Daniel Schorderet ORCID iD: 0000-0002-1331-504X

Isabelle Audo ORCID iD: 0000-0003-0698-5309

Where are the missing gene defects in inherited retinal disorders? Intronic and synonymous variants contribute at least to 4% of *CACNA1F*-mediated inherited retinal disorders

Christina Zeitz¹, Christelle Michiels¹, Marion Neullé¹, Christoph Friedburg², Christel Condroyer¹, Fiona Boyard¹, Aline Antonio^{1,3}, Nassima Bouzidi¹, Milicevic D¹, Robin Veaux¹, Aurore Tourville¹, Axelle Zoumba¹, Imene Seneina¹, Marine Foussard¹, Camille Andrieu³, Markus Preising², Steven Blanchard⁴, Jean-Paul Saraiva⁴, Lilia Mesrob⁵, Edith Le Floch⁵, Claire Jubin⁵, Vincent Meyer⁵, Hélène Blanché⁶, Anne Boland⁵, Jean-François Deleuze^{5,6}, Dror Sharon⁷, Isabelle Drumare⁸, Sabine Defoort-Dhellemmes⁸, Elfride De Baere E⁹, Bart P Leroy^{9,10,11}, Xavier Zanlonghi¹², Ingele Casteels¹³, Thomy J de Ravel¹⁴, Irina Balikova^{10,15}, Rob Koenekoop¹⁶, Fanny Laffargue¹⁷, Rebecca McLean¹⁸, Irene Gottlob¹⁸, Dominique Bonneau^{19,20}, Daniel F. Schorderet^{21,22,23}, Francis Munier²¹, Martin McKibbin²⁴, Katrina Prescott²⁵, Valerie Pelletier^{26,27}, Hélène Dollfuss^{26,27,28}, Yaumara Perdomo-Trujillo²⁶, Céline Faure³, Charlotte Reiff²⁹, Bernd Wissinger³⁰, Isabelle Meunier^{31,32}, Susanne Kohl³⁰, Eyal Banin⁷, Eberhart Zrenner^{30,33}, Bernhard Jurklies³⁴, Birgit Lorenz², José-Alain Sahel^{1,3,35,36,37}, Isabelle Audo^{1,3,38}

¹Sorbonne Université, INSERM, CNRS, Institut de la Vision, Paris, France.

²Department of Ophthalmology, Faculty of Medicine, Justus-Liebig-University Giessen, Germany.

³CHNO des Quinze-Vingts, DHU Sight Restore, INSERM-DGOS CIC 1423, Paris, France.

⁴IntegraGen SA, Genopole Campus, Evry, France.

⁵ Centre National de Recherche en Génomique Humaine (CNRGH), Institut de Biologie François Jacob, CEA, Université Paris-Saclay, Evry, France.

⁶Fondation Jean Dausset-CEPH (Centre d'Etude du Polymorphisme Humain), Paris, France. ⁷Department of Ophthalmology, Hadassah-Hebrew University Medical Center, Jerusalem, 91120, Israel.

⁸Service d'exploration de la vision et neuro-ophtalmologie, CHRU de Lille, Lille, France.

⁹Center for Medical Genetics, Ghent University and Ghent University Hospital, Ghent, Belgium.

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¹⁰Department of Ophthalmology, Ghent University and Ghent University Hospital, Ghent, Belgium.

¹¹Division of Ophthalmology, The Children's Hospital of Philadelphia, Philadelphia, PA, USA.

¹²Clinique Jules Verne, Centre de compétence maladies rares, Nantes, France.

¹³Department of Ophthalmology, University Hospitals Leuven, Leuven, Belgium.

¹⁴Centre for Human Genetics, University Hospitals Leuven, Belgium.

¹⁵Department of Ophthalmology, Queen Fabiola Children's University Hospital, Brussels, Belgium.

¹⁶Department of Ophthalmology, Jewish General Hospital, Montreal, Quebec, Canada.

¹⁷Service de génétique, CHU Clermont-Ferrand, France.

¹⁸Ulverscroft Eye Unit, Department of Neuroscience, Psychology and Behaviour, University of Leicester, Leicester, UK.

¹⁹Département de Biochimie et Génétique, Centre Hospitalier Universitaire, Angers, France.

²⁰Mitovasc, UMR CNRS 6015-INSERM 1083, Université d'Angers, France.

²¹Jules-Gonin Eye Hospital, Department of Ophthalmology, University of Lausanne, Lausanne, Switzerland.

²²IRO-Institute for Research in Ophthalmology, Sion, Switzerland.

²³Faculty of Life Sciences, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland.

²⁴Department of Ophthalmology, St. James's University Hospital, Leeds, United Kingdom.

²⁵Department of Clinical Genetics, Leeds, United Kingdom.

