**Enhancing rare variant interpretation in inherited arrhythmias through quantitative analysis of consortium disease cohorts and population controls**

Roddy Walsh, PhD,a,b Najim Lahrouchi, MD,a,b Rafik Tadros, MD, PhD,c Florence Kyndt, PharmD, PhD,d,b Charlotte Glinge, MD, PhD,e,b Pieter G. Postema, MD, PhD,a,b Ahmad S. Amin, MD, PhD,a,b Eline A. Nannenberg, MD, PhD,f,b James S. Ware, PhD, MRCP,g,h,i Nicola Whiffin, PhD,g,h,i Francesco Mazzarotto, PhD,j,k,g,h Doris Škorić-Milosavljević, MD,a,b Christian Krijger, BSc,a,b Elena Arbelo, MD, PhD,l,m,n Dominique Babuty, MD, PhD,o Hector Barajas-Martinez, PhD,p Britt M. Beckmann, MD,q Stéphane Bézieau, PharmD, PhD,d,b J. Martijn Bos, MD, PhD,r Jeroen Breckpot, MD, PhD,s,b Oscar Campuzano, PhD,t,u,n,v Silvia Castelletti, MD,w Candan Celen, RN,x Sebastian Clauss, MD,q,y,z Anniek Corveleyn, PhD,aa,b Lia Crotti, MD, PhD,w,ab,ac,ad Federica Dagradi, MD,w Carlo de Asmundis, MD, PhD,ae Isabelle Denjoy, MD,af,ag,b Sven Dittmann, PhD,ah,b Patrick T. Ellinor, MD, PhD,ai,aj Cristina Gil Ortuño, MSc,ak,b Carla Giustetto, MD,al Jean-Baptiste Gourraud, MD, PhD,d,b Daisuke Hazeki, MD, PhD,am Minoru Horie, MD, PhD,an Taisuke Ishikawa, DVM, PhD,ao Hideki Itoh, MD, PhD,ap Yoshiaki Kaneko, MD, PhD,aq Jørgen K. Kanters, MD,ar Hiroki Kimoto, MPharm,as Maria-Christina Kotta, MSc, PhD,w,ad Ingrid P.C. Krapels, MD, PhD,at Masahiko Kurabayashi, MD, PhD,aq Julieta Lazarte, MSc,au Antoine Leenhardt, MD,af,ag,b Bart L. Loeys, MD, PhD,av Catarina Lundin, MD, PhD,aw Takeru Makiyama, MD, PhD,ax Jacques Mansourati, MD, PhD,ay Raphaël P. Martins, MD, PhD,az Andrea Mazzanti, MD,ba,b Stellan Mörner, MD, PhD,bb,b Carlo Napolitano, MD, PhD,ba,b Kimie Ohkubo, MD,bc Michael Papadakis, MBBS, MD,bd,be,b Boris Rudic, MD,bf,bg Maria Sabater Molina, PhD,ak,b Frédéric Sacher, MD, PhD,bh Hatice Sahin, RN,x Georgia Sarquella-Brugada, MD, PhD,u,bi,b Regina Sebastiano, BSc,bj Sanjay Sharma, BSc, MBChB, MD,bd,be,b Mary N. Sheppard, MD, FRCPath, FRCPI,bd,be,b Keiko Shimamoto, MD,bk M. Benjamin Shoemaker, MD,bl Birgit Stallmeyer, PhD,ah,b Johannes Steinfurt, MD,bm Yuji Tanaka, MD, PhD,bn David J. Tester, BS,r Keisuke Usuda, MD,bo Paul A. van der Zwaag, MD, PhD,bp Sonia Van Dooren, PhD,bq,b Lut Van Laer, PhD,av Annika Winbo, MD, PhD,br Bo G. Winkel, PhD,e,b Kenichiro Yamagata, MD, PhD,bk Sven Zumhagen, MD,ah,b Paul G.A. Volders, MD, PhD,bs Steven A. Lubitz, MD, MPH,ai,aj Charles Antzelevitch, PhD,p Pyotr G. Platonov, MD, PhD,bt Katja E. Odening, MD,bm,bu Dan M. Roden, MD,bv,bl,bw Jason D. Roberts, MD, MAS,bx Jonathan R. Skinner, MB, ChB, MD,by Jacob Tfelt-Hansen, DMCs,e,bz,b Maarten P. van den Berg, MD, PhD,ca Morten S. Olesen, PhD,cb Pier D. Lambiase, MD, PhD,cc,b Martin Borggrefe, MD,bf,bg Kenshi Hayashi, MD, PhD,bo Annika Rydberg, MD, PhD,cd,b Tadashi Nakajima, MD, PhD,aq Masao Yoshinaga, MD, PhD,bn Johan B. Saenen, MD, PhD,ce Stefan Kääb, MD, PhD,q,y Pedro Brugada, MD, PhD,cf,b Tomas Robyns, MD, PhD,cg,b Daniela F. Giachino, MD, PhD,bj,ch Michael J. Ackerman, MD, PhD,r Ramon Brugada, MD, PhD,ci Josep Brugada, MD, PhD,cj Juan R. Gimeno, MD,ck,b Can Hasdemir, MD,x Pascale Guicheney, PhD,cl Silvia G. Priori, MD, PhD,ba,b Eric Schulze-Bahr, MD, PhD,ah,b Naomasa Makita, MD, PhD,ao Peter J. Schwartz, MD,w,ad Wataru Shimizu, MD, PhD,cm Takeshi Aiba, MD, PhD,bk Jean-Jacques Schott, PhD,d,b Richard Redon, PhD,d,b Seiko Ohno, MD, PhD,cn Vincent Probst, MD, PhD,d,b Nantes Referral Center for inherited cardiac arrhythmia\*, Elijah R. Behr, MA, MBBS, MD,bd,be,b Julien Barc, PhD,co,b Connie R. Bezzina, PhD,a,b.

**Affiliations**

a Amsterdam UMC, University of Amsterdam, Department of Clinical and Experimental Cardiology, Heart Centre, Amsterdam Cardiovascular Sciences, Meibergdreef 9, Amsterdam, Netherlands

b Member of the European Reference Network for rare, low prevalence and/or complex diseases of the heart: ERN GUARD-Heart

c Department of Medicine, Cardiovascular Genetics Center, Montreal Heart Institute and Faculty of Medicine, Université de Montréal, 5000 Belanger, Montreal, QC, Canada

d Université de Nantes, CHU Nantes, CNRS, INSERM, l’institut du thorax, F-44000 Nantes, France

e The Department of Cardiology, The Heart Centre, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark

f Department of Clinical Genetics, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands

g National Heart and Lung Institute, Imperial College London, London, United Kingdom

h Cardiovascular Research Centre, Royal Brompton and Harefield NHS Foundation Trust, London, United Kingdom

i MRC London Institute of Medical Sciences, Imperial College London, London, United Kingdom

j Cardiomyopathy Unit, Careggi University Hospital, Florence, Italy

k Department of Experimental and Clinical Medicine, University of Florence, Italy

l Arrhythmia Section, Cardiology Department, Hospital Clínic, Universitat de Barcelona, Barcelona, Spain

m IDIBAPS, Institut d’Investigació August Pi i Sunyer (IDIBAPS), Barcelona, Spain

n Centro de Investigación Biomédica en Red de Enfermedades Cardiovasculares (CIBERCV), Madrid, Spain

o CHU Tours, Service de Cardiologie, Tours, France

p Lankenau Institute for Medical Research, Wynnewood, PA, USA

q LMU Klinikum, Klinikum der Ludwig Maximilians Universität München, Department of Internal Medicine I, München, Germany

r Department of Cardiovascular Medicine, Division of Heart Rhythm Services, Mayo Clinic, Rochester, MN, USA

