Contribution of Cardiac Sodium Channel β-Subunit Variants to Brugada Syndrome

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Background: Brugada syndrome (BrS) is an inheritable cardiac disease associated with syncope, malignant ventricular arrhythmias and sudden cardiac death. The largest proportion of mutations in BrS is found in the SCN5A gene encoding the α-subunit of cardiac sodium channels (Nav1.5). Causal SCN5A mutations are present in 18–30% of BrS patients. The additional genetic diagnostic yield of variants in cardiac sodium channel β-subunits in BrS patients was explored and functional studies on 3 novel candidate variants were performed.

Methods and Results: The SCN1B–SCN4B genes were screened, which encode the 5 sodium channel β-subunits, in a SCN5A negative BrS population (n=74). Five novel variants were detected; in silico pathogenicity prediction classified 4 variants as possibly disease causing. Three variants were selected for functional study. These variants caused only limited alterations of Nav1.5 function. Next generation sequencing of a panel of 88 arrhythmia genes could not identify other major causal mutations.

Conclusions: It was hypothesized that the studied variants are not the primary cause of BrS in these patients. However, because small functional effects of these β-subunit variants can be discriminated, they might contribute to the BrS phenotype and be considered a risk factor. The existence of these risk factors can give an explanation to the reduced penetrance and variable expressivity seen in this syndrome. We therefore recommend including the SCN1-4B genes in a next generation sequencing-based gene panel. (Circ J 2015; 79: 2118–2129)

Key Words: Brugada syndrome; Functional studies; Patch clamp; SCN1-4B

Brugada syndrome (BrS) is associated with syncope, malignant ventricular arrhythmias and sudden cardiac death (SCD). BrS is diagnosed based on a ST-segment elevation with a type 1 (or coved type) morphology on the electrocardiogram (ECG). This ST-segment elevation can occur spontaneously or can be evoked during a class I antiarrhythmic drug (AAD) test. BrS has a low prevalence of 1-5 per 10,000 in Europe and the USA, and an increased prevalence of 1-2.5 per 1,000 in South-East Asia. BrS is a genetic disease characterized by a dominant inheritance, reduced penetrance and variable expressivity. The predominant gene is SCN5A encoding Nav1.5, the pore-forming α-subunit of the cardiac sodium channels. Loss-of-function mutations in SCN5A can be found in 18–30% of the BrS patients. To date, 19 additional genes have been associated with BrS. These genes are considered to be minor BrS genes because mutations in them are rare.

The cardiac sodium channel consists of the Nav1.5 and associated regulatory proteins. Among these are the β-subunits, a family of 5 proteins: β1, β1b, β2, β3 and β4. These β-subunits interact directly with the Nav1.5 protein and are necessary for its proper function and expression. The β-subunits are encoded...
by 4 genes: SCN1B (encoding 2 isoforms: β1 and β1b), SCN2B, SCN3B and SCN4B.

All β-subunits have been associated with BrS except for β4, which has been associated with long QT syndrome (LQTS). 12-16 In this perspective, we consider these β-subunits suitable candidate genes for genetic testing in BrS patients.

In this study, we want to evaluate the genetic diagnostic yield of SCN1-4B genes by screening a SCN5A mutation-negative BrS population of 74 patients. We found 5 novel β-subunit variants, of which 4 were potentially disease causing. Functional studies were performed on 3 selected variants.

**Methods**

**Population**

This study was performed in accordance with the Declaration of Helsinki and approved by the Ethical committee of the UZ Brussels. Written informed consent was obtained from all patients.

We studied 74 unrelated BrS patients without SCN5A mutation(s) at the outpatient clinic of the UZ Brussels. Clinical diagnosis of BrS was made based on the appearance of a ST-segment elevation of ≥2 mm followed by a negative T-wave in at least 1 precordial lead (V1, V2) positioned in the 2nd, 3rd or 4th intercostal space.1-3 Blood samples for genetic analysis were collected between December 2007 and December 2011. Detailed clinical data were obtained, including symptoms, cardioverter defibrillator (ICD) implantation, standard 12-lead ECG and other cardiac rhythm registrations, results of a class I AAD provocation test, inducibility of sustained ventricular tachycardia/fibrillation (VT/VF) during electrophysiology study (EPS), and family history. ICD implantations at our center were performed conform to the international recommendations at the time of the implant.1,17-20

**Genetic Screening**

Total genomic DNA was isolated from whole blood samples by using standard techniques (Chemagen; PerkinElmer, Zaventem, Belgium).

After excluding mutations in SCN5A all exons, including intron-exon boundaries, of SCN1B, SCN2B, SCN3B and SCN4B were sequenced in both directions. In the case of SCN1B, we included the extended third exon to cover variants in β1b. Primers, PCR conditions and Sanger sequencing are described in Supplementary File 1-1.