²⁶Centre de référence pour les Affections Rares en Génétique Ophtalmologique (CARGO), Hôpital Civil, Strasbourg, France.

²⁷Service de Génétique Médicale, Hôpital de Hautepierre, Strasbourg, France.

²⁸Laboratoire de Génétique Médicale, INSERM U1112, Strasbourg, France.

²⁹Medical Practice Stadttheater, Freiburg, Germany.

³⁰Institute for Ophthalmic Research, Centre for Ophthalmology, University of Tübingen, Tübingen, Germany.

³¹Centre de Référence Maladies Sensorielles Génétiques, Hôpital Gui de Chauliac, Montpellier, France.

³²Montpellier University and INSERM U1051, Institute for Neurosciences of Montpellier, France.

³³Werner Reichardt Center for Integrative Neuroscience, University of Tübingen, Tübingen, Germany.

³⁴Department of Ophthalmology, University of Essen, Essen, Germany

³⁵Fondation Ophtalmologique Adolphe de Rothschild, Paris, France

³⁶Academie des Sciences, Institut de France, Paris, France

³⁷Department of Ophthalmology, The University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, United States

³⁸Institute of Ophthalmology, University College of London, London, United Kingdom

Corresponding author:

Christina Zeitz PhD, DR2

INSERM, UMR_S968

CNRS, UMR_7210

Sorbonne University

Institut de la Vision

Department of Genetics

17, rue Moreau

75012 Paris

France

Tel.: +33 1 53 46 25 40

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Abstract

Inherited retinal disorders (IRD) represent clinically and genetically heterogeneous diseases. To date, pathogenic variants have been identified in ~ 260 genes. Albeit that many genes are implicated in IRD, for 30-50% of the cases the gene defect is unknown. These cases may be explained by novel gene defects, by overlooked copy number variations, by variants in intronic or promoter regions or represent synonymous variants of known genes contributing to the dysfunction of the respective proteins. Patients with one subgroup of IRD, namely incomplete congenital stationary night blindness (icCSNB) show a very specific phenotype. The major cause of this condition is the presence of a hemizygous pathogenic variant in *CACNA1F*. A comprehensive study applying direct Sanger sequencing of gene coding regions, exome and genome sequencing applied to a large cohort of patients with a clinical diagnosis of icCSNB revealed indeed that 7 of 189 *CACNA1F*-related cases have intronic and synonymous disease causing variants leading to missplicing as validated by mini-gene approaches. These findings highlight that gene

locus sequencing may be a very efficient method in detecting disease-causing variants in clinically well characterized patients with a diagnosis of IRD, like icCSNB.

Graphical Abstract

Key words: IRD, icCSNB, *CACNA1F*, gene defect, intronic variants, synonymous variants, min-gene approach

Introduction

Inherited retinal disorders (IRD) are clinically and genetically heterogeneous diseases (Berger, et al., 2010; Sahel, et al., 2014). Today, disease causing variants have been identified in ~ 260 genes (<https://sph.uth.edu/retnet/>). Although variants in 50-70% of cases could explain the disease (e.g. (Daiger, et al., 2018)), for the remaining cases the genetic cause remains to be identified. These cases may be explained by novel gene defects, by overlooked duplications or deletions, by variants in intronic or promoter regions or represented by synonymous variants acting on splicing or regulation in known genes contributing to the dysfunction of the respective proteins (Carss, et al., 2017; Jamshidi, et al., 2018; Van Cauwenbergh, et al., 2017; Van Schil, et al., 2018; Zeitz, et

al., 2013). Congenital stationary night blindness (CSNB) is one form of IRD, which itself is clinically and genetically heterogeneous. It is a non-progressive retinal disorder which can affect visual acuity in dim light, but also day light conditions. In addition, patients frequently present with myopia, nystagmus and strabismus (Zeitzi, et al., 2015). Most of the patients have a normal fundus but show a so-called Schubert Bornschein electroretinogram (ERG), with an electronegative waveform composed of a normal a-wave and a reduced b-wave under scotopic conditions (Schubert and Bornschein, 1952). ERG can further subdivide CSNB into complete and incomplete CSNB (cCSNB and icCSNB) (Miyake, et al., 1986). Patients with icCSNB are characterized by both ON- and OFF-bipolar cell dysfunctions (reviewed in (Zeitzi, et al., 2015)). Pathogenic variants in *CACNA1F* (MIM# 300110) and *CABP4* (MIM# 608965) were identified in patients with icCSNB (reviewed in (Zeitzi, et al., 2015)). Patients with variants in *CACNA2D4* (MIM# 608171) show a phenotype with some overlapping features as patients with icCSNB (reviewed in (Zeitzi, et al., 2015)). Of note, light sensitivity may be one major symptom in patients with variants in *CACNA1F*, *CABP4* and *CACNA2D4* in addition to phenotypic variability sometimes leading to more progressive IRD and thus the term icCSNB may be misleading (reviewed in (Zeitzi, et al., 2015)). More than 150 different variants in *CACNA1F* and only a few in *CABP4* and *CACNA2D4* lead to icCSNB or similar phenotypes (reviewed and summarized in 2015 in (Zeitzi, et al., 2015), additional publications since then (Ba-Abbad, et al., 2016; Hove, et al., 2016; Huang, et al., 2016; Huang, et al., 2015; Patel, et al., 2016; Sun, et al., 2015; Xu, et al., 2015; Zhao, et al., 2015; Zhou, et al., 2015)). The pathogenic variant spectrum comprises missense, nonsense and splice site variants, small insertions and deletions, gross deletions and complex rearrangements with most of the variants representing missense or nonsense variants (HGMD_Pro (Stenson, et al., 2014; Zeitzi, et al., 2015)). Most of the published splice site variants affect canonical splice acceptor or donor sites. Only two variants have been described, which are located $> +3$ or < -3 in the intronic region of *CACNA1F* (Xu, et al., 2015; Zeitzi, et al., 2015). This might be due to selection bias, since variants $> +3$ or < -3 may be classified as variant of unknown significance.

