from the Zurich-inherited arrhythmia programme (ShS ≥ 3.5 points) was also assessed. Six of these subjects had BrS-associated *SCN5A* pathogenic variants, four had no pathogenic mutations detected, and two did not have genetic testing. An additional three BrS patients each were recruited from the University Hospital Münster, Germany and the Mayo Clinic in Rochester, MN, USA. All subjects are summarized according to their ShS in *Table 1*. Endomyocardial biopsy tissue from subjects from an additional inherited arrhythmia centre (Arezzo, Italy) was also assessed, and these subjects are summarized in *Table 2*.

Normal control sera were from Innovative Research Inc., Novi, MI, USA (age 18–61, 39.6 ± 13.9 years; 80% male) and from BBI Solutions, Cardiff, UK (age 24–92, 50.2 ± 18.2 years; all female). In addition, a cohort of subjects from Mayo Clinic, Rochester with hypertrophic cardiomyopathy (Supplementary material online, *Table S1*) and a cohort of subjects from Zurich with dilated cardiomyopathy (Supplementary material online, *Table S2*) served as non-BrS

Table 2 **Clinical characteristics of Brugada Syndrome subjects and controls with myocardium biomarker profiling**

Code	Age	Sex	Spont. type 1	Fever type 1	Drug type 1	VF/VT	Noct. agonal	Arrh. Sync.	Afl/AF <30years	Fam BrS	Fam susp SCD	Fam SCD	Inducibility	ICD	Genetics	Shanghai score	Tissue source	IF stain results
PM control	35–55	M	Acute mixed drug overdose														Ventricle	Speck/Fil
Bx control	35–55	M	0	0	1	0	0	0	0	0	0	0	0	0	0	2	RVOT Bx	Speck (SCN5A)
PM case A	35–55	N	Sudden Cardiac Death during Sleep. Family Identified to have BrS with SCN5A Mutation														RVOT	Aggregates
PM case B	35–55	N															LV	Aggregates
Bx 1	35–55	F	1	0	0	0	1	0	0	1	0	0	0	0	*SCN5A*	8	RVOT Bx	Aggregates
Bx 2	35–55	M	1	0	0	1	0	0	0	0	1	0	1	0	*SCN5A*	8	RVOT Bx	Aggregates
Bx 3	55+	M	1	0	0	0	0	0	0	0	0	0	1	0	0	3.5	RVOT Bx	Aggregates
Bx 4	35–55	F	1	0	0	0	0	1	0	1	0	0	0	0	*SCN5A*	8	RVOT Bx	Aggregates
Bx 5	35–55	F	1	0	0	0	0	0	0	0	0	0	1	1	*SCN4B*	3.5	RVOT Bx	Aggregates
Bx 6	15–35	M	0	0	1	0	0	1	0	0	0	0	1	1	*SCN5A*	4	RVOT Bx	Aggregates
Bx 7	35–55	F	1	0	0	0	1	0	0	1	0	0	1	1	*SCN5A*	8	RVOT Bx	Aggregates
Bx 8	35–55	M	1	0	0	0	0	0	0	0	0	0	0	0	*HCN2*	4	RVOT Bx	Aggregates
Bx 9	35–55	M	1	0	0	0	0	0	0	0	0	0	1	0	*DSP*	3.5	RVOT Bx	Aggregates

Afl/AF, atrial flutter or fibrillation; Arrh. Sync., arrhythmogenic; Fam, familial; Fil, filamentous (for actin); ICD, implantable cardioverter-defibrillator; IF Stain Result, immunofluorescence staining result; Noct, nocturnal; Speck, speckled (for keratin, connexin-43, and SCN5A); Spontan, spontaneous; VF/VT, ventricular fibrillation or tachycardia.

disease controls. Finally, cohorts of ARVC subjects from Zurich and Toronto (Supplementary material online, *Table S3*) also served as non-BrS disease controls.

Two-dimensional gel discovery, identification, and validation of cardiac autoantibodies

Sera obtained from three patients with BrS (discovery cohort) demonstrated a consistent four spot profile of antibodies to low isoelectric pH and low molecular weight proteins on 2D gels (*Figure 1*). Mass spectrometry was used to identify the specific autoantibody protein targets as α-cardiac actin, α-skeletal actin, keratin, and connexin-43 (see Supplementary material online, *Results*). As α-cardiac actin and α-skeletal muscle actin are highly homologous, they likely represent one autoantibody detecting two similar protein targets. Specific monoclonal antibodies against keratins 8, 14, 18, and 24 were assessed against the extracted protein from spot 4 from the 2D gels. Only the specific monoclonal anti-keratin-24 antibody demonstrated binding, confirming that spot 4 was keratin-24 protein.

For validation of this discovered serum autoantibody profile in BrS, we evaluated additional 18 sera from BrS patients (Zurich inherited arrhythmia programme: $n = 12$, ERN Guard Heart outpatient clinic of the University Hospital Münster: $n = 3$ and $n = 3$ participants from Mayo Clinic), all with probable/definite BrS (ShS $\geq$ 3.5; see *Table 1*). These sera were evaluated with the same 2D gels and western blots similarly used for the discovery cohort. All showed the identical four-spot autoantibody profile (antibodies to α-cardiac actin, keratin-24, and connexin-43 proteins) as shown in the discovery cohort (*Figure 2* and Supplementary material online, *Figure S2*) and the results were independently confirmed by western blot to have antibodies to α-cardiac actin, α-skeletal actin, keratin-24, and connexin-43 proteins (*Figure 3* and Supplementary material online, *Figure S2*).

Autoantibody assessments by ELISA

ELISAs of BrS serum from the discovery cohort, the validation cohort and controls detected again antibodies against each protein (α-cardiac actin, α-skeletal actin, keratin-24, and connexin-43; *Figure 4*). In each case, ELISA optical densities from the discovery and validation cohort were equivalent, and markedly increased over the baseline optical densities as seen in control samples. These differences were statistically significant, and did not change for any group comparisons, even after computing the Hodges–Lehmann estimator for the group differences.

Immunofluorescence staining of myocardial tissues

To examine whether BrS patient sera bind to the target proteins in cardiac tissue, we double stained normal cardiac tissue with BrS patient sera and commercial antibodies against α-cardiac actin, keratin-24, and connexin-43. Co-localization of staining patterns of BrS serum and all the three commercial antibodies clearly demonstrated co-staining of α-cardiac actin, keratin-24, and connexin-43 (*Figure 5*).