Results were compared to the reference sequence (NM_001037 for β1, NM_199037 for β1b, NM_004588 for β2, NM_018400 for β3 and NM_174934 for β4) using SeqPilot (version 4.0.1 build 502; JSI Medical systems GmbH, Kippenheim, Germany) and Variant Reporter v1.1 (Life Technologies).

Human Genome Variant Society (HGVS) nomenclature was used (Alamut version 2.3 rev. 1; Interactive Biosoftware, Rouen, France).

Novel variants were submitted to the Leiden Open Variation Database (LOVD, http://www.LOVD.nl)

**In Silico Prediction of the Variants**

Variants were compared to online databases: 1,000 genomes, dbSNP, HGMD, LOVD, Cardiac arrhythmia database and literature (consulted October 2014). Minor allele frequency (MAF) was obtained from 1,000 genomes (1,000 genomes browser released October 2013; Ensembl v73, consulted June 2014). Variants were analyzed with different prediction tools: (1) Functional effects: GVGD classes (version October 2013), SIFT (version May 2009, consulted in June 2014) and Mutation Taster (built NCBI37/Ensembldb); (2) Conservation of amino acids: orthologue alignments (hg19, through Ensembl Compara database); and (3) Splice predictions: SpliceSiteFinder-like, MaxEntScan, GeneSplicer, Known constitutive signals, ESEFinder, RESCUE-ESE.

All prediction tools were consulted via Alamut (performed August 2014).

Variants were classified according to the system suggested by Hofman et al.21 This classification is based on the results of various prediction tools and categorizes the variants according to these results into 5 classes: (1) not pathogenic; (2) unlikely pathogenic (VUS1; variant of unknown significance 1); (3) unknown pathogenicity (VUS2); (4) likely pathogenic (VUS 3); and (5) pathogenic.

For novel variants in untranslated regions (UTRs), sequence comparison between different species was performed using Clustal Omega v2.122 (consulted in March 2014).

To predict putative microRNA binding, microRNA.org (August 2010 release, via Alamut, consulted in August 2014) and TargetScanHuman 6.2 prediction of microRNA targets23 (consulted in March 2014), were used.

**Vectors and Site-Directed Mutagenesis**

The β1b-WT vector is a pcDNA3.1 vector containing green fluorescent protein (GFP)19 and the wild-type SCN1Bβ sequence.24 The β1b-del-ext vector was created based on the β1b-WT vector; genomic DNA of a control sample was used as a template to amplify the extended 3’ terminal region, and the deletion was introduced using site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit; Agilent Technologies, Santa Clara, CA, USA).

A β3-WT-containing, pReceiver-M61 vector with GFP (GeneCopoeia Inc, Rockville, MD, USA) was used as a template to create the mutated β3-Gln101Iys (site-directed mutagenesis).

The SCN4Bprom-MT vector was constructed using the Gluc-ON™Promoter Reporter Clone containing the SCN4B promoter region (c.−1333 to c.−1; GeneCopoeia Inc) using site-directed mutagenesis.

A detailed description of plasmid construction can be found in Supplementary File 1-1.

The pZOOM-Kv4.3 vector was a kind gift from Prof. Kristine Callo (University of Copenhagen, Denmark).

The pcDNA3.1-Nav1.5 vector was described previously.13,24

**Cells and Transfection**

Human embryonic kidney cells (HEK cells, tsa201) and H9c2 cells (rat cardiomyocyte-like cells) were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum, 1% antibiotics (penicillin/streptavidin) and 1% Glutamax (all from Invitrogen, Carlsbad, CA, USA) at 37°C and 5% CO2.

HEK cells were used for patch clamp experiments. Cells were transfected using GeneCellin transfection reagent (BioCellChallenge, Toulon, France) in experiments with Nav1.5 or Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) in experiments with Kv4.3. The following molar ratios were used: Nav1.5/β1b-WT or β1b-del-ext 1:2.3; Kv4.3/β1b-WT or β1b-del-ext 1:2.3, and Nav1.5/β3-WT or β3-Gln101Iys 1:4; to ensure saturation of the α-subunit with β-subunits.24 A maximum of 2 µg DNA per transfection was used.

For promoter studies, HEK cells transfected with polyethyleneimine (Polysciences, Eppelheim, Germany; 1 µg or 3 µg of DNA) or H9c2 cells transfected with Lipofectamine 2000 (0.5 µg of DNA) were used.
**Table 1. Patient Characteristics**