Although for most of the patients with icCSNB the genetic cause has been solved, for a few cases the gene defect still needs to be identified. The purpose of the study presented herein was to identify the genetic cause in patients of our icCSNB cohort of 189 cases and investigate if genetically unsolved patients with a clear icCSNB phenotype may reveal overseen variants in *CACNA1F*. Those may be found in intronic regions or represent synonymous variants affecting splicing. Putative candidate variants were validated by mini-gene approaches for seven cases and the prevalence of these causes determined in *CACNA1F*-mediated IRD.

Materials and Methods

Clinical examination and DNA collection

The 189 male index cases with icCSNB from a large world-wide multi-center cohort were clinically investigated as previously described (Zeitzi, et al., 2015). Prior to genetic

testing, informed consent was obtained from each participant. Research procedures adhered to the tenets of the Declaration of Helsinki and were approved by the local Ethics Committees of the different centers. Blood samples of index patients and available family members were collected for genetic research and genomic DNA was extracted by standard methods over a period of 18 years.

Targeted Sanger sequencing

All patients diagnosed with icCSNB were initially screened for variants in *CACNA1F* (NM_005183.3). More specifically, variant analysis of the coding and flanking intronic regions (~50 bps) by direct Sanger sequencing of *CACNA1F*, *CABP4* (NM_145200.3) and *CACNA2D4* (NM_172364.4) was performed in all cases of a clear icCSNB phenotype. In addition, index male patients suggestive for an X-linked mode of inheritance and a clinical diagnosis of CSNB of unspecified type, icCSNB or cCSNB, were screened for variants in *CACNA1F* and/or *NYX* (MIM# 300278, NM_022567.2) by the same sequencing method. Detailed protocols are available on request. Putative disease-causing variants identified by targeted next generation sequencing (NGS), exome sequencing (ES) and genome sequencing (GS) were validated by Sanger sequencing. Similarly, co-segregation analyses in all available family members were performed by Sanger sequencing.

Targeted next generation sequencing and exome sequencing

After exclusion of variants in known genes underlying icCSNB by Sanger sequencing, all family members of family A and B (Figure 1) were investigated by targeted NGS and ES by a company (Integrigen, Evry, France) (Audo, et al., 2012; Zeitz, et al., 2013). Exons of DNA samples were captured and investigated as shown before with in-solution enrichment methodology (SureSelect Clinical Research Exome (Family A) and SureSelect Human All Exon V5 (Family B), Agilent, Massy, France) and NGS (Illumina HISEQ, Illumina, San Diego, CA, USA)(Zeitz, et al., 2013). For all subjects of family A, overall ES coverage of the captured regions was 86,67% for 25x depth of coverage resulting in a mean sequencing depth of 92x per base. For all subjects of family B, overall ES coverage of the captured regions was 87,25 % for 25x depth of coverage resulting in a mean sequencing depth of 75%. Base calling was performed with Real Time Analysis software (Illumina)(Zeitz, et al., 2013). Genetic variation annotations were realized by an in-house pipeline (IntegraGen), and results were provided per sample or family in tabulated text files. After exclusion of variants in *CACNA1F* and in *NYX*, the index patient of family C (CIC05614, Family C, III.1, Figure 1) was investigated for variants in exons of 254 genes known to be associated with IRD or candidate genes by targeted NGS as previously described (Audo, et al., 2012).