We assessed myocardium from a BrS decedent and biopsies from nine BrS subjects. Each protein demonstrated abnormal aggregates within the sarcoplasm of BrS myocardium (*Figure 6* and Supplementary material online, *Figure S4*), as compared to normal tissue where α-cardiac actin expressed as filaments, and keratin-24 and connexin-43 demonstrated fine speckled staining. Sodium channel protein Type 5 subunit alpha demonstrated similar large aggregates of staining within BrS cardiomyocytes. These aggregates were most convincing for keratin-24 and the cardiac sodium channel, and particle size analysis for both of these proteins separated Brugada decedent/biopsy patients from controls with 89% sensitivity (see Supplementary material online, *Figure S5*).

Discussion

This study identified that autoantibodies to cardiac and skeletal α-actins, keratin-24, and connexin-43 are consistently present in the sera of patients with probable/definite BrS (as defined by ShS $\geq$ 3.5), in both the discovery cohort and validation cohort. These autoantibodies were specifically detected in BrS patients and were absent in two independent sets of controls but also from patients with hypertrophic, dilated, and arrhythmogenic cardiomyopathies. The detection of these autoantibodies may provide a reliable biomarker assay in patients undergoing evaluation for BrS. Moreover, this antibody profile encompassing four proteins was present independent of an identified underlying genetic cause for BrS.

The myocardium from patients with BrS demonstrated aggregates of each target protein (α-actin, keratin-24, connexin-43) within the cytoplasm, as opposed to diffuse reticular or speckled patterns seen in normal myocardium.

Cardiomyocytes have a cytoskeleton composed of actin microfilaments, tubulin microtubules, and IFs (usually desmin in cardiomyocytes, although expression of keratin occurs under cellular stress[31]). Actin isoforms in heart are highly homologous, and include abundant sarcomeric cardiac α-actin, as well as lesser amounts of skeletal muscle α-actin, and β and γ-actins that contribute to the cytoskeleton, including subcortical actin anchoring ion channels.[30] Cytoskeletal actins contribute to actin 'rest stops,' which redirect trafficking of connexin-43 along microtubule networks,[33,34] and similar processes traffic sodium channel complexes.

Reduction of sodium current density in BrS may arise from defects in channel trafficking, or non-conducting channels, with *SCN5A* loss-of-function pathogenic variants showing both features. Other proposed genetic causes of BrS show reduced trafficking, such as the *SCN3B* p.V110I mutation,[35] and the recently described *RRAD* p.Arg211His mutation.[36] This *RRAD* gene variant demonstrated both abnormal distribution of actin and reduced sodium current, implicating cytoskeletal disturbances in abnormal channel trafficking. It is intriguing that myocardial tissue from all BrS subjects sampled, regardless of genetic cause, demonstrates cytoplasmic clustering of actin and keratin, components of the cytoskeletal network, as well as connexin-43 which depends on the cytoskeletal network for trafficking to the sarcolemma, and particularly to the intercalated disk. We also observed aggregates of SCN5A in BrS myocardium, supporting our view that trafficking defects are implicated.

The mechanism by which autoimmune antibodies to the cardiac proteins actin, keratin, and connexin-43 develop remains unclear. These three protein targets are all low molecular weight acidic proteins and demonstrate altered expression within BrS myocardium. Elimination of these altered expressed proteins may involve