| Characteristic | Variables of the SCN5A-negative BrS study population | All SCN1–4B variants (n=74) | SCN1B variants (n=47) | SCN2B variants (n=51) | SCN3B variants (n=67) | SCN4B variants (n=7) |
|----------------|-----------------------------------------------------|-----------------------------|----------------------|----------------------|----------------------|---------------------|
| Mean age (years) | 47.69±14.56 | 46.87±12.49 | 46.65±14.37 | 47.72±14.74 | 47.43±18.47 |
| Age range (years) | [10–81] | [21–75] | [10–81] | [10–81] | [21–81] |
| Ethnic group, n (%) | | | | | | |
| Caucasian | 65 (88) | 39 (83) | 43 (84) | 65 (97) | 6 (86) |
| African | 3 (4) | 3 (6) | 3 (6) | 3 (4) | 1 (14) |
| Asian | 2 (3) | 2 (4) | 2 (4) | 2 (3) | 0 (0) |
| Unknown | 4 (5) | 3 (6) | 3 (6) | 3 (4) | 0 (0) |
| ECG features, n (%) | | | | | | |
| Spont. type 1 ECG | 13 (18) | 11 (23) | 10 (20) | 12 (18) | 0 (0) |
| Dynamic ECG | 24 (32) | 17 (36) | 20 (39) | 21 (31) | 2 (29) |
| Pos. Prov. Test | 72 (97) | 46 (98) | 49 (96) | 65 (97) | 7 (100) |
| Clinical presentation, n (%) | | | | | | |
| Syncope | 37 (50) | 22 (47) | 25 (49) | 32 (48) | 2 (29) |
| Spont. AF | 11 (15) | 7 (15) | 9 (18) | 11 (16) | 0 (0) |
| Spont. VF | 7 (9) | 4 (8) | 7 (14) | 7 (10) | 0 (0) |
| Spont. VT | 6 (8) | 4 (8) | 4 (8) | 5 (7) | 0 (0) |
| ACA | 4 (5) | 3 (6) | 4 (8) | 4 (6) | 0 (0) |
| EPS performed | 62 (84) | 41 (87) | 42 (82) | 56 (84) | 6 (86) |
| EPS positive | 12 (16) | 8 (17) | 6 (12) | 10 (15) | 1 (14) |
| ICD | 44 (59) | 28 (60) | 28 (55) | 38 (57) | 2 (29) |
| Family history, n (%) | | | | | | |
| BrS | 23 (31) | 15 (32) | 16 (31) | 20 (30) | 3 (43) |
| SCD | 39 (53) | 23 (49) | 29 (57) | 36 (54) | 5 (71) |
| Syncope, VT/VF, ACA | 18 (24) | 7 (15) | 12 (24) | 16 (24) | 1 (14) |

Age is indicated as mean±SD. Values in parentheses (except for age range) are percentages. ACA, aborted cardiac arrest; AF, atrial fibrillation; BrS, Brugada syndrome; ECG, electrocardiogram; EPS, electrophysiological study; ICD, implantable cardioverter defibrillator; pos. prov. test, a positive class I AAD provocative test; SCD, sudden cardiac death; spont, spontaneous; VF, sustained ventricular fibrillation; VT, sustained ventricular tachycardia.

**Electrophysiological Studies**

Experiments were performed at room temperature, 48 h after transfection using standard whole-cell patch clamp techniques. An Axopatch 200B amplifier and Digidata 1440A acquisition system (Molecular Devices, Sunnyvale, CA, USA) were used to obtain the data.

Data were filtered at 5 kHz and sampled at 5–20 kHz; 80–90% series resistance compensation was used. Recording voltages were not corrected for junction potential.

Solutions and patch clamp protocols are described in **Supplementary File 1-1**.

**Luciferase Assays**

The medium of transfected HEK or H9c2 cells was harvested 48 or 72 h after transfection and frozen at −20°C until the luciferase assay was performed.

The luciferase assay was performed according to the manufacturer’s instructions (Secrete-Pair™ Dual Luminescence and Gaussia Luciferase Assay Kits; GeneCopoeia Inc) using a GloMax 96 Microplate Luminometer (Promega, Madison, WI, USA) or a Spectramax M3 (Molecular Devices).

**Next Generation Sequencing (NGS)**

Library preparation was performed by using the KAPA Low-Throughput Library preparations kit (KAPA Biosystems Inc, Wilmington, MA, USA) or NebNext Ultra DNA Library prep Kit for Illumina (New England Biolabs Inc). Target enrichment was performed by using the SeqCap EZ Human Exome v3.0 kit (Roche NimbleGen Inc, Madison, WI, USA). Sequencing (paired-end 2×200 base pairs) was performed on an Illumina HiSeq 1500 (Illumina Inc) sequencing platform at 75× average coverage.

Reads were mapped to the human genome (Hg19, BWA-mem 0.7.5). Variants were called using GATK version 2.8 and annotated with Annovar (version August 2013; ljb23 version 2.3 database).

Eighty-eight arrhythmia genes were analyzed (gene panel available on request). Variant filtering was performed by using Microsoft Excel (Microsoft Office 2007) and confirmed by Sanger sequencing. To exclude NGS sequencing errors, 3 in-house control samples of healthy individuals were used. A more detailed description can be found in **Supplementary File 1-1**.