Genome sequencing

After exclusion of variants in the coding regions of *CACNA1F* and *NYX* by Sanger sequencing and in 254 genes known to be associated with IRD by targeted NGS, all family members of family C were investigated by genome sequencing (GS) by the “Centre National de Recherche en Génomique Humaine” (Institut de Biologie François Jacob, CEA, Evry, France). After a complete quality control, genomic DNA (1µg) was

used to prepare a library for GS, using a kit (Illumina TruSeq DNA PCR-Free Library Preparation Kit, Illumina Inc., CA, USA) according to the manufacturer's instructions. After normalization and quality control, qualified libraries were sequenced on a HiSeq. 2000 platform (Illumina Inc) as paired-end 100 bp reads. At least 3 lanes of a HiSeq. 2000 flow cell were used for each sample, in order to reach an average sequencing depth of 30 x per sample. Sequence quality parameters were assessed throughout the sequencing run and standard bioinformatics analysis of sequencing data was based on a pipeline (Illumina) to generate .fastq files for each sample. Variant calling and annotation was performed as follows: .fastq files alignment on the human genome (GRCh37) [and decoy (Heng Li's hs37d5 genome for 1000genomes project, ftp://ftp.ncbi.nlm.nih.gov/1000genomes/ftp/technical/reference/phase2_reference_assembly_sequence/hs37d5.fa.gz)] was performed using bwa software (mem+default option, <https://github.com/lh3/bwa> version 0.7.12) (Li and Durbin, 2009). Duplicate sequences were referenced and eliminated from the .bam files using Sambamba tools (<http://lomereiter.github.io/sambamba/docs/sambamba-view.html>). An additional step of realignment was performed on the .bam file using GATK programs (RealignerTargetCreator/IndelRealigner). Coverage analyses was generated using an in-house pipeline based on metrics generated by Bedtools programs (<http://code.google.com/p/bedtools/>) (Quinlan and Hall, 2010). The identification of variants was performed using 4 programs: UnifiedGenotyper and HaplotypeCaller from GATK (McKenna, et al., 2010), Platypus (<http://www.well.ox.ac.uk/platypus>) (Rimmer, et al., 2014) and Samtools (<http://samtools.sourceforge.net/>). Results generated by these 4 programs were then grouped in a .vcf file. Annotation of the vcf file was carried out using snpEff and snpSift (<http://snpeff.sourceforge.net> and <http://snpeff.sourceforge.net/SnpSift.html>) (Cingolani, et al., 2012) based on data available in the Ensembl database (<http://www.ensembl.org/index.html>) and dbNSFP database (<https://sites.google.com/site/jpopgen/dbNSFP>). *De novo*, hemizygous, homzygous and compound heterozygous SNPs were identified by an in-house program.

Haplotyping:

In order to compute haplotypes of the X chromosome, VCFtools we first used (<http://vcftools.sourceforge.net/>) to convert the .vcf file of the family into the transposed linkage file format, readable by the PLINK software (<https://www.cog-genomics.org/plink2>) (Danecek, et al., 2011) . After selection of the SNPs with known genetic distances on the X-chromosome (38071 SNPs), haplotype analysis was performed applying Merlin (<http://csg.sph.umich.edu/abecasis/merlin/index.html>) (Abecasis, et al., 2002) using the minx option designed for X- chromosome haplotyping. The analysis was carried out on six family members of Family C: the father and the maternal grandparents of the affected nephew had been created with missing genotype values, so that Merlin could take into account all familial relationships.

To be able to visualize the haplotypes with HaploPainter (<http://haploPainter.sourceforge.net/index.html>) (Thiele and Nurnberg, 2005), they were computed with Merlin on a smaller region of the X-chromosome (48.7Mb) including 10000 SNPs with known genetic distances, centered on the position of the variant of interest c.522-16G>A .

Filtering to determine putative disease-causing variants

Initially, very stringent filtering criteria were used to select most likely pathogenic variant(s): only nonsense, missense, canonical splice site variants (intronic region $\leq +$ and $- 3$) or small deletions or insertions (InDels), leading to a frameshift, with a minor allelic frequency ≤ 0.005 in Exome Variants Server (EVS, <http://evs.gs.washington.edu/EVS/>), HapMap (<http://hapmap.ncbi.nlm.nih.gov/>), 1000Genomes (<http://www.1000genomes.org/>), Exome Aggregation Consortium (ExAC, <http://exac.broadinstitute.org/>) and Genome Aggregation Database (gnomAD <http://gnomad.broadinstitute.org/>) were considered to be putative disease-causing. Variant pathogenicity including splice site effect prediction was used with programs available within the Alamut software (Interactive Biosoftware, Rouen, France; <http://www.interactive-biosoftware.com/alamut-visual>), including Polymorphism Phenotyping v2 (PolyPhen2, <http://genetics.bwh.harvard.edu/pph2/>) and Sorting Intolerant From Tolerant (SIFT, <https://sift.bii.a-star.edu.sg/>). Polyphen2 scores ranging from 0.0 to 0.15 are predicted to be benign, ranging from 0.15 to 1.0 are possibly damaging and ranging from 0.85 to 1.0 are more confidently predicted to be damaging. SIFT Scores range from 0 to 1. The amino acid substitution is predicted to be damaging if the score is ≤ 0.05 , and tolerated if the score is > 0.05 . Variants were only kept if at least one of the programs predicted the variant to be disease causing. Subsequently the same filters were used but also taking into consideration intronic and synonymous exonic variants. The following splice site predictions programs are integrated in the Alamut software: MaxEntSca is a method based on the Maximum Entropy principle (Yeo and Burge, 2004). NNPLICE (available at the Berkeley Drosophila Genome Project web site: http://www.fruitfly.org/seq_tools/splice.html) is a prediction method based on neural networks (Reese, et al., 1997). HSF, Human Splicing Finder is based on position weight matrices with some position-dependent logic (Desmet, et al., 2009). As higher the numbers are as more is the splice site predicted to be affected.