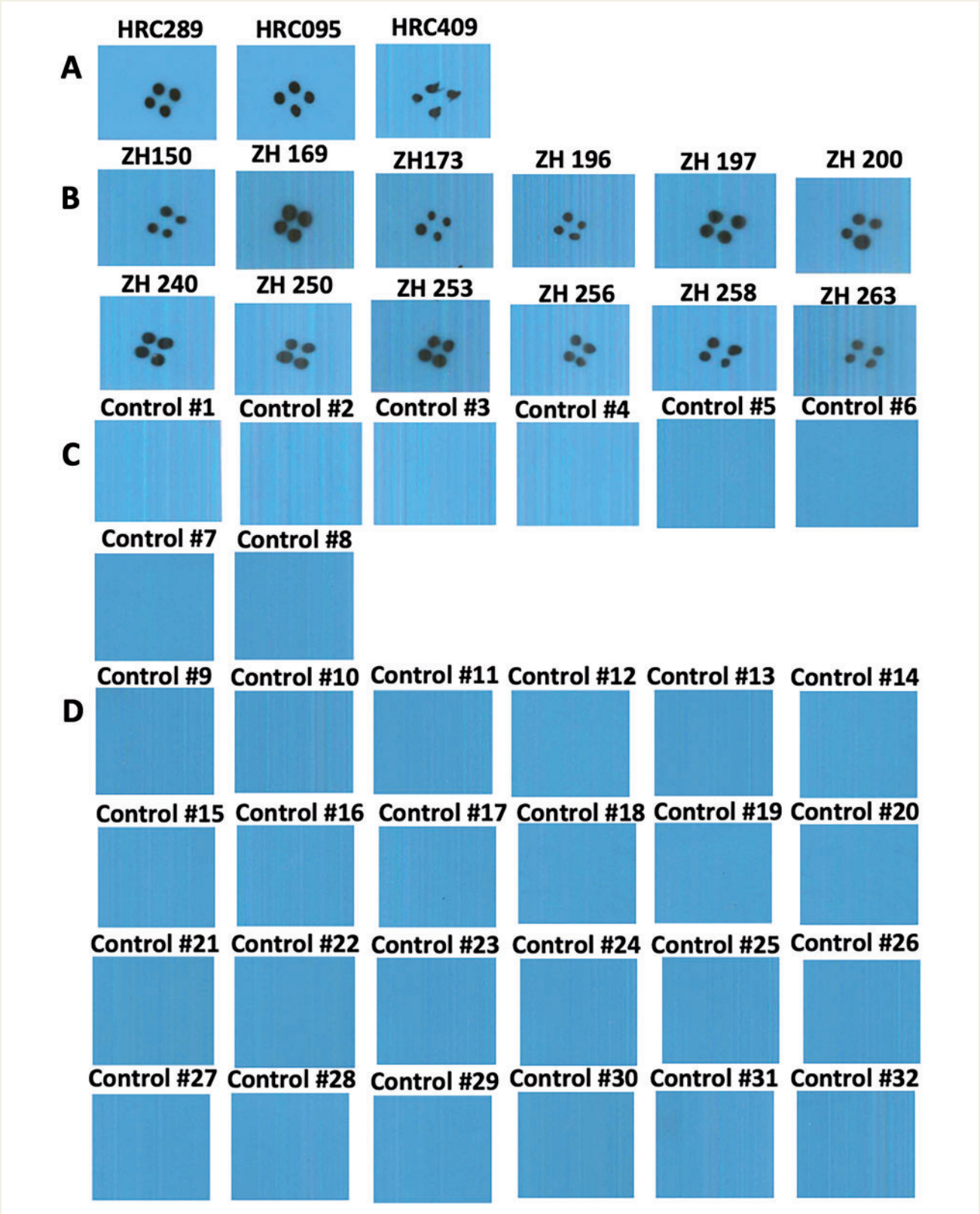


Figure 2 Two-dimensional blots from (*A*) (The Hospital for Sick Children) discovery cohort; (*B*) (Zurich) validation cohort demonstrate consistent and specific antibodies to four myocardial proteins in BrS subjects (ShS $\geq$ 3.5), whereas these are not present in (*C*) (commercial source) discovery control subjects or (D) (second commercial source) validation control subjects.

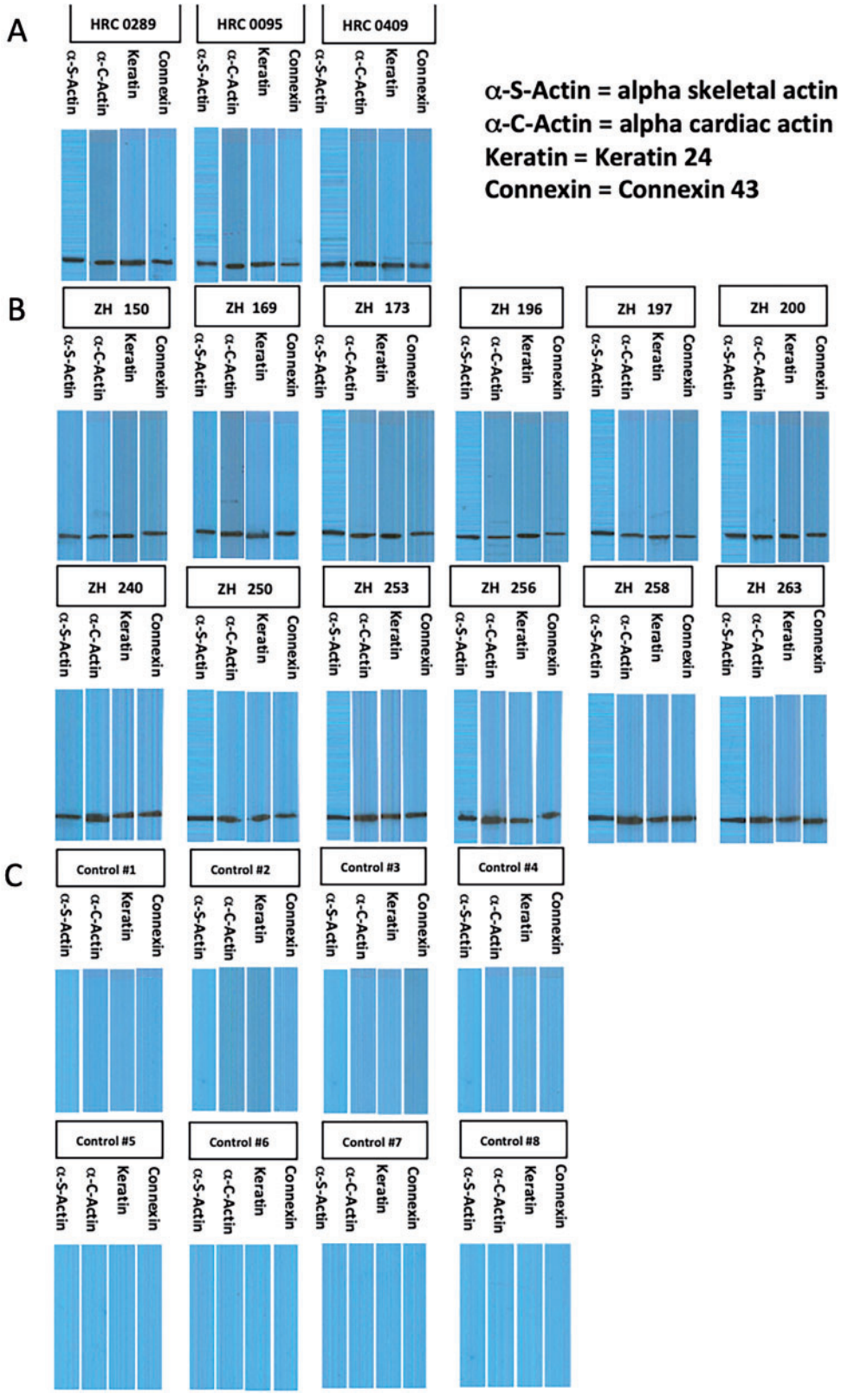


Figure 3 Confirmatory Western blots from (*A*) (The Hospital for Sick Children) discovery cohort, and (*B*) (Zurich) validation cohort demonstrate consistent and specific antibodies to α-cardiac actin, keratin, and connexin-43 in BrS subjects (ShS ≥ 3.5), but not in (*C*) eight control subjects.