s Department of Human Genetics, University Hospitals Leuven, Leuven, Belgium

t Cardiovascular Genetics Center, University of Girona-IDIBGI, Girona, Spain

u Medical Science Department, School of Medicine, University of Girona, Girona, Spain

v Biochemistry and Molecular Genetics Department, Hospital Clinic, University of Barcelona-IDIBAPS, Barcelona, Spain

w Istituto Auxologico Italiano, IRCCS - Center for Cardiac Arrhythmias of Genetic Origin, Via Pier Lombardo 22, 20135 Milan, Italy

x Ege University School of Medicine, Department of Cardiology, Izmir, Turkey

y German Centre for Cardiovascular Research (DZHK), Partner Site Munich, Munich Heart Alliance (MHA), Munich, Germany

z Walter Brendel Centre of Experimental Medicine, Ludwig-Maximilians University Munich (LMU), Munich, Germany

aa Center for Human Genetics, University Hospitals Leuven, Leuven, Belgium

ab Istituto Auxologico Italiano, IRCCS, Department of Cardiovascular, Neural and Metabolic Sciences, San Luca Hospital, Milan, Italy

ac Department of Medicine and Surgery, University of Milano-Bicocca, Milan, Italy

ad Istituto Auxologico Italiano, IRCCS - Laboratory of Cardiovascular Genetics, via Zucchi 18, 20095 Cusano Milanino (MI), Italy

ae Heart Rhythm Management Center, Postgraduate program in Cardiac Electrophysiology and Pacing Universitair Ziekenhuis, Brussel-Vrije Universiteit Brussel, ERN Heart Guard Center Laarbeeklaan 101, 1090, Brussels, Belgium

af CNMR Maladies Cardiaques Héréditaires Rares, Hôpital Bichat, Université de Paris, F-75018, Paris, France

ag AP-HP, Service de Cardiologie, Hôpital Bichat, F-75018, Paris, France

ah Institute for Genetics of Heart Diseases, University Hospital Münster, Germany

ai Cardiac Arrhythmia Service and Cardiovascular Research Center, Massachusetts General Hospital, Boston, MA, USA

aj Cardiovascular Disease Initiative, Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, MA, USA

ak Cardiogenetic Laboratory, Inherited Cardiac Disease Unit, University Hospital Virgen de la Arrixaca-IMIB, Murcia, Spain

al Division of Cardiology, University of Torino, Department of Medical Sciences, “Città della Salute e della Scienza” Hospital, C.so A.M. Dogliotti 14, 10126 Torino, Italy

am Department of Pediatrics, Kagoshima City Hospital, Kagoshima, Japan

an Center for Epidemiologic Research in Asia, Shiga University of Medical Science, Seta-Tsukinowa-cho, Otsu, Japan

ao Omics Research Center, National Cerebral and Cardiovascular Center, 6-1 Kishibe-Shimmachi, Suita, Osaka, 564-8565, Japan

ap Division of Patient Safety, Hiroshima University Hospital 1-2-3 Kasumi, Minami-ku, Hiroshima, Japan

aq Department of Cardiovascular Medicine, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan

ar Laboratory of Experimental Cardiology, Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark

as Department of Molecular Physiology, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4, Sakamoto, Nagasaki, 852-8523, Japan

at Department of Clinical Genetics, Maastricht University Medical Center+, P.O. Box 5800, 6202 AZ Maastricht, The Netherlands

au Department of Medicine and Robarts Research Institute, Schulich School of Medicine and Dentistry, Western University, London, Ontario, Canada

av Center of Medical Genetics, Faculty of Medicine and Health Sciences, University of Antwerp and Antwerp University Hospital, Antwerp, Belgium

aw Department of Clinical Genetics and Pathology, Lund University, Lund, Sweden

ax Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto, Japan

ay CHU Brest, Service de Cardiologie, Brest, France

az Univ Rennes, CHU Rennes, INSERM, Rennes, France

ba Molecular Cardiology, ICS Maugeri, IRCCS and Department of Molecular Medicine, University of Pavia, Pavia, Italy

bb Centre for Cardiovascular Genetics, Department of Public Health and Clinical Medicine, Umeå University, 901 85 Umeå, Sweden

bc Department of Cardiovascular Medicine, Nihon University, Tokyo, Japan

bd Molecular and Clinical Sciences Research Institute, St. George’s, University of London, London, United Kingdom

be Cardiology Clinical Academic Group, St. George’s University Hospitals’ NHS Foundation Trust, London, United Kingdom

bf Department of Medicine, University Medical Center Mannheim, Mannheim, Germany

bg German Center for Cardiovascular Research (DZHK), Partner Site Heidelberg/Mannheim, Germany

bh Hôpital Cardiologique du Haut-Lévêque, LIRYC Institute, Université Bordeaux, Bordeaux, France

bi Arrhythmias Unit, Hospital Sant Joan de Déu, University of Barcelona, Barcelona, Spain

bj Medical Genetics, San Luigi Univesity Hospital, regione Gonzole 10, 10043 Orbassano (TO), Italy

bk Department of Cardiovascular Medicine, National Cerebral and Cardiovascular Center, 6-1 Kishibe-shimmachi, Suita, 564-8565, Japan

bl Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, USA

bm Department of Cardiology and Angiology I, Heart Center University of Freiburg, Medical Faculty, Freiburg, Germany

bn Department of Pediatrics, National Hospital Organization Kagoshima Medical Center, Kagoshima, Japan

bo Department of Cardiovascular Medicine, Kanazawa University Graduate School of Medical Sciences, 13-1 Takara-machi, Kanazawa, Japan

bp University of Groningen, University Medical Center Groningen, Department of Genetics, Groningen, The Netherlands

bq Centre for Medical Genetics, research group Reproduction and Genetics, research cluster Reproduction, Genetics and Regenerative Medicine, Vrije Universiteit Brussel (VUB), Universitair Ziekenhuis Brussel (UZ Brussel), Brussels, Belgium

br Department of Physiology, University of Auckland, Auckland, New Zealand

bs Department of Cardiology, CARIM, Maastricht University Medical Center+, P.O. Box 5800, 6202 AZ Maastricht, The Netherlands

bt Department of Cardiology, Clinical Sciences, Lund University, Lund, Sweden

bu Department of Cardiology, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland

bv Department of Biomedical Informatics, Vanderbilt University Medical Center, Nashville, TN, USA

bw Department of Pharmacology, Vanderbilt University Medical Center, Nashville, TN, USA

bx Section of Cardiac Electrophysiology, Division of Cardiology, Department of Medicine, Western University, London, Ontario, Canada

by Cardiac Inherited Disease Group, Starship Childrens Hospital, Auckland, New Zealand

bz Department of Forensic Medicine, Faculty of Medical Sciences, University of Copenhagen, Denmark

ca University of Groningen, University Medical Center Groningen, Department of Cardiology, Groningen, The Netherlands

cb Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark

cc Barts Heart Centre, Barts Health NHS Trust & Institute of Cardiovascular Science, University College London, London, United Kingdom

cd Department of Clinical Sciences, Paediatrics, Umeå University, 901 85 Umeå, Sweden

ce Department of Cardiology, University of Antwerp, Antwerp University Hospital, Antwerp, Belgium

cf Heart Rhythm Management Center, UZ Brussel-VUB, Laarbeeklaan 101, 1090, Brussels, Belgium

cg Department of Cardiovascular Diseases, University Hospitals Leuven, Leuven, Belgium

ch Medical Genetics Dept. Clinical and Biological Sciences, Univestity of Torino, regione Gonzole 10, 10043 Orbassano (TO), Italy

ci Hospital Trueta, CiberCV, University of Girona, IDIBGI, Girona, Spain

cj Cardiovascular Institute, Hospital Clinic, University of Barcelona, Barcelona, Spain

ck Inherited Cardiac Disease Unit, Cardiology Department. University Hospital Virgen de la Arrixaca, Murcia, Spain

cl INSERM, Sorbonne University, UMRS 1166, Institute of Cardiometabolism and Nutrition (ICAN), Paris, France

cm Department of Cardiovascular Medicine, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo, 113-8603, Japan

cn Department of Bioscience and Genetics, National Cerebral and Cardiovascular Center, 6-1 Kishibeshinmachi, Suita, Japan

co Université de Nantes, CNRS, INSERM, l’institut du thorax, F-44000 Nantes, France

\* A list of authors and their affiliations appears at the end of the paper.