Candidate gene analysis

Candidate genes harboring putative pathogenic variants predicted either by polyphen2 or SIFT passing our stringent filtering were investigated for their retinal or ocular expression in transcriptomic databases as previously described (Zeit, et al., 2015).

Mini-gene approach

To test the pathogenic effect on splicing for the intronic and synonymous variants identified in *CACNA1F* a mini-gene approach as described before (Orhan, et al., 2013) was used. Surrounding exons and introns of the variants identified in the patients was amplified on patient and control genomic DNA (Figures 1 and 3, Supp. Figure S1, Supp. Table S1). More specifically: for Family A with a variant in intron 11 (c.1496+5G>C, 132.01, III.1, Family A, Figure 1) intron 9 - intron 12 was amplified. For Family B with a variant in intron 36 (c.4294-9G>A, 8798, II.1, Family B, Figure 1) intron 33 - intron 39 was amplified. For Family C with a variant in intron 4 (c.522-16G>A CIC04152, II.3 and CIC05614, III.1, Family C, Figure 1) intron 2 - intron 6 was amplified. For Family D with a variant in intron 2 (c.276-15C>A, 5875, II.1, Family D, Figure 1) 5'UTR - intron 4 was amplified. For Family E with a variant in intron 17 (c.2321+5G>T, CH880, II.1, Family E, Figure 1) intron 16 - intron 19 was amplified. For Family F with a variant in exon 5 (c.646C>T, CIC05358, II.1, Family F, Figure 1) intron 2 - intron 6 was amplified.

For Family G with a variant in exon 14 (c.1719G>A, 1101_1, II.1, Family G, Figure 1) intron 12 - intron 14 was amplified. The amplicons were either subcloned in a vector (pCR®-Blunt vector, Invitrogen, Thermo Fischer Scientific, Villebon-sur- Yvette, France) and subsequently cloned in an overexpression vector (pBudCE4.1 vector, Invitrogen) or directly cloned in this overexpression vector. The inserts were validated by Sanger sequencing (Supplementary Table S1). Transient transfection studies were performed in COS-1 cells (cell lines derived from monkey kidney tissue) in 6-well plates and total RNA was extracted using a kit (RNeasy Mini Qiagen, Hilden, Germany). Reverse transcription was performed using a reverse transcriptase (Quanti Tect® Reverse Transcription Kit, Qiagen, Courtaboeuf, France). To analyze the *in vitro* splicing products, a PCR was performed using oligonucleotides specific to exons surrounding the variants in *CACNA1F* (Supp. Table S2) with a DNA polymerase (HOT FIREPol®, Solis BioDyne, Tatu, Estonia). Ten microliters of the RT-PCR products were investigated by electrophoresis on a 2% agarose gel and visualized with a gel documentation machine (Molecular Imager® Gel Doc™ XR+ Sytem with Image Lab™ Software, BIO RAD, Life Science, Marnes-la-Coquette, France). Prominent bands were either purified (NucleoSpin Gel or PCR Clean-up®, Machery-Nagel) or directly cloned in a vector (Topo-TA dual cloning vector, pCR™II-TOPO® vector, Invitrogen, Thermo Fischer Scientific, Villebon sur Yvette, France). Several clones per condition were picked, plasmids prepared and sequenced with vector or internal primers (Supp. Tables S1 and S2).