**Statistical Analysis**

P-values were calculated using the N−1 $\chi^2$ test for binary data or the Student’s t-test for continuous data of the population screening. OriginPro 8 software (OriginLab Corporation, Northampton, MA, USA) was used to analyze the patch clamp data. An ANOVA with the Bonferroni post-hoc test was used to determine significance.

Values are given as mean ± standard error of mean (SEM) unless indicated otherwise. Results are considered significant when P<0.05.

**Results**

**Population Screening**

Our patient population consisted of 74 unrelated SCN5A mutation-negative BrS patients (65% male, average age±SD...
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47.69±14.56 years), 20% of the patients displayed a spontaneous type 1 ECG. Patient characteristics were stratified based on the variants present in the β-subunits (Table 1). No significant differences were observed between the populations (Table 1).

A total of 30 sequence variations were found (Table 2). A study of the literature and various databases did not reveal any known mutations among the variants. Five of these 30 variants were novel variants.

When compared to the normal population, the MAF of 5 single nucleotide polymorphisms (SNPs) were found to be statistically different (Table 2). All variants were fed into multiple in silico prediction tools. These results were used to classify the variants according to the system suggested by Hofman et al (Table 2). 21

Additional In Silico Prediction Analysis of Candidate Variants

Further in silico analysis was performed on novel variants and variants classified as VUS 2 (unknown pathogenicity).

The 2 base pair deletion (Figure 1A) in β1b (SCN1Bb, c.622_623delCT, p.Leu208Valfs*99) causes a frameshift, which abolishes the normal stop codon and introduces a novel stop codon further downstream. This completely changes the C-terminal part of the protein starting at amino acid 208. The second variant in β1 could potentially affect both isoforms of SCN1B.

In β1, this variant (c.448+444G>A, Figure 1A) was not predicted to have an effect on splicing. In β1b, the same variant is located in the 3'UTR (c.*85G>A, Figure 1A). Prediction tools for microRNA binding did not reveal any potential effect on the electrophysiology of the heart (Supplementary File 1-2). In addition, this nucleotide was not conserved (Figure 1B). The SCN2B c.–212C>T variant (Figure 1A) is a conserved nucleotide (Figure 1C). The β3 missense variant (c.301C>A, p.Gln101Lys, Figure 1A) involves a conserved amino acid. This change is predicted to be disease causing by all in silico prediction tools. The SCN4B promoter variant

### Table 2. Variants Found in the Study Population

| Gene   | cDNA   | Protein            | rs-number   | MAF study population | MAF general population | Hofman classification |
|--------|--------|--------------------|-------------|----------------------|------------------------|-----------------------|
| SCN1B  |        |                    |             |                      |                        |                       |
| exon 1 | 40+15G>T | Leu208Valfs*99   | rs72556351  | 0.122                | 0.168                  | Not pathogenic        |
| exon 1 | 207+55G>A |                   | rs147990128 | 0.007                | 0.003                  | Not pathogenic        |
| exon 3B| 622_623delCT | Leu208Valfs*99 | rs55742440   | 0.007                | VUS2                   |
|        | 629T>C   | Leu210Pro          | rs67701503   | 0.203*               | 0.133                  | Not pathogenic        |
|        | 749G>C   | Arg250Thr          | rs67486287   | 0.203*               | 0.129                  | Not pathogenic        |
|        | 769G>A   | Gly257Arg          | rs72558028   | 0.007                | NA                     | VUS1                  |
|        | *85G>A   |                   | rs375266320  | 0.007                | NA                     | VUS2                  |
| exon 4 | 501T>C   | Ile167Ile          | rs16969930   | 0.027                | 0.014                  | Not pathogenic        |
| exon 5 | 591–25T>G |                   | rs28365107   | 0.014                | 0.006                  | Not pathogenic        |
|        | 591–14C>A|                   | rs28365109   | 0.014                | 0.006                  | Not pathogenic        |
|        | *5+31G>A |                   | rs28365108   | 0.014                | 0.001                  | Not pathogenic        |
| SCN2B  |        |                    |             |                      |                        |                       |
| exon 1 | –212C>T  |                   | rs72544143   | 0.007                | VUS2                   |
| exon 2 | 70+11_70+12insTC |                 | rs645675    | 0.027**              | 0.201                  | Not pathogenic        |
| exon 4 | 449–12C>A |                   | rs8192613    | 0.588**              | 0.463                  | Not pathogenic        |
|        | *38C>T   |                   | rs8192614    | 0.068                | 0.072                  | Not pathogenic        |
| SCN3B  |        |                    |             |                      |                        |                       |
| exon 1 | –763G>A  |                   | rs12420563   | 0.169                | 0.148                  | Not pathogenic        |
|        | –962G>C  |                   | rs7483687    | 0.169                | 0.16                   | Not pathogenic        |
|        | –234G>A  |                   | rs3851104    | 0.169                | 0.153                  | Not pathogenic        |
|        | –181A>G  |                   | rs3851103    | 0.169                | 0.154                  | Not pathogenic        |
|        | 55+44C>T |                   | rs3851102    | 0.162                | 0.158                  | Not pathogenic        |
| exon 3 | 301C>A   |                   | rs14848744   | 0.007                | 0.002                  | Not pathogenic        |
|        | 390G>A   |                   | rs1275085    | 0.108                | 0.068                  | Not pathogenic        |
|        | 438C>T   |                   | rs1148110    | 0.372*               | 0.289                  | Not pathogenic        |
| SCN4B  |        |                    |             |                      |                        |                       |
| exon 1 | –137T>C  |                   | rs72544155   | 0.020                | 0.001                  | Not pathogenic        |
| exon 2 | 174C>T   |                   | rs45539032   | 0.014                | 0.031                  | Not pathogenic        |
| exon 3 | 295G>A   |                   | rs72544155   | 0.020                | 0.001                  | Not pathogenic        |