**Address for correspondence**

Roddy Walsh, Department of Clinical and Experimental Cardiology, Heart Centre, Academic Medical Centre, Meibergdreef 9, 1105 AZ, Amsterdam, Netherlands.

phone: +31205664682, email: [r.t.walsh@amsterdamumc.nl](mailto:r.t.walsh@amsterdamumc.nl), twitter: @roddywalsh

**Abstract**

**Purpose**: Stringent variant interpretation guidelines can lead to high rates of variants of uncertain significance (VUS) for genetically heterogeneous disease like Long QT syndrome (LQTS) and Brugada syndrome (BrS). Quantitative and disease-specific customisation of ACMG/AMP guidelines can address this false negative rate.

**Methods**: We compared rare variant frequencies from 1847 LQTS (*KCNQ1*/*KCNH2*/*SCN5A*) and 3335 BrS (*SCN5A*) cases from the International LQTS/BrS Genetics Consortia to population-specific gnomAD data and developed disease-specific criteria for ACMG/AMP evidence classes – rarity (PM2/BS1 rules) and case enrichment of individual (PS4) and domain-specific (PM1) variants.

**Results**: Rare *SCN5A* variant prevalence differed between European (20.8%) and Japanese (8.9%) BrS patients (p=5.7x10-18) and diagnosis with spontaneous (28.7%) versus induced (15.8%) Brugada type 1 ECG (p=1.3x10-13). Ion channel transmembrane regions and specific N-terminus (*KCNH2*) and C-terminus (*KCNQ1*/*KCNH2*) domains were characterised by high enrichment of case variants and >95% probability of pathogenicity. Applying the customised rules, 17.4% of European BrS and 74.8% of European LQTS cases had (likely) pathogenic variants, compared to estimated diagnostic yields (case excess over gnomAD) of 19.2%/82.1%, reducing VUS prevalence to close to background rare variant frequency.

**Conclusion**: Large case/control datasets enable quantitative implementation of ACMG/AMP guidelines and increased sensitivity for inherited arrhythmia genetic testing.

**Keywords:** variant interpretation, LQTS, Brugada, ACMG/AMP guidelines

**Introduction**

The accurate interpretation of genetic variants remains one of the key challenges in clinical genetic testing for inherited cardiac conditions. It carries major clinical implications due to both the danger of assigning causality to variants that are in fact benign (false positive findings) as well as the possibility that variants that are truly causal are considered as “variants of uncertain significance”. The relatively high rate of rare benign variants in many genes (revealed by population sequencing studies such as gnomAD), and the subsequent debunking of many gene and variant associations with disease in the scientific literature1,2, has highlighted the extent of this challenge and the danger of false positive classifications.

Variant interpretation guidelines produced by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) in 2015 have brought much needed consistency and stringency to variant classification in both clinical and research laboratories3. However, standard implementation of such guidelines may lead to overly conservative interpretation and a substantial rate of variants of uncertain significance (VUS) in clinical genetic testing4. Interpretation is particularly challenging for non-truncating variants, i.e. those causing minor alterations in the protein sequence while preserving the reading frame (the majority of which are missense variants) whose functional consequences are harder to predict. For diseases with high allelic heterogeneity (i.e. thousands of potential disease-causing variants) and where non-truncating variants are the predominant pathogenic variant class, this can correspond to a high rate of false negative results, evidenced when the frequency of VUS in cases exceeds the background rare variant frequency1.

Brugada syndrome (BrS) and Long QT syndrome (LQTS) are inherited disorders that can cause severe arrhythmogenic events and sudden cardiac death in young individuals. Pathogenic variants in three genes account for the vast majority of genotype-positive cases for these conditions. These 3 genes code for -subunits of voltage-gated ion channels expressed in cardiomyocytes: *SCN5A* underlying the cardiac depolarizing sodium current (*I*Na), and *KCNH2* and *KCNQ1* underlying the rapid (*I*Kr) and slow (*I*Ks) components of the repolarizing potassium delayed rectifier current. Rare coding variants in *SCN5A* cause BrS by a loss-of-function mechanism while LQTS is caused by functional loss-of-function variants in *KCNQ1* (LQT1) and *KCNH2* (LQT2) or gain-of-function variants in *SCN5A* (LQT3).

Genetic testing is well established for BrS and LQTS, to inform clinical management and facilitate cascade screening in families to identify at-risk carriers5. Despite this, variant interpretation remains challenging for non-truncating variants that comprise the majority of causative variants in all three genes. For example, a recent reappraisal of all *SCN5A* variants implicated in BrS using contemporary ACMG/AMP guidelines found that only 17% of missense variants could be classified as (likely) pathogenic6. While this may partly reflect some erroneous disease associations in earlier reports, it highlights the difficulty in identifying pathogenic missense variants with these guidelines.

The classes of evidence used for variant interpretation can be broadly grouped based on their interpretative power and the frequency with which they can be applied (Figure 1). For non-truncating variants not common in the population, supporting evidence, such as variant frequency in the population and computational predictions of variant effect, can be universally applied but has limited ability to distinguish between pathogenic and benign rare variation. These require informative variant-specific evidence to establish pathogenicity. However, due to the high allelic heterogeneity of these disorders, small pedigree sizes that preclude conclusive co-segregation analysis and limited accessibility of functional assays, a large proportion of likely causative variants detected in patients with LQTS and BrS are unable to be classified as pathogenic. To address this false negative rate and improve the detection of pathogenic non-truncating variants, a third evidence category specific to gene-disease dyads can be applied – case-control studies that identify specific variants or classes of variants highly enriched in disease. Several disease and gene-specific implementations of the ACMG/AMP guidelines have been proposed by ClinGen and independent studies detailing how the rules, including those related to variant enrichment in cases over controls, should be applied for the particular genetic characteristics of each disease (Table S1)7.

We have previously described a quantitative implementation of variant class enrichment evidence for sarcomeric genes involved in hypertrophic cardiomyopathy (HCM)4. By identifying gene regions highly enriched for rare variants in HCM cohorts compared to population controls, we were able to substantially decrease the rate of VUS in HCM cases using adapted ACMG/AMP guidelines (14-20% increase in actionable HCM variants). Particular ion channel domains are also known to be enriched for cases variants, as previously demonstrated for LQTS8,9 and BrS10. Here, using large case cohorts from international consortia (1847 LQTS and 3335 BrS cases), we apply and refine our ACMG/AMP guidelines adaptation to the three major disease genes of BrS and LQTS. We demonstrate how we can identify and classify high confidence pathogenic variant classes in specific clinical genetics contexts, and apply this evidence within the ACMG/AMP framework to enable an improved balance between sensitivity and specificity in clinical genetic testing for these conditions.

**MATERIALS AND METHODS**

*Patient and control cohorts*

The primary arrhythmia cohorts comprised patients diagnosed with BrS or LQTS recruited by the International BrS Genetics Consortium and the International LQTS Genetics Consortium, respectively5,11. The BrS cohort consisted of 2400 unrelated cases of European origin from 23 predominantly European referral centres and 935 unrelated cases from 5 Japanese centres. The LQTS cohort consisted of 1394 unrelated cases of European origin and 453 unrelated cases from Japan (Table S2). All ECGs were centrally assessed by a genetic cardiac electrophysiologist prior to inclusion. For European cases, genetic ancestry was confirmed using genotypic principal component analysis. All subjects or their guardians provided informed consent and the study was approved by local ethical review committees of the participating centres.