Results

Phenotype of patients with icCSNB and variants in *CACNA1F*

The phenotype of all patients investigated herein was either reported as showing icCSNB or was not specified in detail. Targeted Sanger sequencing, NGS or ES revealed in 182 cases variants in *CACNA1F* (Table 1). This includes patients previously published and reviewed (Zeitz, et al., 2015) but also novel cases harboring known and 63 novel variants in *CACNA1F* (Table 1). The variant spectrum comprises missense, splicing, small deletion and insertion variants as previously reported (Zeitz, et al., 2015), all predicted to be pathogenic by different *in silico* analyses. Albeit they occurred rarely in the general populations, a few appeared 1-3 times hemizygous in the control population (gnomAD, Table 1), which might be due to undiagnosed cases in the latter group. The seven remaining cases (Family A, B, C, D, E and F) did not reveal obvious disease causing variants in *CACNA1F*, *CABP4* nor *CACNA2D4*. However the clinical diagnosis pointed indeed to the icCSNB phenotype. The detailed clinical description of the male index 132.01 of Family A is given as an example (Figure 2). He showed a normal a-wave in scotopic and photopic conditions with a reduced b-wave resulting in an electronegative ERG waveform. The light-adapted 3.0 ERG and light-adapted 30Hz-flicker ERG responses were severely reduced and delayed. At the age of 14 he revealed a best corrected visual acuity (BCVA) of 0.5 oculus uterque (o.u), spherical equivalent (s.e.). - 11.75/ -10.9 and at 26 years of 0.1/0.32, s.e.- 16/ -16 with fundus changes typical for myopia associated with icCSNB at both ages (Figure 2). The index patient 8798 of family B was also reported to have icCSNB. The phenotype of index patient CIC04152 of family C was initially unclear, since only the ERG of the young nephew, CIC05614 was

available (Audo, et al., 2012). More recent investigations however, revealed for both affected individuals of family C phenotypic characteristics in keeping with icCSNB. The sporadic cases, 5875 of family D, CH880 of family E (Zeitz, et al., 2015) and CIC05358 of family F were reported to have icCSNB. Similarly, the sporadic case 1100_1 of family G showed an icCSNB phenotype as well.

Genetic investigation of patients and validation of splice site defect

Direct sequencing of the coding regions of known genes underlying CSNB in index patients of family A, B and C did not identify any disease-causing variant.

Family A

ES on all available family members of family A (Figure 1) applying stringent filtering investigating exons and canonical intronic regions identified only variants for which the predictions were benign. However, when intronic variants beyond 3 nucleotides from exon boundaries were kept and selecting specifically variants in genes shown to be mutated in icCSNB, a c.1496+5G>C variant in intron 11 of *CACNA1F* was identified, which was never reported before. It co-segregated with the phenotype, as confirmed by Sanger sequencing (Figure 1, Table 2). Different programs predicted that this variant influences the donor splice site (Table 2, Figure 3, Family A). A mini-gene approach including flanking intronic regions of genomic sequences encompassing exon 10, 11 and 12 and subsequent RT-PCR experiments revealed a different transcript profile in the mutant setting when compared to the control (Figure 3, Family A). Although, both the control and the mutant mini-gene construct seem to lead to products lacking exon 11 (120 bp), it was only in the control that a transcript harboring the correctly spliced exon 10, 11 and 12 (211 bp) was present. These findings were confirmed by Sanger sequencing. Other higher molecular weight bands, similarly or differentially present were not further investigated. Indeed, at the donor splice site, variants affecting the G residue at position 1 are the most common, followed by variants at position 5. Variants at these two positions are thought to significantly reduce the pairing of the donor splice site with the complementary site in the small nuclear ribonucleoprotein particle U1snRNP, which is one of the first steps in the complex process of mRNA splicing (Kramer, 1996; Wittop Koning and Schumperli, 1994). These findings suggest that the icCSNB phenotype in this patient is due to the absence of the correctly spliced *CACNA1F* transcript encompassing exons 10, 11 and 12.

Family B

ES performed on the affected index, on one unaffected sibling and on the parents of Family B (Figure 1) applying stringent filtering investigating exons and canonical intronic regions, did not identify any putative disease causing variant underlying the icCSNB phenotype. However, many candidate genes were investigated in more detail (Supp. Tables S3-S5). At one point compound heterozygous variants in *UNC80* (MIM# 612636, MIM# 616801) (Perez, et al., 2016; Shamseldin, et al., 2016), recently associated with a severe syndrome including an eye phenotype were considered as a possible cause for icCSNB (Supp. Table S5). However, due to the absence of other severe phenotypes in

our patient with icCSNB, we do not consider that variants in *UNC80* were responsible for the isolated ocular phenotype observed in our patient. Thus, in the absence of promising disease causing variants, we softened our stringent filtering now including intronic variants beyond 3 nucleotides from exon boundaries and filtering specifically genes recently shown to be mutated in icCSNB or showing a similar phenotype. Indeed, a c.4294-9G>A variant in intron 36 of *CACNA1F* was identified, which had never been reported before (Table 2) and cosegregated with the phenotype (Figure 1, Table 2). Different programs predicted this variant to influence splicing by creating a novel splice acceptor site (Table 2, Figure 3, Family B). A mini-gene approach including flanking intronic regions of genomic sequences encompassing exon 34 to 39 and subsequent RT-PCR using primers located in exon 36 and 37 revealed a similar transcript, but a slightly larger mutant amplicon with a size of 295 bp instead of the expected size of 288 bp (Figure 3). Purification and sequencing of these transcripts revealed that the c.4294-9G>A *CACNA1F* variant created a novel acceptor splice site leading to 7 additional nucleotides from intron 36 (CCCACAG) as predicted for the variant (Figure 3, Family B, Table 2). In addition, amplicons containing exon 36, intron 36 and exon 37 were detected in both, mutant and control transcripts (Figure 3, Family B, 517 bp) indicating that in this *in vitro* system not all control transcripts show correctly spliced exon 36 to exon 37.