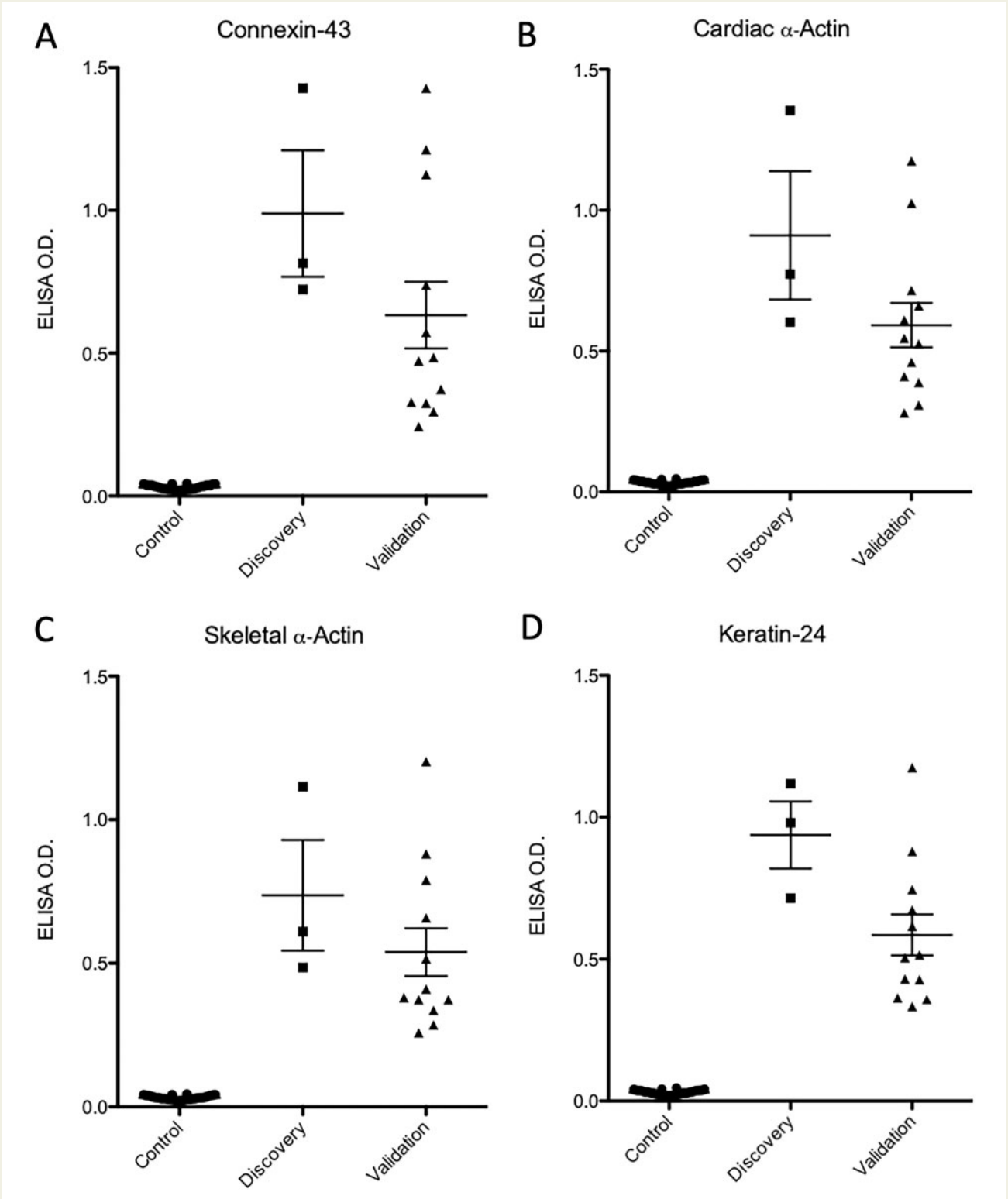


Figure 4 Enzyme-linked immuno-sorbent assays (ELISA) demonstrate antibodies to (*A*) connexin-43, (*B*) cardiac α-actin, (*C*) skeletal α-actin, and (*D*) keratin-24 and in discovery and validation cohorts vs. controls.

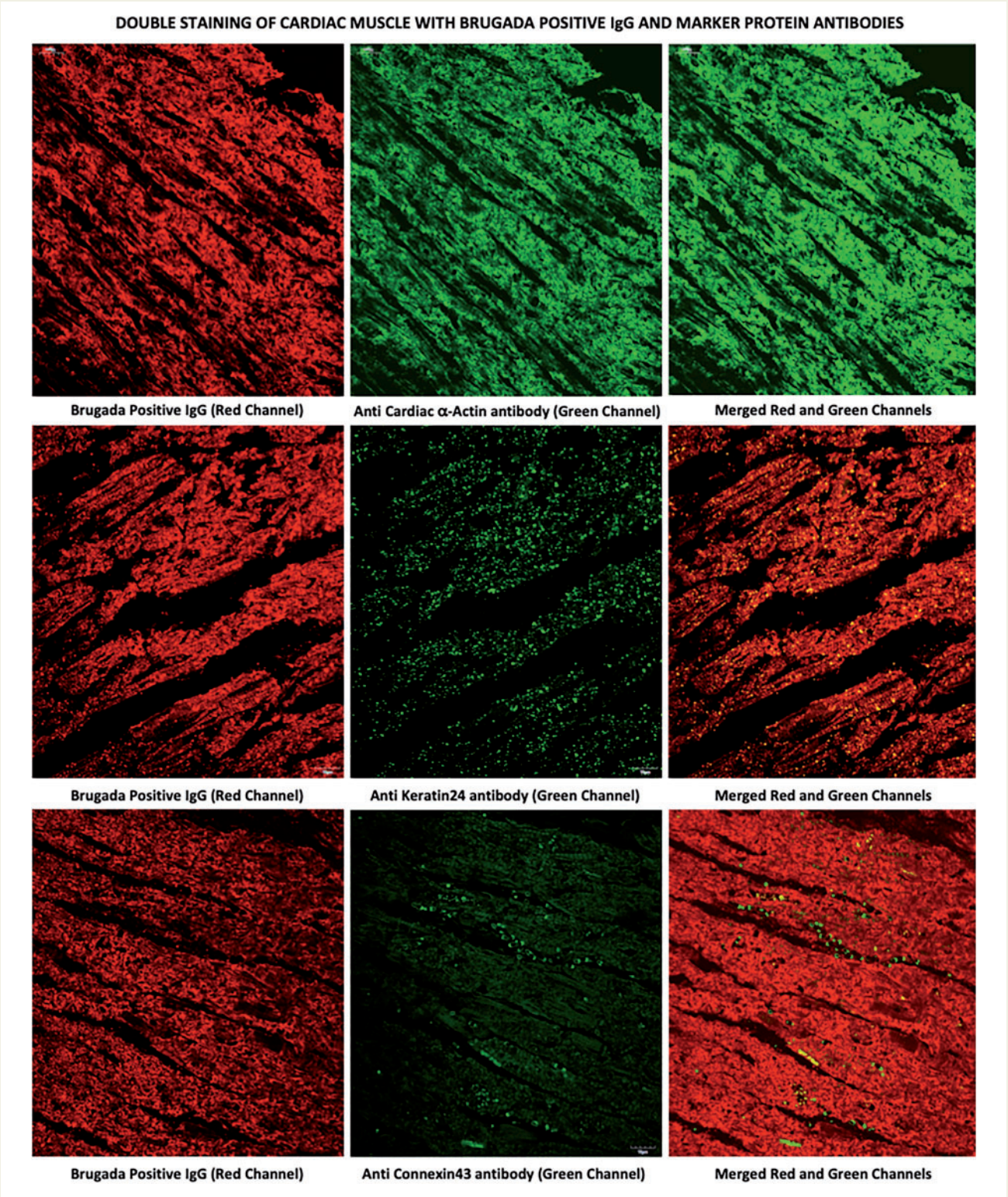


Figure 5 Staining of normal tissue with serum from a patient with BrS (red in left panels), commercial antibodies to α-actin, keratin-24, and connexin-43 (green in top, mid, and bottom centre panels, respectively), and merged (yellow) overlap of BrS serum and commercial antibody staining (right panels).