Rare variant data from diagnostic sequencing of *SCN5A* (BrS) and *KCNQ1*, *KCNH2* and *SCN5A* (LQTS) were provided by the different centres and curated based on the following canonical transcripts: ENST00000155840 (*KCNQ1*), ENST00000262186 (*KCNH2*) and ENST00000333535 (*SCN5A*). Details of all rare variants are provided in Tables S3/S4. Three additional arrhythmia-related datasets detailed in Supplemental Methods and Table S2 were used for further analyses as described in Results.

The gnomAD dataset (version 2.1) comprising 125748 individuals with exome sequencing was used for population controls, with the following population-specific subsets used for the European and Japanese cases respectively: non-Finnish European (gnomAD-NFE) comprising 56885 individuals and East Asian (gnomAD-EAS) comprising 9197 individuals.

*Defining population frequency thresholds for rare variants*

The maximum credible population allele frequency thresholds for rare, pathogenic variants were calculated as previously described12:

where the maximum allelic contribution is the maximum proportion of cases potentially attributable to a single causative variant in these cohorts.

Population frequency thresholds were calculated on the basis of two estimates of penetrance – 50% and 10% - for both BrS and LQTS. For BrS, we used a disease prevalence of 1/1000 and a maximum allelic contribution of 0.01 (based on *SCN5A*:c.4813+5\_4813+6insGTGG, attributable to 15/2400 or 0.6% (0.3-1.0%) of cases in this study). The thresholds for BrS were calculated as 1.0x10-5 and 5.0x10-5 respectively. For LQTS, we used a disease prevalence of 1/2000 and maximum allelic contribution of 0.022 (based on *KCNQ1*:c.1032G>A, attributable to 29/1847 or 1.6% (1.1-2.2%) of cases in this study). The thresholds for LQTS were calculated as 1.1x10-5 and 5.5x10-5 respectively.

*Defining case-enriched regions in arrhythmia genes*

To define the relative enrichment of rare variants in cases over controls for arrhythmia gene regions, we used the etiological fraction (EF), which in the context of Mendelian disease estimates the proportion of cases with a rare variant of a particular class in whom that variant is disease-causing. The EF is derived from the odds ratio (OR) by the formula: EF=(OR-1)/OR, with 95% confidence intervals (CI) calculated as described in Supplemental Methods. For the three arrhythmia genes (*KCNQ1*/*KCNH2*/*SCN5A*), EFs were calculated for rare non-truncating (missense and inframe insertions/deletions) variants by comparing arrhythmia cohorts with population-relevant gnomAD controls. EFs were calculated for specific gene/protein regions in *KCNQ1*/*KCNH2*/*SCN5A*, defined according to recognised domains in the respective UniProt (version 207) entries (details in Results and Supplemental Methods), as well as the *KCNQ1* C-terminus highly conserved regions defined by Kapplinger at al.9 and the *KCNH2* N-terminus cluster based on distribution of LQTS variants in this study. Regions with poor coverage in gnomAD and founder/recurrent case variants that may inflate regional EFs were masked from this analysis (see details in Supplemental Methods).

*Variant interpretation with standard ACMG/AMP guidelines*

Case variants were classified according to ACMG/AMP guidelines using the CardioClassifier application13 with adaptations for the following rules: (1) Variant population frequency (PM2/BS1 rules) used gnomAD exomes filtering allele frequencies (FAF) with thresholds defined in this study. (2) Loss-of-function evidence (PVS1) was implemented according to recommendations from the ClinGen Sequence Variant Interpretation Workgroup14. (3) Functional evidence rules (PS3/BS3) for BrS/*SCN5A* were applied using published data curated by Denham et al6 and a high-throughput functional study by Glazer et al15. Note, evidence relating to co-segregation (PP1/BS4), *de novo* inheritance (PS2/PM6) and functional data for LQTS (PS3/BS3) could not be applied due to the lack of curated resources. While this will affect the yield of (likely) pathogenic variants, co-segregation evidence is not expected to be particularly informative for BrS as family pedigrees tend to be of limited sizes with numerous examples of non-Mendelian segregation6,16.

*Variant interpretation with case-control evidence*

We then implemented refined ACMG/AMP guidelines based on the case-control evidence we generated to assess the effect on the yield of (likely) pathogenic variants. For the enrichment of classes of rare non-truncating variants (PM1 rule), EF data from the protein region analysis was applied. *PM1\_strong* was applied for rare variants in regions with an EF≥0.95 (equivalent to OR≥20), *PM1\_moderate* where 0.95>EF≥0.90 (20>OR≥10) and *PM1\_supporting* where 0.90>EF≥0.80 (10>OR≥5), as previously described4. The EF threshold of 0.95 implies a 95% or greater prior probability of pathogenicity and therefore the likely pathogenic classification for variants with EF≥0.95 (PM1\_strong, PM2) aligns with variant interpretation guidelines3. Individual variants significantly enriched in cases over population-specific gnomAD controls were identified (minimum case count of 3 and Fisher’s exact p-value <0.05 with Bonferroni multiple testing correction for the number of case variants assessed for each condition). The PS4 rule strength was applied based on the lower boundary of the 95% CI of the OR, ≥20, ≥10 and ≥5 for strong, moderate and supporting evidence respectively (equivalent to the PM1 OR thresholds). See Supplemental Methods for full details of how each ACMG/AMP rule was applied.

**RESULTS**

*Defining population frequency thresholds for rare variants*

To define appropriate population allele frequency thresholds for BrS and LQTS (i.e. the frequency above which variants are unlikely to be causative), we computed disease odds ratios (OR) across different frequency bins (comparing non-truncating variants from European arrhythmia case cohorts and gnomAD-NFE population controls). Disease-specific frequency thresholds were calculated as described in Methods for variant penetrance estimates of 10% and 50% (5.0x10-5 and 1.0x10-5 for BrS, 5.5x10-5 and 1.1x10-5 for LQTS respectively), which were used as upper frequency thresholds along with 1x10-3 (BA1 benign variant threshold13) (Figure 2A and Table S5).

Variants in the second rarest bin, i.e. corresponding to estimated penetrance between 10% and 50%, were either not significantly enriched in cases or had substantially lower ORs compared to the rarest bin (where penetrance ≥50%) – 10-50%:3.8 (2.2-6.6) and >50%:155.8 (128.9-188.2) for *KCNQ1*/LQTS, 10-50%:2.4 (1.2-4.5) and >50%:55.5 (46.0-67.0) for *KCNH2*/LQTS, 10-50%:1.0 (0.5-1.9) and >50%:7.9 (6.2-10.0) for *SCN5A*/LQTS and 10-50%:2.7 (1.9-3.6) and >50%:18.6 (16.0-21.7) for *SCN5A*/BrS. No significant enrichment of variants with frequencies between 1x10-3 and 5x10-5/5.5x10-5 was observed for any gene.

These results indicate that while some variants with a population frequency above the more stringent thresholds may be disease-causing, the lower signal-to-noise ratio and presumably lower penetrance of such variants suggest that a higher burden of proof should be required to classify them as (likely) pathogenic (e.g. demonstration of functional effects or statistical enrichment in cases). For subsequent analysis in this study, we used the higher, more inclusive thresholds (based on estimated penetrance of 10%), for describing the prevalence of rare variants in arrhythmia cohorts. However, the more stringent thresholds (based on estimated penetrance of ≥50%) were used when applying the PM2/PM1 variant classification rules to minimise false positive classifications. The PM2 rule (rarity in population) was therefore applied to variants with a gnomAD exomes FAF<1.0x10-5 (BrS) and 1.1x10-5 (LQTS) and the BS1 rule (frequency too high for the disorder) was applied to variants with a FAF>5.0x10-5 (BrS) and 5.5x10-5 (LQTS), with a recommended “grey zone”7,17 where neither is applied for the potential low penetrance variants.