The minor allele frequency (MAF) of the study, which was significantly different to the general population, is indicated: *P<0.05, **P<0.01. Novel variants are indicated with a $-sign. The MAF of variant c.449–12C>A in SCN2B and c.558–45G>C in SCN3B correlates with the C and G frequencies, respectively. Variants in SCN1B exon 3 are only given as variants in exon 3B (NM_199037) because they all occur in the intronic region of β1 (NM_001037). MAF, minor allele frequency; VUS1, unlikely pathogenic; VUS2, unclear pathogenicity (according to Hofman et al21).
Some symptoms and family information

The patient carrying the deletion in β1b complained of dizziness and presyncope during a febrile episode. Two of her brothers suffer from palpitations (Figure 2A). They could not be examined in our hospital as they live abroad. The SCN1Bb c.*85G>A variant was found in a patient with a history of syncope during exercise. The patient reported multiple cases of SCD in the family (Figure 2B).

The patient with the SCN2B promoter variant had a syncope while sitting at the breakfast table. Neither his brothers nor children display cardiac symptoms. One son tested negative (c.−137T>C, Figure 1A) is a conserved nucleotide, located in a stretch of 11 conserved nucleotides (Figure 1D).

The novel variant in exon 3 of β4, c.295G>A (p.Asp99Asn, Figure 1A), is predicted to be not pathogenic (Table 2).

Figure 1. Novel β-subunit variants. (A) SCN1B and SCN1Bb: (a) p.Leu208Valfs*99, (b) c.*85G>A; SCN2B: (c) c.−137T>C and (f) p.Asp99Asn. Blue boxes represent untranslated regions (UTRs), white boxes represent the exons. Result of the Clustal Omega conservation study of (B) the SCN1B 3'UTR (C) the SCN2B and (D) the SCN4B promoter region. The start codon is indicated with a box, the changed nucleotide is in red and conserved regions are indicated with an asterisk (*).
for BrS (Figure 2C).

The β3-p.Gln101Lys variant was found in a patient who had syncope while driving his car and during a sauna visit. The patient displayed a spontaneous type 2 BrS ECG and was diagnosed after a positive class I AAD provocation test. He has neither siblings nor children (Figure 2D). The patient reported SCD of a maternal and paternal aunt at the age of 77 and 68 years, respectively.

A second promoter variant (SCN4B, c.−137T>C) was found in a patient who had an episode of syncope during the day, sitting at his desk. Both his father and grandfather died suddenly in the fifth decade of their life. His brother and 3 daughters all tested negative for BrS (Figure 2E).

Based on the results of the prediction tools and family information, we selected 3 novel variants for further functional study: β1b p.Leu208Valfs*99, β3 Gln101Lys and SCN4B c.−137T>C.

Functional Effects of β1b-del-ext

Cellular electrophysiology was performed to study the effect of β1b p.Leu208Valfs*99 on the sodium current (I\textsubscript{Na}). HEK cells were transfected with Nav1.5 in combination with wild-type (Nav1.5+β1b-WT) or p.Leu208Valfs*99 (Nav1.5+β1b-del-ext) β1b. No significant difference was seen in the peak I\textsubscript{Na} density (Table 3, Figures 3A,B). The inactivation voltage-dependence showed a significant negative shift (P<0.01) and the activation curve displayed a small positive shift (P<0.01, Table 3, Figure 3C) due to the variant. There was a signifi-
No difference could be found in the recovery from inactivation, voltage-dependent and time-dependent slow inactivation, or the τ of inactivation between the wild-type and variant β1b (Table 3, Supplementary File 1-3).