Family C

Sanger and targeted NGS of index patient CIC04152 of family C for which the phenotype was corrected from cCSNB to icCSNB did not identify the genetic cause (Audo, et al., 2012). To identify a putative novel gene defect underlying icCSNB, GS of all available family members showing X-linked mode of inheritance was performed (Figure 1). Selecting only variants on the X-chromosome identified in the index patient 155554 variants. By including only variants being hemizygous in the affected individuals and heterozygous in the sister of the index patient 34918 variants were found. Of those 3543 variants showed a minor allele frequency (MAF) ≤ 0.001 . Keeping only variants in genomic sequences of coding genes reduced the number of variants to 904 (data not shown). Interestingly one variant was indeed identified in *CACNA1F* (Table 2). This c.522-16G>A variant in intron 4 of *CACNA1F* had never been reported before. This variant co-segregated with the phenotype (Figure 1). Different programs predicted this variant to influence splicing (Table 2). A novel 3' acceptor splice site in intron 4 was predicted (c.522-14) (Supp. Figure S1, Family C). Similarly, haplotype analyses using data obtained from GS supported that the c.522-16G>A *CACNA1F* variant is most likely disease causing, as it lies in a region of at least 1.6 Mb, containing 100 SNPs, which is shared by the affected index and affected nephew, which is transmitted by the mother of the index patient (Supp. Figure S2). A mini-gene approach including flanking intronic regions of genomic sequences encompassing exon 3 to 6 and subsequent RT-PCR experiments revealed a different transcript profile in the mutant transcript when compared to the control (Supp. Figure S1, Family C). Sequencing of cloned products revealed different sequences. Interestingly, the expected transcript of the control transcript harboring exon 3, 4, 5 and 6 (533 bp) was almost invisible on an agarose gel and only one sequenced clone showed the expected sequence. Both, the control and mutant seem to reveal predominant products lacking exons 4 and 5 (250 bp). But only in the control a transcript harboring exon 3, 5 and 6 (393 bp) (lacking exon 4) was present,

while in the mutant situation transcripts harboring 14 nucleotides of intronic sequence of intron 4 (407 bp) were detected as predicted by a software (Supp. Figure S1, top, table 2). In addition, a transcript harboring 93 nucleotides of intronic sequence of intron 4 (486 bp) including the c.522-16G>A variant was found, indicating that the variant indeed creates alternative acceptor splice sites and thus different most likely disease-causing transcripts leading to altered CACNA1F proteins are generated.

Family D, E, F and G

Due to the observation that flanking intronic sequences in *CACNA1F* might be the cause of icCSNB, re-investigation of former directly sequenced patients with icCSNB was performed. This identified indeed two additional intronic variants in Family D and Family E, c.276-15C>A in intron 2 and c.2321+5G>T in intron 17, (previously described as: c.2288+5G>T in (Zeitz, et al., 2015)) (Figure 1, Table 2), respectively and two synonymous variants in Family F and G, c.646C>T in exon 5 and c.1719G>A in exon 14 (Figure 1, Table 2), respectively. None of these variants have been reported before. Below we show in detail that all variants were confirmed to result in missplicing of the *CACNA1F* transcript.

Family D

The c.276-15C>A variant in intron 2 was predicted to influence splicing (Table 2). Importantly one exon splicing enhancer binding site seems to be affected by this variant (Supp. Figure S1, Family D). A mini-gene approach including flanking intronic regions of genomic sequences encompassing exon 1 to 4 and subsequent RT-PCR experiments revealed a different expression profile at the presence of the variants when compared to control (Supp. Figure S1, Family D). Only the control revealed a transcript, where exons 1, 2, 3 and 4 were correctly spliced (581 bp). Instead, in the mutant situation, a transcript harboring exon 1, exon 2, a part of intron 2, exon 3 and exon 4 (636 bp) was detected, indicating that the initial donor and acceptor sites were not used but alternative ones in intron 2 (Supp. Figure S1, Family D). In addition, alternative splicing for both, mutant and control transcripts were observed including products lacking exon 2 and harboring a small part of intron 2 (355 bp). Indeed, the naturally occurring 5' donor splice site does not represent the conserved "GT" but instead a "GC". Other bands were present but no sequencing data was obtained. Together, these findings confirm that the splice sites seem to be impacted by the intron 2 variant, explaining the icCSNB phenotype.