*Prevalence of rare variants in primary arrhythmia cohorts*

The prevalence of rare (FAF<5x10-5) *SCN5A* variants in the BrS cohort was significantly different between the European and Japanese cohorts, detected in 20.8% and 8.9% of cases respectively (p=5.7x10-18) (Figure 2B, Table S6). Non-truncating variants accounted for 71.0% and 86.7% of rare variants detected in the European and Japanese BrS cases respectively. The prevalence of rare (FAF<5.5x10-5) variants in *KCNQ1*, *KCNH2* and *SCN5A* in the LQTS cohort was high (as expected) and similar between European and Japanese cohorts, detected in 85.4% and 85.9% of cases respectively (p=0.63). The relative proportions of truncating and non-truncating variants in *KCNQ1* and *KCNH2* however are marginally different between the two populations (p=0.007) (Figure 2B).

*Assessing enrichment of rare variants in cases in arrhythmia gene regions*

We next assessed variant distribution to identify gene/protein regions enriched for rare (corresponding to an estimated penetrance of ≥50% as described above) non-truncating variants in cases compared to gnomAD (Figure 3A), as defined by the etiological fraction (EF).

For BrS and LQTS, EFs were calculated separately by population, with European cases versus gnomAD-NFE and Japanese cases versus gnomAD-EAS (Tables 1,2). For BrS, the yield of rare *SCN5A* variants was significantly higher for cases diagnosed with a spontaneous compared to a drug-induced type 1 ECG pattern (28.7% vs 15.8% in the European cases, p=1.3x10-13), therefore EFs were also calculated for European cases sub-classified by ECG pattern (Table 1). For LQTS, as clinical genetic tests are often referred based on suspected rather than confirmed diagnosis, we also calculated EFs from a published referral cohort of 2500 cases18, where the yield of rare variants in the three LQTS genes (33.6%) reflects a lower proportion of definitive cases (Table 2).

Variants were highly enriched in cases in the transmembrane regions of all three genes (with the exception of *SCN5A* variants in LQTS cases) and select N/C-terminus domains in *KCNQ1* and *KCNH2* (Tables 1,2). The regional EFs produced by this analysis, and consequently the strength of the evidence applied (PM1 rule), display marked variability by ancestry (BrS), diagnostic certainty (LQTS) and diagnostic criteria (BrS spontaneous/drug-induced type 1 ECG pattern), highlighting the importance of considering these contexts for variant interpretation.

*Identification of specific variants enriched in arrhythmia case cohorts*

Based on European and Japanese case-control comparisons as described in Methods, 48 individual variants are enriched in BrS cases (with 2, 26 and 20 variants activating the PS4 strong, moderate and supporting rules respectively) (Figure S1, Table S9) and 108 variants are enriched in LQTS cases (with 43, 54 and 11 variants activating the PS4 strong, moderate and supporting rules respectively) (Figure S2, Table S10).

*ACMG/AMP classification of rare variants in arrhythmia cohorts*

We classified the variants in the BrS and LQTS cohorts according to the ACMG/AMP guidelines, using the CardioClassifier application13 (with adaptations as described in Methods). The frequency of rare variants in gnomAD estimates the rate of rare benign variants in both population and arrhythmia cohorts, and can be used to assess the sensitivity (and specificity) of variant classification approaches. In particular, the excess of VUS over this gnomAD frequency denotes the false negative rate in clinical genetic testing, i.e. the proportion of cases with variants that are expected to be disease-causing but for which insufficient evidence currently exists to classify them as (likely) pathogenic.

For BrS, classification was performed before and after inclusion of the case-control evidence described above (PM1/PS4 rules), to assess the effect of these approaches on the diagnostic yield. With the basic ACMG/AMP rules (including functional evidence but excluding segregation data), 9.1% of European BrS cases had a rare non-truncating *SCN5A* VUS compared to 1.5% in gnomAD, highlighting a large false negative rate. After applying the case-control PM1/PS4 evidence, the proportion of cases with VUS was reduced to 3.1% (Figure 3B). Given the more modest EFs associated with variants found solely in Japanese BrS cases, the VUS rate was reduced from 4.4% to 3.0% in Japanese BrS patients (compared to a 1.7% background rate in gnomAD-EAS) (Figure 3C).

For LQTS, familial co-segregation and functional evidence are critical for the classification of non-truncating variants (all were VUS with the basic ACMG/AMP classification) but given the extensive allelic heterogeneity (504 distinct rare non-truncating variants in 1847 cases) and the lack of high-throughput functional studies or curated resources, it was not feasible to include this evidence here. However, using the case-control PM1/PS4 evidence, the proportion of cases with VUS was reduced to 2.33% for *KCNQ1* (compared to a gnomAD background frequency of 0.53%), 3.68% for *KCNH2* (gnomAD=0.77%) and 3.14% for *SCN5A* (gnomAD=1.5%) (Figure 3D).

Of the variants upgraded from VUS to (likely) pathogenic, only 11.1% of BrS and 36.1% of LQTS variants had a non-conflicting pathogenic or likely pathogenic classification in ClinVar (Tables S11/S12).

*Re-classification of published BrS SCN5A variants*

We also re-analysed the set of published BrS-implicated *SCN5A* variants that were classified with contemporary ACMG/AMP guidelines by Denham et al6, where 72% (231/321) of non-truncating *SCN5A* variants were classified as VUS. Applying the case-control (PS4 and PM1 based on all European BrS cases) and frequency (PM2/BS1) evidence described here, 152 of these VUS (65.8%) were upgraded to (likely) pathogenic (Table S13).

*Application to Sudden Arrhythmic Death Syndrome (SADS)*

As well as clinical genetic testing for individuals with BrS/LQTS, sequencing of the major arrhythmia genes is also often performed for SADS cases, i.e. sudden death in usually non-elderly individuals with a structurally normal heart, including the coronary arteries. We assessed the enrichment of rare variants (FAF<1x10-5) in *KCNQ1*, *KCNH2*, *SCN5A* and *RYR2* in a cohort of 505 SADS cases compared to gnomAD to calculate EFs in the context of SADS and identify interpretable variant classes19. The yield of rare non-truncating variants in the SADS cohort was low - *KCNQ1* (1.4%), *KCNH2* (1.8%), *SCN5A* (1.8%) and *RYR2* (5.0%), with EFs for most protein regions either too low for PM1 rule application (EF<0.8) or based on very limited case variants. The exception are variants in the pathogenic hotspots of *RYR2* identified in our previous study4, although the EF (0.88) yields only PM1\_supporting evidence (Table S14).

**Discussion**

In this study we have shown that large cohorts of genetically characterised arrhythmia patients can be used to develop disease- and gene-specific quantitative applications of the ACMG/AMP framework and increase the sensitivity of genetic testing in these conditions. Through case-control analysis with gnomAD population data, we defined appropriate arrhythmia-specific population frequency thresholds to define variant rarity and then developed rules for variant enrichment evidence - the association of individual variants with disease (PS4) and enrichment of region-specific variants in case cohorts (PM1). The latter allows us to distinguish between highly pathogenic non-truncating variant classes (with some approaching truncating variant ORs) that can be confidently classified as likely pathogenic even when further corroborating evidence is absent and those with lower probabilities that require additional evidence to inform pathogenicity. By incorporating this evidence into ACMG/AMP guidelines, the prevalence of VUS in the three arrhythmia genes are reduced close to the background rare variant frequency (Figure 3).