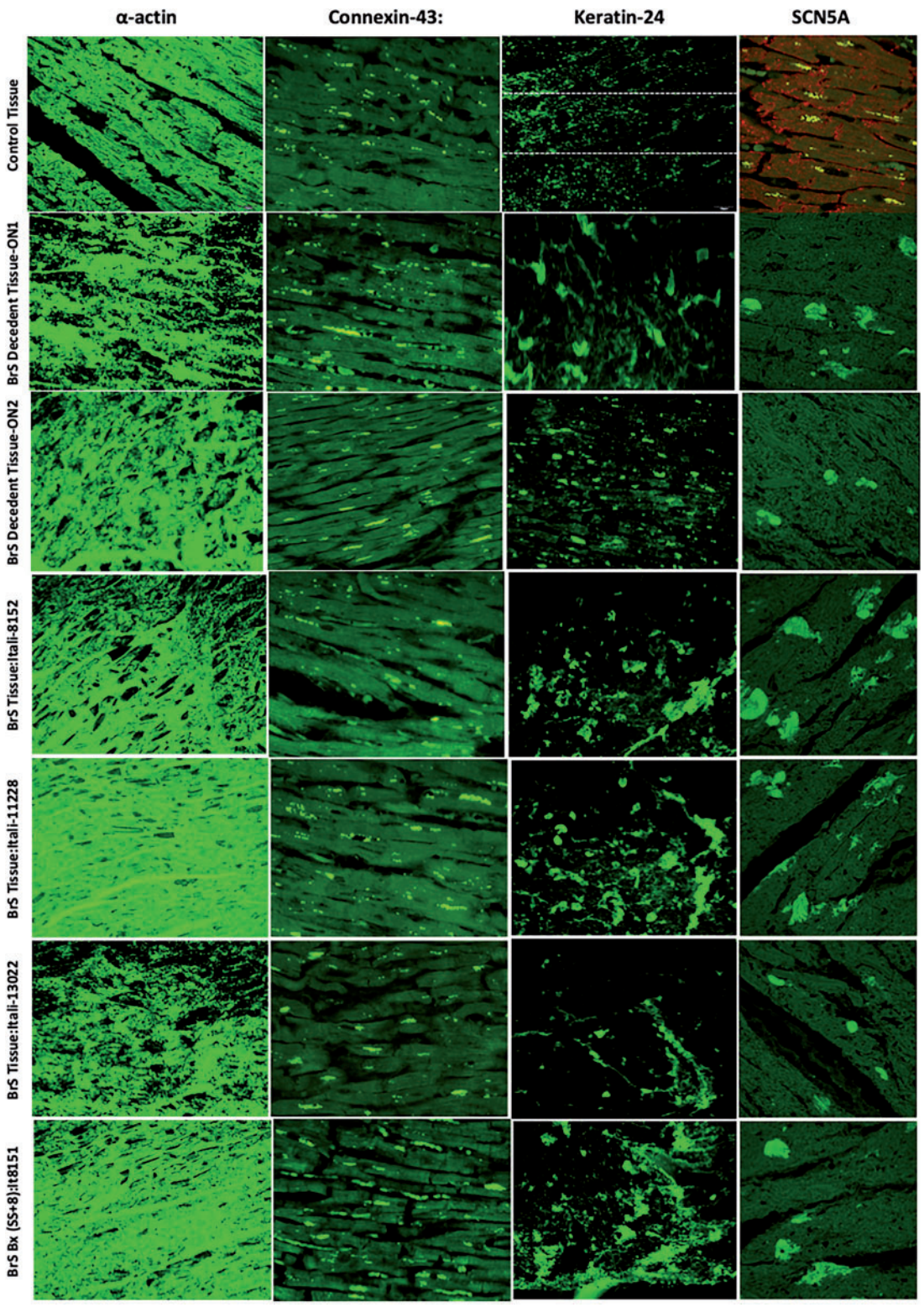
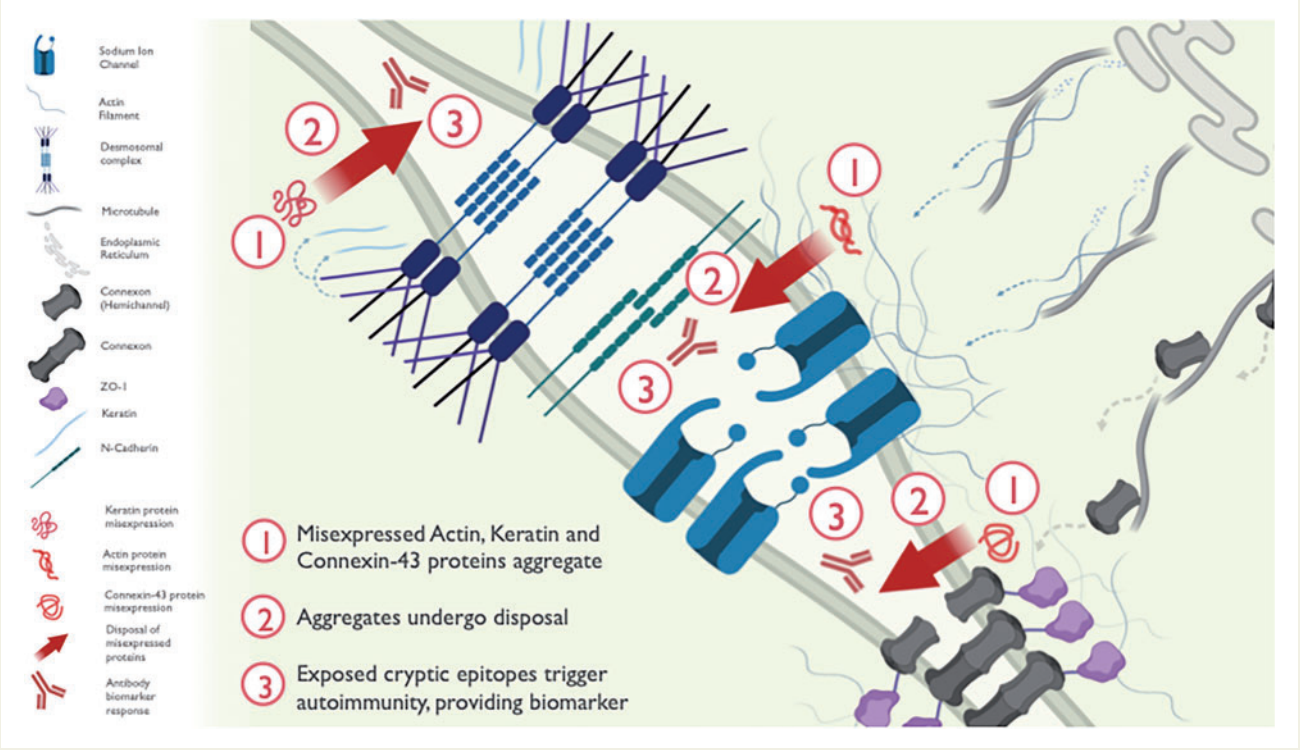