Functional Effects of β3-Gln101Lys
To assess the functional effects of β3-Gln101Lys on the Nav1.5 function, HEK cells were transfected with Nav1.5 and β3 wild-type (Nav1.5+β3-WT) or β3-Gln101Lys (Nav1.5+β3-Gln101Lys). No difference in the peak I (ms) density was observed (Table 3, Figures 3E,F). Analyzing the inactivation voltage-dependence, a shift to more negative potentials was observed with β3-Gln101Lys (P<0.01, Table 3, Figure 3G). β3-Gln101Lys also shifted the activation curve to more negative potentials (P<0.01, Table 3, Figure 3G). These differences in activation and inactivation caused small changes in the window current (Figure 3H). No differences were observed when comparing recovery from inactivation, τ of inactivation, and voltage-dependent or time-dependent slow inactivation (Table 3, Supplementary File 1-3).

Promoter Study of β4, c.-137T>C
HEK cells transfected with either the wild-type (SCN4Bprom-WT) or variant promoter (SCN4Bprom-MT) were studied 48 h and 72 h after transfection (Figure 3I,J). These experiments did not reveal any significant difference in promoter activity. Because HEK cells are a non-cardiac lineage, the experiment was repeated in rat cardiomyocyte-like H9c2 cells. These cells are more likely to express the correct transcription factors and therefore would better mimic the physiological conditions. However, similar to the HEK cells, no difference could be detected between the wild-type and the variant promoter (Figure 3K).

Arrhythmia Gene Panel Analysis
Because functional analysis did not indicate any of the variants as the sole cause of BrS, the patients were screened for a set of 88 cardiac arrhythmia genes using massive parallel sequencing (MPS). Only coding variants or variants affecting splice sites with a MAF of less than 5% were considered. A relative high MAF cut-off value was used to avoid the exclusion of variants associated with BrS, because they can be found in 4.35% of the overall population. In a first step, we analyzed the 20 genes already described in BrS; subsequently the other arrhythmia associated genes were studied (Figure 4A).

MPS detected 15 additional variants (Table 4); 5 novel and 10 previously described. All variants were further classified according to Hofman et al (Table 4). Six variants were classified as VUS1 (unlikely pathogenic), 7 as VUS2 (unclear pathogenicity) and 5 as not pathogenic.

In patient 1, we found 8 variants, including the β1b p.Leu208Valfs*99 (Table 4). Four variants were classified as possibly pathogenic: 3 missense mutations (SCN7A, ZFHX3 and DSG2) and the β1b deletion. Except for SCN1Bβ1b None of the genes have been described in BrS; no functional studies concerning these variants were described.

The β3-p.Gln101Lys carrying patient (patient 2) had 4 additional missense variants (GJA5, AKAP9, HCN4 and ZFHX3); none of them are classified as possibly pathogenic (Table 4). The HCN4 gene is associated with BrS and other arrhythmias. The variant found here has not been described in BrS, but in sudden infant death syndrome (SIDS) and sudden unexplained death in epilepsy. Its BrS disease-causing effect is questionable due to a lack of further functional evidence and the non-pathogenic Hofman score.

Besides the studied promoter variant, 4 additional missense variants were found in patient 3 (CACNB2, ZFHX3, DSC2 and KCNE1). The CACNB2 gene is associated with BrS; however, this particular mutation seems to be benign, as it was present in 1 of our technical control samples. KCNE1 is associated with LQTS. Changes of this particular amino acid
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Figure 3. Results of functional studies. Patch clamp results: Effects of the β1b-del-ext on Nav1.5: (A) current voltage (I–V) relationship, normalized for the cell capacitance, (B) representative whole cell patch clamp recordings. (C) Combined graph of activation and inactivation voltage-dependence. The conductance (G), normalized for the maximal conductance (Gmax), is displayed as a function of the applied voltage. For the inactivation curve; the peak current (I), normalized for the maximal current (Imax), is plotted as a function of the applied voltage during P1. (D) Window current. Effects of the β3-Gln101Lys on Nav1.5: (E) Current voltage (I–V) relationship, (F) representative recordings. (G) Activation and inactivation voltage-dependence, (H) window current. All graphs show mean±SEM. Patch clamp protocols are shown as insets. (A–D): Nav1.5+β1b-WT (circles) and Nav1.5+β1b-del-ext (triangles). (E–H): Nav1.5+β3-WT (circles) and Nav1.5+β3-Gln101Lys (triangles). Promoter-reporter study: Expression of the reporter gene in human embryonic kidney (HEK) cells transfected with (I) 1 µg (n=6) or (J) 3 µg of plasmid (n=6) or (K) H9c2 cells (n=2). NT, non-transfected cells. Values represent relative expression: the reporter gene was corrected for both transfection efficiency and highest value to compare between experiments performed on different days. Data are shown as mean±SEM. Squares indicate results 48 h after transfection; circles 72 h after transfection. **P<0.01.
Screening of the 4 \(\beta\)-subunits leads to a potential supplemental genetic diagnostic yield of 5.40% (4 out of 74 patients) based on exclusion of common variants, the results of in vitro prediction tools and Hofman classification. The prevalence of potential disease-associated variants in the \(\beta\)-subunit genes is thus comparable to other minor BrS genes. This correlates with what has been described in the literature.