As the domain-based PM1 rule adaptations depend on the case-control signal-to-noise ratio, these should be applied taking into account factors that affect this ratio (see Tables 1 and 2 for how to apply this evidence). These include patient ethnicity due to differences in rare variant population frequencies and disease genetic architecture, such as observed here between European and Japanese BrS cases. Diagnostic criteria such as spontaneous or induced type 1 ECG pattern in BrS patients may also influence diagnostic yield and hence the interpretability of detected variants. It is also critical to account for phenotype uncertainty in the individual being tested. Here, we provide guidelines for LQTS in the context of either a definitive diagnosis of disease or referral for genetic testing based on suspicion of disease, and demonstrate that this approach has limited utility for SADS genetic testing, given the extremely heterogeneous aetiology underlying these cases.

We applied this method to rare variants defined with stringent population frequency thresholds, based on the published framework12 and estimating penetrance of at least 50%. Such ultra-rare variants have the strongest case-control signals (Figure 2A), allowing us to identify interpretable variants with high confidence and minimise the risk of false positive classifications. We also observed a modest case excess of variants with population frequencies corresponding to lower estimated penetrance (10%-50%). Some of these variants, if detected in patients, may well contribute to the disease phenotype as genetic risk factors with modest effect sizes. However, given the much reduced signal-to-noise ratio, and the likelihood that many will be benign variants, a higher evidence threshold should be applied for establishing any pathogenic role. For this reason, we have not applied the PM1 or PM2 rules to such variants – more direct evidence such as enrichment of the specific variant in cases and validation with established functional assays is required.

Demonstrating significant enrichment of specific variants in disease cases is also potentially powerful evidence for pathogenicity when pedigree or functional data is not available. However, defining the significance of an observed enrichment and applying the appropriate evidence strength can be challenging. While most disease-specific ACMG/AMP framework implementations have proposed specific numbers of previously described cases for each evidence level (in the absence of large sequenced case cohorts), these can be arbitrary and do not demonstrate statistical enrichment (Table S1). The approach we describe here utilises large multi-centre arrhythmia cohorts to identify significantly enriched variants compared to population-matched gnomAD controls, adjusts for the disparity in cohort size between cases and controls and applies conservative thresholds for the different evidence levels to minimise any false positive classifications. These methods can also easily incorporate any newly sequenced cohorts to identify additional enriched variants in the main arrhythmia genes. The specific PS4 variant evidence (detailed in Tables S9/S10) can be applied to all BrS/LQTS patients regardless of ethnicity or clinical context.

*Limitations*

The same primary arrhythmia cohorts were used to calculate regional EFs and then test the effect of PM1 evidence on variant classification. However, when accounting for population and disease diagnosis, we are confident that the observed variant frequencies will be consistent with other equivalent cohorts. We provide variant interpretation guidelines based on analysis of European and Japanese arrhythmia patients, therefore these should be used with caution for other ethnicities if there is an expectation of different case/control rare variant frequencies in other population groups. Large-scale sequencing of disease and population datasets for other populations groups remains a critical need, especially as quantitative classification methods like these are likely to be particularly beneficial for non-European populations, given the increased difficulty of identifying pathogenic variants for such patients using standard guidelines20. For LQTS we were unable to fully assess the additive effect of these methods on ACMG/AMP variant classification, as this is dependent on segregation and functional evidence for which comprehensive curated datasets are not available.

*Conclusion*

Through analysis of large sequenced case and control cohorts, we have developed quantitative and gene/disease-specific applications of ACMG/AMP variant interpretation guidelines for major arrhythmia genes, that improve the balance between sensitivity and accuracy of clinical genetic testing and reduce the false negative rate associated with these genetically heterogeneous diseases. The findings also highlight that population and phenotype context are critical for effectively interpreting the clinical effect of rare variation. This study will have significant clinical impact in improving the diagnosis, treatment and family screening of BrS/LQTS patients who currently receive uncertain genetic test results.

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**CONSORTIUM**

**Nantes Referral Center for inherited cardiac arrhythmia:**

Alain Al Arnaout, MD, PhD,cp Mathieu Amelot, MD, PhD,cq Frédéric Anselme, MD, PhD,cr Dominique Babuty, MD, PhD,o Olivier Billon, MD, PhD,cs Pascal Defaye, MD, PhD,ct Jean-Marc Dupuis, MD, PhD,cu Jean-Baptiste Gourraud, MD, PhD,d,b Laurence Jesel, MD, PhD,cv Gabriel Laurent, MD, PhD,cw Jacques Mansourati, MD, PhD,ay Raphaël P. Martins, MD, PhD,az Philippe Maury, MD, PhD,cx Jean-Luc Pasquie, MD, PhD,cy Vincent Probst, MD, PhD,d,b Frédéric Sacher, MD, PhD,bh Francois Wiart, MD, PhD,cz

cp Service de cardiologie, GH La Rochelle, 17019 La Rochelle, France

cq Cardiology department, CH de Le Mans, Le Mans, France

cr Cardiology department, CHU de Rouen, Rouen, France

cs Chd Les Oudairies, La Roche Sur Yon, France

ct CHU Grenoble, Service de Cardiologie, Grenoble, France

cu Service de cardiologie, CHU d'Angers, 49100 Angers, France

cv Service de cardiologie, CHRU de Strasbourg, 67091 Strasbourg, France

cw Department of Cardiology, University Hospital, 21000 Dijon, France

cx CHU Toulouse, Service de Cardiologie, Toulouse, France

cy Department of Cardiology, Arnaud de Villeneuve Hospital, Montpellier University Hospital Center, Montpellier, France