Figure 6 Expression of α-cardiac actin, keratin-24, connexin-43, and SCN5A in control tissue (top row) vs. BrS tissue from a post-mortem case (PM Case A and B) and endomyocardial biopsies from BrS subjects (remaining rows). Biopsies from four BrS subjects are shown here, with an additional five shown in Supplementary material online, *Figure S5*. Staining of normal cardiac tissue demonstrates a fine reticular patter of actin staining, and fine speckled patterns of staining for connexin-43, keratin-24, and SCN5A. In contrast, cardiac tissues from BrS patients demonstrate aggregates of actin, connexin-43, keratin-24, and SCN5A staining.



Take home figure (1) As part of the pathophysiology of Brugada syndrome, actin, keratin, and connexin-43 proteins are misexpressed and form aggregates within cardiomyocytes. (2) These protein aggregates undergo cell disposal. (3) As a result, cryptic epitopes of these proteins are exposed to the immune system, resulting in the generation of specific autoantibodies, creating a biomarker profile for the disease.

mechanisms that result in extracellular exposure of regions not being recognized as 'self' (cryptic epitopes), resulting in the autoimmune response.

Brugada syndrome is being recognized increasingly as a disorder of the right ventricular outflow tract epicardium where (in animal studies) connexin-43 is less abundant.[37,38] In human post-mortem SCD hearts with familial BrS (matched to homograft control hearts), connexin-43 signal is reduced, regardless of mutation status.[25] We identified anti-connexin-43 antibodies in the sera of all BrS participants (regardless of genetic cause), and abnormal connexin-43 protein expression in BrS myocardium, with intracellular clustering as compared to normal control myocardial tissue. Whether anti-connexin-43 antibodies contribute to the pathophysiology of BrS disease or are just a marker upon exposure of these proteins during the disease course remains unknown.

Our results are promising for the development of a highly sensitive and specific serological biomarker test for BrS. The identification of autoimmunity in BrS may pave the way for novel therapeutic avenues, such as immunosuppression.

Limitations

This initial study assessed only patients with probable or definite BrS based on 2015 consensus conference criteria (ShS $\geq$ 3.5). We did not design our study to assess whether our biomarker identifies other loss of function *SCN5A* phenotypes. Additional studies, such as assessing gene-positive relatives, subjects converting with provocative testing, and/or prospective studies of relatives will be required to determine if this biomarker profile will be predictive for disease.

Conclusion

Our discovery of autoantibodies against α-cardiac actin, α-skeletal muscle actin, keratin-24, and connexin-43 will enable the development of a new serological biomarker test for BrS. The assay is negative in unaffected and control individuals and also negative in three forms of cardiomyopathy, but further assessment is required among other myocardial diseases to determine its exact or exclusive specificity to BrS. Further research is required to elucidate the pathophysiology of this autoimmune response, its sensitivity in predicting disease risk, and its utility in directing or monitoring new therapies.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Acknowledgements

The authors are grateful to Stephen W. Scherer and Ronald D. Cohn for manuscript review, and to the many patients who participated as subjects for this study.

Funding

This study was funded by a Waugh Family Innovation grant from the Labatt Family Heart Centre (2019–2021), a Freeman Innovation Award from the Heart and Stroke Richard Lewar Centre of Excellence (2019), the Caitlyn Elizabeth Morris Memorial Foundation, the Alex Corrance Memorial Foundation, and Meredith Cartwright to R.M.H. M.F. is supported by a Carter Heart Rhythm Fellowship. A patent application covering this work has been submitted. The Zurich ARVC Program is supported by grants from the Georg und Bertha Schwyzer-Winiker Foundation, the Baugarten Foundation, the Swiss National Science Foundation, and the Swiss Heart Foundation. M.P was funded by a 3-year Telethon grant (GGP10186). J.M.B. and M.J.A. were supported by the Mayo Clinic Windland Smith Rice Comprehensive Sudden Cardiac Death Program.

Conflict of interest: none declared.