A functional study of the variant \(\beta\)1b showed a significant (p.Arg67) to other amino acids have been found in patients with LQTS and sudden unexplained death; however, the variant described here has not been reported yet. Two variants were predicted to be possibly pathogenic: the \(SCN4B\) promoter variant and the \(KCNE1\) missense variant. Additional familial segregation analysis was performed in the proband and 2 of his daughters (Figure 4B). Each variant observed in the proband was also present in one of the 2 BrS negative daughters, but never in both daughters; one daughter carried 2 of the 5 variants and the other daughter carried the 3 other variants.

**Discussion**

Screening of the 4 \(\beta\)-subunits leads to a potential supplemental genetic diagnostic yield of 5.40% (4 out of 74 patients) based on exclusion of common variants, the results of in vitro prediction tools and Hofman classification. The prevalence of potential disease-associated variants in the \(\beta\)-subunit genes is thus comparable to other minor BrS genes. This correlates with what has been described in the literature. A functional study of the variant \(\beta\)1b showed a significant...
| Chromosome (chr) | Reference allele | Alternative allele | Gene | Effect | cDNA | Protein |
|------------------|------------------|-------------------|------|--------|------|---------|
| Patient 1        |                  |                   |      |        |      |         |
| chr1             | T                | C                 | RYR2 | Missense | 13969T>C | Tyr4657His |
| chr2             | A                | C                 | SNTG2| Missense | 149A>C  | Glu504Ala  |
| chr2             | T                | C                 | SCNTA| Missense | 3805A>G  | Met1269Val |
| chr10            | C                | T                 | ANK3 | Missense | 6955G>A  | Asp2319Asn |
| chr14            | –                | G                 | MYH7 | Splicing | 3337-3dupG | –         |
| chr16            | A                | C                 | ZFHX3| Missense | 5516T>G  | Val1839Gly |
| chr18            | A                | C                 | DSG2 | Missense | 2098A>C  | Ser700Arg  |
| chr19<sup>3</sup> | CT               | –                 | SCN1B| Frameshift | 622_623delCT | Leu208Valafs*99 |
| Patient 2        |                  |                   |      |        |      |         |
| chr1             | G                | A                 | GJA5 | Missense | 793C>T  | Pro265Ser |
| chr7             | C                | T                 | AKAP9| Missense | 139C>T  | His477Tyr |
| chr11<sup>4</sup> | G                | T                 | SCN3B| Missense | 301C>A  | Gin101Lys |
| chr15            | C                | T                 | HCN4 | Missense | 2275G>A  | Val759Ile |
| chr16            | C                | G                 | ZFHX3| Missense | 6042G>C  | Gin2014His |
| Patient 3        |                  |                   |      |        |      |         |
| chr10            | C                | G                 | CACNB2| Missense | 1651C>G  | Arg551Gly |
| chr11<sup>4</sup> | A                | G                 | SCN4B| Promoter function | –137T>C | –         |
| chr16            | C                | A                 | ZFHX3| Missense | 1563G>T  | Lys521Asn |
| chr18            | C                | T                 | DSC2 | Missense | 2393G>A  | Arg799Gln |
| chr21            | C                | A                 | KCNE1| Missense | 200G>T   | Arg67Leu  |

Variants were filtered according to the arrhythmia gene panel. The variants that were studied with patch clamp or promoter studies are indicated with a $-sign. ARVD, arrhythmogenic right ventricular dysplasia; BV, benign variant; BV?, probably benign variant; found in a technical control; CCD, cardiac conduction disease; CPVT, catecholaminergic polymorphic ventricular tachycardia; DCM, dilated cardiomyopathy; ERS, exercise-related syncope; HCM, hypertrophic cardiomyopathy; IVF, idiopathic ventricular fibrillation; JLNS, Jervell and Lange-Nielsen syndrome; LQTS, long QT syndrome; LVNC, left ventricular non-compaction; MPS, massive parallel screening; SD, sudden death; SIDS, sudden infant death syndrome; SQTS, short QT syndrome; SSS, sick sinus syndrome; SUDEP, sudden unexplained death in epilepsy; ToF, tetralogy of Fallot. Variants are classified according to Hofman et al. Other abbreviations as in Tables 1,2.
shift of the activation and inactivation voltage-dependence compared to the wild-type. However, no significant decrease in \( I_{Na} \) was found. Based on these results, we assume that this mutation is not the major cause of BrS in this patient.

It is surprising that the deletion, creating a completely different and extended C-terminus, only causes small effects on the Nav1.5 function. The frameshift changes 2 amino acids already associated with an impaired function of \( \beta 1b \). A known BrS and SIDS-associated mutation \(^{12} \) (p.Arg214Gln) and a LQT3-associated mutation \(^{24} \) (p.Pro213Tyr). Both missense mutations have been shown to nearly obliterate the effect of \( \beta 1b \) on Nav1.5. \(^{13,24} \) indicating the importance of the C-terminus.