cz Service de cardiologie, CHU de La Réunion, 97400 Saint-Denis, Reunion

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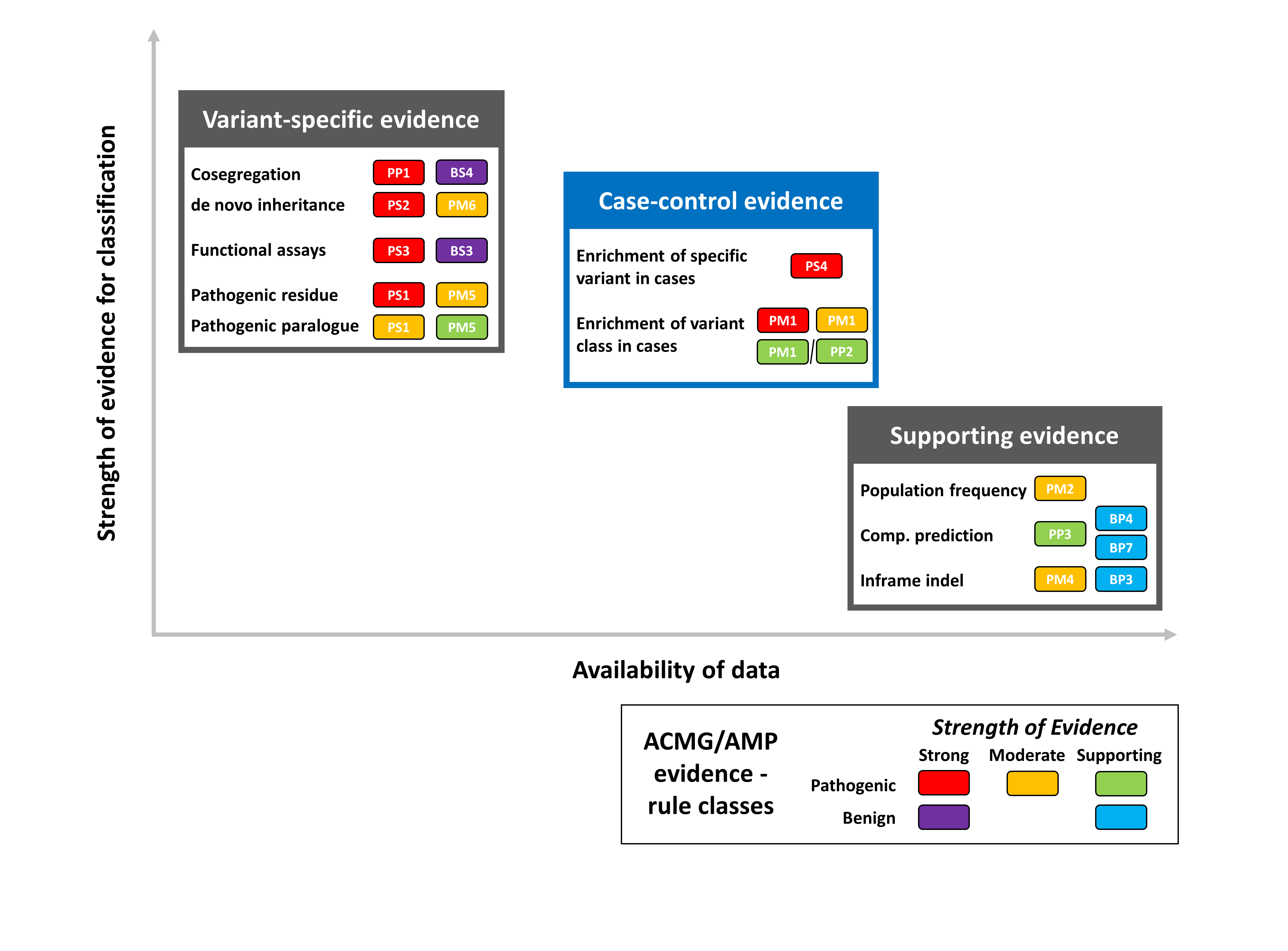
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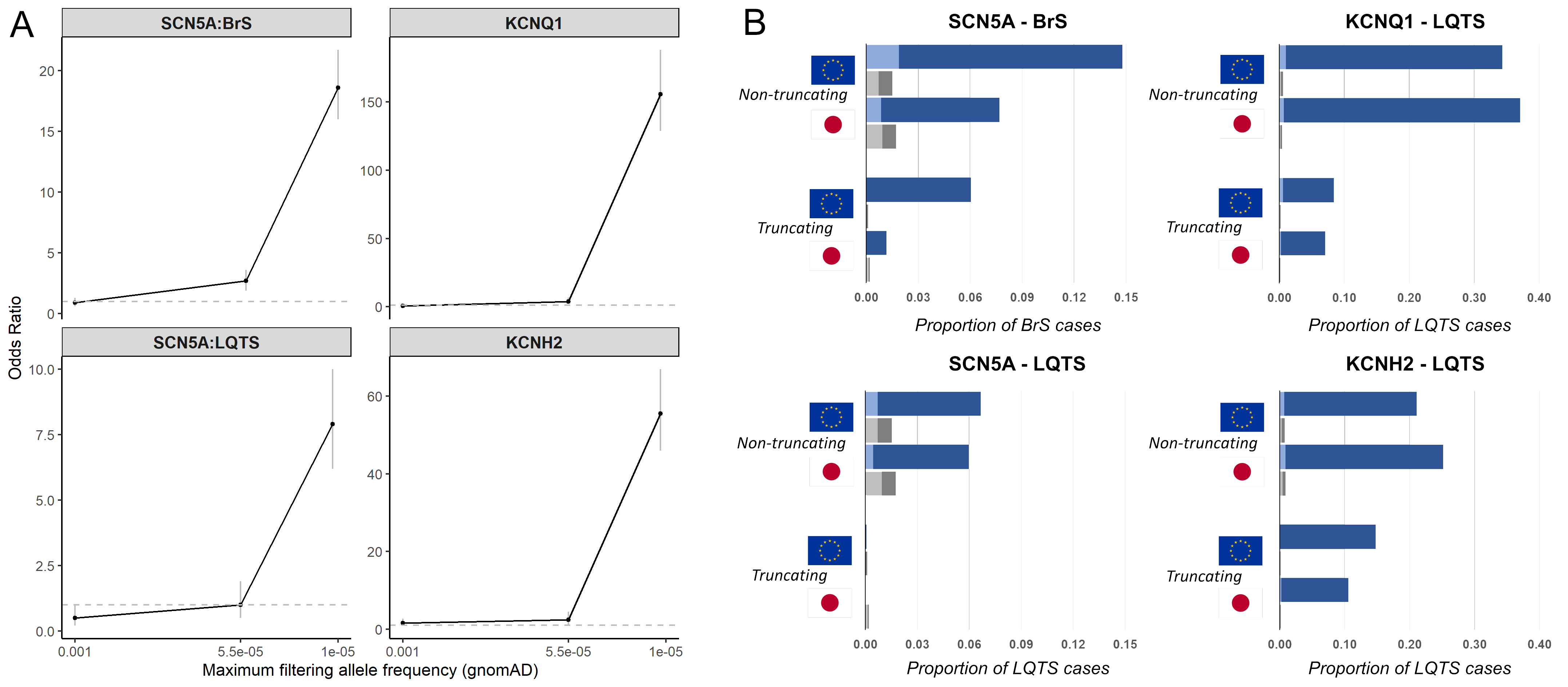
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**FIGURES**



*Figure 1: For rare non-truncating variants in autosomal dominant disease, evidence classes and ACMG/AMP rules (rule codes from Richards et al*3*) can be broadly grouped by their power to distinguish between pathogenic and benign variants (y-axis) and the likelihood that such evidence will be available (x-axis). Variant-specific evidence (such as co-segregation in family pedigrees) is powerful but often unavailable for genetically heterogeneous diseases. Supporting evidence (such as population frequency) can be applied to most variants but is rarely sufficient for definitive classification. If available, data from case-control studies, relating to enrichment of specific variants or classes of variants, provides powerful gene/disease-specific evidence and helps to address the high false negative rate associated with stringent contemporary guidelines.*



*Figure 2: (A) The odds ratio for disease-association for LQTS (KCNQ1, KCNH2, SCN5A) and BrS (SCN5A) stratified by filtering allele frequency, based on the prevalence of rare variants in the European arrhythmia cohorts and gnomAD exomes. Data for each bin is plotted at the upper frequency cut-off. Error bars represent 95% confidence intervals. The dashed grey line indicates an OR of 1. (B) Proportion of cases in the BrS and LQTS European and Japanese cohorts with rare non-truncating (missense and inframe insertions/deletions) and truncating (frameshift, nonsense, splice) variants (blue) and comparison to the frequency of such variants in population-specific gnomAD datasets (grey). The darker shades indicate the rarest variants corresponding to an estimated penetrance of ≥50% (FAF<1.0x10-5 and 1.1x10-5 for BrS and LQTS respectively), while the lighter shades represent variants in the 10-50% penetrance range.*

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*Figure 3: (A) Distribution of rare, non-truncating variants in the primary BrS and LQTS cohorts across the domains of KCNQ1, KCNH2 and SCN5A and equivalent variant classes in gnomAD (full dataset). Domain coordinates are derived from UniProt entries, with the exception of the KCNQ1 C-terminus highly conserved regions (from Kapplinger et al*9*) and the KCNH2 N-terminus cluster (based on variant distribution observed in this cohort and published referral cohort*18*). Regions with poor coverage in gnomAD exome sequencing, and therefore excluded from EF calculations, are in white. Darker grey indicates higher variant density (overlapping variants not plotted separately). The coordinates describe amino-acid position. (B-D) Effect of case-control evidence (PM1/PS4 rules) on ACMG/AMP classification of rare non-truncating variants. For BrS, the proportion of cases with pathogenic, likely pathogenic and VUS variants are displayed before and after use of these evidence classes, for European (B) and Japanese (C) cases. Classification using case-control evidence for both European and Japanese LQTS cases is shown in (D). The sensitivity of variant classification methods can be measured by comparison to the rate of rare benign variation in gnomAD (grey) – any excess beyond this is expected to reflect pathogenic variation in cases and therefore represents the target cohort yield of pathogenic variants.*