References

1. Brugada P, Brugada J. Right bundle branch block, persistent ST segment elevation and sudden cardiac death: a distinct clinical and electrocardiographic syndrome. A multicenter report. *J Am Coll Cardiol* 1992;**20**:1391–1396.
2. Vutthikraivit W, Rattanawong P, Putthapiban P, Sukhumthammarat W, Vathesatogkit P, Ngarmukos T, Thakkinstian A. Worldwide prevalence of Brugada syndrome: a systematic review and meta-analysis. *Acta Cardiol Sin* 2018;**34**:267–277.
3. Tohyou Y, Nakazawa K, Ozawa A, Tanaka O, Watanuki M, Takagi A, Akagi T, Masui Y, Matumoto N, Miyake F, Murayama M. A survey in the incidence of right bundle branch block with ST segment elevation among normal population. *Jpn J Electrocardiol* 1995;**15**:223–226.
4. Antzelevitch C, Brugada P, Brugada J, Brugada R, Shimizu W, Gussak I, Perez Riera AR. Brugada syndrome: a decade of progress. *Circ Res* 2002;**91**:1114–1118.
5. Brugada R, Campuzano O, Sarquella-Brugada G, Brugada P, Brugada J, Hong K. Brugada syndrome. In: MP Adam, HH Ardinger, RA Pagon, SE Wallace, LJH Bean, K Stephens, A Amemiya, eds. *GeneReviews®*. University of Washington: Seattle, WA; 1993; 1–28.
6. Chen Q, Kirsch GE, Zhang D, Brugada R, Brugada J, Brugada P, Potenza D, Moya A, Borggrefe M, Breithardt G, Ortiz-Lopez R, Wang Z, Antzelevitch C, O'Brien RE, Schulze-Bahr E, Keating MT, Towbin JA, Wang Q. Genetic basis and molecular mechanism for idiopathic ventricular fibrillation. *Nature* 1998;**392**:293–296.
7. Yamagata K, Horie M, Aiba T, Ogawa S, Aizawa Y, Ohe T, Yamagishi M, Makita N, Sakurada H, Tanaka T, Shimizu A, Hagiwara N, Kishi R, Nakano Y, Takagi M, Makiyama T, Ohno S, Fukuda K, Watanabe H, Morita H, Hayashi K, Kusano K, Kamakura S, Yasuda S, Ogawa H, Miyamoto Y, Kapplinger JD, Ackerman MJ, Shimizu W. Genotype-phenotype correlation of SCN5A mutation for the clinical and electrocardiographic characteristics of probands with brugada syndrome: a Japanese Multicenter Registry. *Circulation* 2017;**135**:2255–2270.
8. Kapplinger JD, Wilde AAM, Antzelevitch C, Benito B, Berthet M, Brugada J, Brugada P, Fressart V, Guerchicoff A, Guicheney P, Kamakura S, Koopmann TT, Miyamoto M, Pollevick GD, Pfeiffe rR, Probst V, Salisbury BA, Schulze-Bahr E, Shimizu W, Towbin JA, Vatta M, Zumhagen S, Schott JJ, Brugada R, Ackerman MJ. A worldwide compendium of putative Brugada syndrome associated mutations in the SCN5A encoded cardiac sodium channel. *Heart Rhythm* 2009;**5S(6)**: S392–S393.
9. Crotti L, Marcou CA, Tester DJ, Castelletti S, Giudicessi JR, Torchio M, Medeiros-Domingo A, Simone S, Will ML, Dagradi F, Schwartz PJ, Ackerman MJ. Spectrum and prevalence of mutations involving BrS1- through BrS12-susceptibility genes in a cohort of unrelated patients referred for Brugada syndrome genetic testing: implications for genetic testing. *J Am Coll Cardiol* 2012;**60**: 1410–1418.
10. Kapa S, Tester DJ, Salisbury BA, Harris-Kerr C, Pungliya MS, Alders M, Wilde AA, Ackerman MJ. Genetic testing for long-QT syndrome: distinguishing pathogenic mutations from benign variants. *Circulation* 2009;**120**:1752–1760.
11. Ackerman MJ, Splawski I, Makielski JC, Tester DJ, Will ML, Timothy KW, Keating MT, Jones G, Chadha M, Burrow CR, Stephens JC, Xu C, Judson R, Curran ME. Spectrum and prevalence of cardiac sodium channel variants among Black, White, Asian, and Hispanic individuals: implications for arrhythmogenic susceptibility and Brugada/long QT syndrome genetic testing. *Heart Rhythm* 2004;**1**: 600–607.
12. Hosseini SM, Kim R, Udupa S, Costain G, Jobling R, Liston E, Jamal SM, Szybowska M, Morel CF, Bowdin S, Garcia J, Care M, Sturm AC, Novelli V, Ackerman MJ, Ware JS, Hershberger RE, Wilde AAM, Gollob MH; National Institutes of Health Clinical Genome Resource Consortium. Reappraisal of reported genes for sudden arrhythmic death: an evidence-based evaluation of gene validity for Brugada syndrome. *Circulation* 2018;**138**:1195–1205.
13. Cerrone M. Controversies in Brugada syndrome. *Trends Cardiovasc Med* 2018;**28**: 284–292.
14. Veltmann C, Schimpf R, Echternach C, Eckardt L, Kuschyk J, Streitner F, Spehl S, Borggrefe M, Wolpert C. A prospective study on spontaneous fluctuations between diagnostic and non-diagnostic ECGs in Brugada syndrome: implications for correct phenotyping and risk stratification. *Eur Heart J* 2006;**27**:2544–2552.
15. Shimizu W, Matsuo K, Takagi M, Tanabe Y, Aiba T, Taguchi A, Suyama K, Kurita T, Aihara N, Kamakura S. Body surface distribution and response to drugs of ST segment elevation in Brugada syndrome: clinical implication of eighty-seven-lead body surface potential mapping and its application to twelve-lead electrocardiograms. *J Cardiovasc Electrophysiol* 2000;**11**:396–404.
16. Govindan M, Batchvarov VN, Raju H, Shanmugam N, Bizrah M, Bastiaenen R, Kiotsekoglou A, Camm J, Behr ER. Utility of high and standard right precordial leads during ajmaline testing for the diagnosis of Brugada syndrome. *Heart* 2010; **96**:1904–1908.
17. Rivero-Ayerza M, Brugada R, Brugada J, Geelen P, Brugada P. Electrocardiogram of Brugada syndrome and its dynamic patterns. In: M Malik, AJ Camm, eds. *Dynamic Electrocardiography*. NY: Futura/Blackwell Publishing; 2004, pp. 417–424.
18. Wilde AA, Antzelevitch C, Borggrefe M, Brugada J, Brugada R, Brugada P, Corrado D, Hauer RN, Kass RS, Nademanee K, Priori SG, Towbin JA; Study Group on the Molecular Basis of Arrhythmias of the European Society of Cardiology. Proposed diagnostic criteria for the Brugada syndrome. *Eur Heart J* 2002;**23**:1648–1654.
19. Antzelevitch C, Brugada P, Borggrefe M, Brugada J, Brugada R, Corrado D, Gussak I, LeMarec H, Nademanee K, Perez Riera AR, Shimizu W, Schulze-Bahr E, Tan H, Wilde A. Brugada syndrome: report of the second consensus conference. *Heart Rhythm* 2005;**2**:429–440.
20. Antzelevitch C, Yan GX, Ackerman MJ, Borggrefe M, Corrado D, Guo J, Gussak I, Hasdemir C, Horie M, Huikuri H, Ma C, Morita H, Nam GB, Sacher F, Shimizu W, Viskin S, Wilde AA. J-Wave syndromes expert consensus conference report: emerging concepts and gaps in knowledge. *Heart Rhythm* 2016;**13**:e295–e324.
21. Wilde AA, Postema PG, Di Diego JM, Viskin S, Morita H, Fish JM, Antzelevitch C. The pathophysiological mechanism underlying Brugada syndrome: depolarization versus repolarization. *J Mol Cell Cardiol* 2010;**49**:543–553.
22. Corrado D, Nava A, Buja G, Martini B, Fasoli G, Oselladore L, Turrini P, Thiene G. Familial cardiomyopathy underlies syndrome of right bundle branch block, ST segment elevation and sudden death. *J Am Coll Cardiol* 1996;**27**:443–448.
23. Saguner AM, Ganahl S, Kraus A, Baldinger SH, Akdis D, Saguner AR, Wolber T, Haegeli LM, Steffel J, Krasniqi N, Luscher TF, Tanner FC, Brunckhorst C, Duru F. Electrocardiographic features of disease progression in arrhythmogenic right ventricular cardiomyopathy/dysplasia. *BMC Cardiovasc Disord* 2015;**15**:4.
24. Papavassiliu T, Veltmann C, Doesch C, Haghi D, Germans T, Schoenberg SO, van Rossum AC, Schimpf R, Brade J, Wolpert C, Borggrefe M. Spontaneous type 1 electrocardiographic pattern is associated with cardiovascular magnetic resonance imaging changes in Brugada syndrome. *Heart Rhythm* 2010;**7**:1790–1796.
25. Nademanee K, Raju H, de Noronha SV, Papadakis M, Robinson L, Rothery S, Makita N, Kowase S, Boonmee N, Vitayakritsirikul V, Ratanarapee S, Sharma S, van der Wal AC, Christiansen M, Tan HL, Wilde AA, Nogami A, Sheppard MN, Veerakul G, Behr ER. Fibrosis, connexin-43, and conduction abnormalities in the Brugada syndrome. *J Am Coll Cardiol* 2015;**66**:1976–1986.
26. Pieroni M, Notarstefano P, Oliva A, Campuzano O, Santangeli P, Coll M, Nesti M, Carnevali A, Fraticelli A, Iglesias A, Grassi S, Brugada R, Bolognese L. Electroanatomic and pathologic right ventricular outflow tract abnormalities in patients with Brugada syndrome. *J Am Coll Cardiol* 2018;**72**:2747–2757.
27. Chatterjee D, Fatah M, Akdis D, Spears DA, Koopmann TT, Mittal K, Rafiq MA, Cattanach BM, Zhao Q, Healey JS, Ackerman MJ, Bos JM, Sun Y, Maynes JT, Brunckhorst C, Medeiros-Domingo A, Duru F, Saguner AM, Hamilton RM. An autoantibody identifies arrhythmogenic right ventricular cardiomyopathy and participates in its pathogenesis. *Eur Heart J* 2018;**39**:3932–3944.
28. Agullo-Pascual E, Cerrone M, Delmar M. Arrhythmogenic cardiomyopathy and Brugada syndrome: diseases of the connexome. *FEBS Lett* 2014;**588**:1322–1330.
29. Vermij SH, Abriel H, van Veen TA. Refining the molecular organization of the cardiac intercalated disc. *Cardiovasc Res* 2017;**113**:259–275.
30. Steele DF, Fedida D. Cytoskeletal roles in cardiac ion channel expression. *Biochim Biophys Acta* 2014;**1838**:665–673.
31. Papathanasiou S, Rickelt S, Soriano ME, Schips TG, Maier HJ, Davos CH, Varela A, Kaklamanis L, Mann DL, Capetanaki Y. Tumor necrosis factor-alpha confers