\( \beta 1b \) can also associate with the cardiac potassium channel Kv4.3, \(^{12} \) which is responsible for \( I_{Ks} \). However, no differences in \( I_{Ks} \) caused by the variant \( \beta 1b \) compared to the wild type \( \beta 1b \) were detected.

The \( \beta 3 \)-Gln101Lys electrophysiology studies displayed negative shifts in activation and inactivation voltage-dependence. However, this was not further supported by a decrease of \( I_{Na} \), indicating that the global effect of this variant is too small to be the single cause of BrS in this patient. This was unexpected since all in silico prediction tools classified this variant as disease causing. Two mutations associated with BrS have been described in the \( \beta 3 \) protein: p.Lys101Pro \(^{14} \) and p.Val110Ile \(^{15} \), the latter is located close to the region of our variant, which suggested that this might be an important region.

The \( \beta 4 \)-subunit has not been associated with BrS yet, but it is linked with LQT3. \(^{16} \) Little is known about the structure and function of the promoter regions of the \( \beta \)-subunits. The SCN4B c.−137T>C promoter variant was selected based on its location in a stretch of 11 conserved nucleotides. Neither HEK cells nor H9c2 cells showed any difference in expression caused by this mutation in a stretch of 11 conserved nucleotides. Neither HEK cells nor H9c2 cells showed any difference in expression caused by the variant \( \beta 1b \) compared to the wild type \( \beta 1b \) were detected.

The \( \beta 4 \)-subunit has not been associated with BrS yet, but it is linked with LQT3. \(^{16} \) Little is known about the structure and function of the promoter regions of the \( \beta \)-subunits. The SCN4B c.−137T>C promoter variant was selected based on its location in a stretch of 11 conserved nucleotides. Neither HEK cells nor H9c2 cells showed any difference in expression caused by this variant, which suggests that this nucleotide and/or region is not involved in the promoter function of SCN4B.

Although Juang et al. \(^{33} \) demonstrated a high concordance rate (83.3%) between in silico prediction tools and the results of in vitro functional experiments for SCN5A, this rate does not seem applicable to the \( \beta \)-subunits as shown by our study; this is possibly due to the fact that \( \beta \)-subunits are not as well characterized and studied as the \( \alpha \)-subunit. \(^{33} \) MPS analysis revealed that, at least in patients 1 and 2, the other identified variants were not better potential disease-causing candidates. Further familial segregation analysis could add support, but unfortunately no family samples were accessible.

In patient 3, the familial segregation analysis revealed discordant results, indicating that none of the variants alone are the major cause of BrS in the patient. These findings can, however, not rule out a cumulative effect of the variants in the father.

**Limitations of the Study**

Only limited familial segregation studies could be performed due to the small pedigrees and restricted accessibility of family samples. Patch clamp recordings were performed at room temperature for technical reasons.

**Conclusions**

Our study demonstrates that the classification of variants, based on prediction tools alone, should be interpreted with caution. It stresses the importance of familial segregation analysis and functional studies. Unfortunately, it is often not possible to assess multiple family members. Certainly in such situations, functional studies are of great added value.

Our electrophysiological experiments demonstrate that the variants studied here cannot solely explain the existence of BrS in these patients. This supports the concept of BrS as a complex genetic disorder rather than a clear monogenic disease. \(^{24} \) The discordant familial segregation in patient 3 adds support to the hypothesis of an additive and/or synergistic effect of genetic variants. The genetic background, possibly together with environmental factors, could explain the reduced penetrance and variable expressivity that is seen in BrS.

We suggest including these SCN1-4B genes together with the other minor and major BrS genes in MPS-based gene panels and whole exome genetic analysis (Figure 4A).

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**Conflicts of Interest**

The authors declare no conflicts of interest.

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Supplementary Files

Supplementary File 1

1. Methods

Table S1. Primers and PCR conditions for the SCN1B to SCN4B genes

2. Potential changes in microRNA binding due to the β1b *85G>A variant

Table S2. Potential changes in microRNA binding sites due to the β1b *85G>A variant

3. Additional patch clamp results

Figure S1. Electrophysiological effects of β1b-del-ext on the α-subunit of the cardiac sodium channel (Nav1.5)

Table S3. Electrophysiological effects of β1b-del-ext on the α-subunit of the cardiac sodium channel (Nav1.5)

Figure S2. Electrophysiological effects of β1b-del-ext on the transient outward current-potassium channel (Kv4.3)

Table S4. Electrophysiological effects of β1b-del-ext on the transient outward current-potassium channel (Kv4.3)

Figure S3. Electrophysiological effects of β3-Gln101Lys on the α-subunit of the cardiac sodium channel (Nav1.5)

Table S5. Electrophysiological effects of β3-Gln101Lys on the α-subunit of the cardiac sodium channel (Nav1.5)

Please find supplementary file(s):
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