**TABLES**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| ***SCN5A* Region/Domain** | **Amino acid residues** | **European BrS spontaneous** **Type 1 ECG vs gnomAD-NFE** | | |  | **European BrS induced Type 1 ECG vs gnomAD-NFE** | | |  | **European BrS all cases vs gnomAD-NFE** | | |  | **Japanese BrS cases vs gnomAD-EAS** | | |
| **gnomAD** | **BrS** | **EF** | **gnomAD** | **BrS** | **EF** | **gnomAD** | **BrS** | **EF** | **gnomAD** | **BrS** | **EF** |
| Transmembrane regions | 132-410, 718-938, 1207-1466, 1530-1771 | 0.31% | 14.67% | 0.982 | 0.31% | 7.78% | 0.964 | 0.31% | 10.58% | 0.974 | 0.37% | 4.28% | 0.918 |
| Transmembrane - pore segments | 273-389, 857-917, 1354-1444, 1675-1748 | 0.09% | 8.22% | 0.989 | 0.09% | 3.89% | 0.977 | 0.09% | 5.58% | 0.984 | 0.17% | 2.14% | 0.924 |
| Transmembrane - other regions | - | 0.21% | 6.44% | 0.969 | 0.21% | 3.89% | 0.947 | 0.21% | 5.00% | 0.959 | 0.20% | 2.14% | 0.908 |
| N-terminus | 1-131 | 0.05% | 0.78% | 0.931 | 0.05% | 0.49% | 0.889 | 0.05% | 0.58% | 0.908 | 0.07% | 0.11% | 0.373 |
| Interdomain Linkers | 411-717, 939-1206, 1467-1529 | 0.32% | 1.00% | 0.684 | 0.32% | 0.69% | 0.544 | 0.32% | 0.79% | 0.600 | 0.24% | 0.43% | 0.448 |
| C-terminus | 1772-2016 | 0.11% | 0.67% | 0.833 | 0.11% | 0.35% | 0.679 | 0.11% | 0.46% | 0.757 | 0.12% | 0.43% | 0.713 |

*Table 1: Etiological fraction (EF) values for gene regions/domains in SCN5A based on comparison of rare (FAF<1x10-5) non-truncating variants in BrS and gnomAD population cohorts. EF values are coloured according to the PM1 rule activated – strong (red), moderate (orange), supporting (green) and none (black). The comparisons shown are European BrS cases with a spontaneous* *Type 1 ECG (n=900) vs gnomAD-NFE, European BrS cases with an induced Type 1 ECG (n=1440) vs gnomAD-NFE, all European BrS cases (n=2400) vs gnomAD-NFE and Japanese BrS cases (n=935) vs gnomAD-EAS (see Table S7 for full details). This evidence should be used only for non-truncating SCN5A variants detected in patients with BrS as follows: 1) check the variant is rare (gnomAD FAF<1x10-5), 2) select the appropriate comparison depending on patient ethnicity (European ancestry or Japanese) and type 1 ECG pattern of the patient (spontaneous, induced or use “all cases” if unknown), 3) select the PM1 evidence level (based on EF) depending on the SCN5A region/domain where the variant is located.*

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Gene** | **Region/Domain** | **Amino acid residues** | **European LQTS cohort vs gnomAD-NFE** | | |  | **Japanese LQTS cohort vs gnomAD-EAS** | | |  | **Referral LQTS cohort vs gnomAD-ALL** | | |
| **gnomAD** | **LQTS** | **EF** | **gnomAD** | **LQTS** | **EF** | **gnomAD** | **LQTS** | **EF** |
| *KCNQ1* | Transmembrane/Linker/Pore | 122-348 | 0.10% | 22.31% | 0.997 | 0.11% | 26.05% | 0.997 | 0.10% | 7.68% | 0.988 |
| N-terminus | 71-121 | 0.04% | 0.65% | 0.940 | 0.02% | 0.00% | - | 0.03% | 0.24% | 0.887 |
| C-terminus: Highly conserved regions | 349-391, 509-575, 585-607 | 0.05% | 8.68% | 0.995 | 0.05% | 5.74% | 0.992 | 0.05% | 3.24% | 0.986 |
| C-terminus: Other regions | 392-508, 576-584, 608-676 | 0.11% | 0.86% | 0.879 | 0.07% | 0.88% | 0.923 | 0.10% | 0.44% | 0.765 |
|  | | | | | | | | | | | | | |
| *KCNH2* | Transmembrane/Linker/Pore | 404-659 | 0.07% | 10.83% | 0.994 |  | 0.06% | 12.36% | 0.996 |  | 0.06% | 3.68% | 0.985 |
| N-terminus: Cluster | 1-130 | 0.03% | 4.38% | 0.994 | 0.02% | 5.74% | 0.996 | 0.03% | 1.72% | 0.984 |
| N-terminus: Other regions | 131-156, 306-403 | 0.11% | 0.93% | 0.878 | 0.06% | 1.10% | 0.949 | 0.11% | 0.48% | 0.773 |
| C-terminus: cNBD | 742-842 | 0.04% | 2.15% | 0.981 | 0.02% | 2.43% | 0.991 | 0.03% | 0.60% | 0.946 |
| C-terminus: Other regions | 660-741, 843-1159 | 0.20% | 2.08% | 0.906 | 0.24% | 2.65% | 0.913 | 0.20% | 0.80% | 0.751 |
|  | | | | | | | | | | | | | |
| *SCN5A* | Transmembrane/Linker/Pore | 132-410, 718-938, 1207-1466, 1530-1771 | 0.33% | 1.79% | 0.818 |  | 0.38% | 2.43% | 0.848 |  | 0.34% | 1.84% | 0.820 |
| N-terminus | 1-131 | 0.05% | 0.07% | 0.247 | 0.07% | 0.00% | - | 0.06% | 0.20% | 0.678 |
| Interdomain linkers | 411-717, 939-1206, 1467-1529 | 0.30% | 0.65% | 0.540 | 0.24% | 0.88% | 0.734 | 0.28% | 1.00% | 0.725 |
| C-terminus | 1772-2016 | 0.11% | 0.93% | 0.879 | 0.13% | 0.66% | 0.798 | 0.11% | 0.76% | 0.857 |

*Table 2: Etiological fraction (EF) values for gene regions/domains in KCNQ1, KCNH2 and SCN5A based on comparison of rare (FAF<1.1x10-5) non-truncating variants in LQTS and gnomAD population cohorts. EF values are coloured according to the PM1 rule activated – strong (red), moderate (orange) and supporting (green). The comparisons shown are European LQTS cases (n=1394) vs gnomAD-NFE, Japanese LQTS cases (n=453) vs gnomAD-EAS and the published LQTS referral cohort (n=2500) vs gnomAD-ALL.* *It is uncertain whether the absence of KCNQ1 N-terminus variants in Japanese cases reflects a genuine difference between populations or is due to technical sequencing issues for this GC-rich region (see Table S8 for full details). This evidence should be used only for non-truncating KCNQ1, KCNH2 or SCN5A variants detected in patients with (or being genetically tested for) LQTS as follows: 1) check the variant is rare (gnomAD FAF<1.1x10-5), 2) select the appropriate comparison – patients diagnosed with LQTS of European ancestry, patients diagnosed with LQTS of Japanese ancestry or individuals of any ethnicity referred for LQTS genetic testing, 3) select the PM1 evidence level (based on EF) depending on the gene and region/domain where the variant is located.*