cardioprotection through ectopic expression of keratins K8 and K18. *Nat Med* 2015;**21**:1076–1084.
32. Tsikitis M, Galata Z, Mavroidis M, Psarras S, Capetanaki Y. Intermediate filaments in cardiomyopathy. *Biophys Rev* 2018;**10**:1007–1031.
33. Basheer WA, Xiao S, Epifantseva I, Fu Y, Kleber AG, Hong T, Shaw RM. GJA1-20k arranges actin to guide Cx43 delivery to cardiac intercalated discs. *Circ Res* 2017;**121**:1069–1080.
34. Epifantseva I, Shaw RM. Intracellular trafficking pathways of Cx43 gap junction channels. *Biochim Biophys Acta Biomembr* 2018;**1860**:40–47.
35. Ishikawa T, Takahashi N, Ohno S, Sakurada H, Nakamura K, On YK, Park JE, Makiyama T, Horie M, Arimura T, Makita N, Kimura A. Novel SCN3B mutation associated with brugada syndrome affects intracellular trafficking and function of Nav1.5. *Circ J* 2013;**77**:959–967.
36. Belbachir N, Portero V, Sayed A, Gourraud ZR, Dilasser JB, Jesel F, Guo L, Wu H, Gaborit H, Guilluy N, Girardeau C, Bonnaud A, Simonet S, Karakachoff F, Pattier M, Scott S, Burel C, Marionneau S, Chariau C, Gaignerie C, David A, Genin L, Deleuze Jf E, Dina C, Sauzeau V, Loirand G, Baro I, Schott JJ, Probst V, Wu JC, Redon R, Charpentier F, Le Scouarnec S. RRAD mutation causes electrical and cytoskeletal defects in cardiomyocytes derived from a familial case of Brugada syndrome. *Eur Heart J* 2019;**40**:3081–3094.
37. Poelzing S, Akar FG, Baron E, Rosenbaum DS. Heterogeneous connexin43 expression produces electrophysiological heterogeneities across ventricular wall. *Am J Physiol Heart Circ Physiol* 2004;**286**:H2001–H2009.
38. Ou B, Nakagawa M, Kajimoto M, Nobe S, Ooie T, Ichinose M, Yonemochi H, Ono N, Shimada T, Saikawa T. Heterogeneous expression of connexin 43 in the myocardium of rabbit right ventricular outflow tract. *Life Sci* 2005;**77**:52–59.