Family E

Different splice programs predicted that the c.2321+5G>T variant in intron 17 influences splicing (Table 2, Supp. Figure S1, Family E). A mini-gene approach including flanking intronic regions of genomic sequences encompassing exon 17 to 19 and subsequent RT-PCR experiments revealed indeed a different expression profile at the presence of the variant when compared to control (Figure 3, Family E). A transcript encompassing exon 17, 18 and 19 was only revealed in the control. Other higher molecular bands, similarly or differentially present were not further followed up. These findings suggest that the

icCSNB phenotype in this patient is due to the absence of correctly spliced *CACNA1F* transcript encompassing exons 17, 18 and 19.

Family F

The c.646C>T synonymous variant in exon 5 was predicted to influence splicing (Table 2, Supp. Figure S1, Family F). A mini-gene approach including flanking intronic regions of genomic sequences encompassing exon 3 to 6 and subsequent RT-PCR experiments revealed a different transcript profile in the presence of the variant when compared to control (Supp. Figure S1, Family F). For the synonymous variant, c.646C>T in exon 5 the splice site prediction program indicated a putative novel 5' donor site in exon 5. Interestingly, neither mutant nor control revealed a transcript encompassing exons 3, 4, 5 and 6 (533 bp). A similar finding was already observed for family C, where only one control clone revealed this expected transcript. One alternative splicing product encompassing exon 3 and 6 (250 bp) was mainly detected in the control. Even more interestingly, while the control showed a transcript harboring exons 3, 5 and 6, the synonymous variant in exon 5 lead to an alternative splicing harboring a shorter exon 5 (c.645_664del) (373 bp) as predicted by the splice prediction program (Supp. Figure S1, family F).

Family G

A novel splice acceptor was predicted by the c.1719G>A synonymous variant in exon 14 (Table 2, Supp. Figure S1, Family G). A mini-gene approach including flanking intronic regions of genomic sequences encompassing exon 13 and 14 and subsequent RT-PCR experiments revealed a different transcript profile in the presence of the variant when compared to control (Figure 3, Family G). While both, control and mutant revealed an alternative spliced product containing intron 13 (1305 bp), only the control revealed a product in which exon 13 and exon 14 are correctly spliced (383 bp). The patient transcript revealed a product lacking 36 nucleotides as predicted by the splice prediction program (347 bp).

Discussion

To our knowledge, this is the largest cohort of *CACNA1F*-mutated patients with IRD ever reported. Our study identified 189 patients with known and novel variants in *CACNA1F* and draws attention to non-coding variants in that gene. Albeit a large portion of this cohort was already reviewed in 2015 (Zeitz, et al., 2015), still novel cases with known but also 70 novel variants are reported herein. This includes also a few variants occurring 1-3 times at a hemizygous state in the control population (gnomAD) from South Asia, non-Finish-Europeans and Latinos. This holds true for seven identified variants (c.148C>T, p.Arg50* (1x), c.1537C>T, p.Arg513* (1x), the c.2577-1G>A (1x), c.3433G>A, p.Glu1445Lys (3x), the c.4041+1G>A (1x), c.5479C>T, p.Arg1827* (1x) and c.5641C>G, p.Pro1881Ala (1x)). However, icCSNB, as a congenital stationary disorder, might be undiagnosed in these cases in the control population. Thus, prevalence data in control populations needs to be investigated with caution when working with milder phenotypes as found in cases with icCSNB.

Our work also identified seven patients with non-coding variants in *CACNA1F* previously missed by standard exon-focused sequencing methods. When analyzing non-progressive or progressive IRD applying stringent filtering, we would have missed these variants proven to be pathogenic. It needs to be noted that all variants were predicted to alter splicing, which could be validated by mini-gene approaches. However, this is an *in vitro* approach, which also leads to indistinguishable missplicing in control and mutant transcripts. This might be due to artefacts of the minigene system or alternative splicing (Baux, et al., 2017; Cooper, 2005). It would be interesting to confirm the predicted effect of the variants in photoreceptor precursor cells (Albert, et al., 2018) or even retinal organoids from patient derived iPS cells.

In total 4% of our patients of this cohort have such intronic or synonymous variants resulting in missplicing of *CACNA1F* (Table 2). Even more non-coding variants may be detected in unsolved cases, if targeted genomic sequencing of *CACNA1F* is applied. In our cohort at least nine other cases with a clear diagnosis of icCSNB are present, lacking variants in the coding and flanking intronic regions of the known genes.

Our study herein highlights the importance of an accurate phenotyping protocol which, depending on the phenotype, ideally points to only one or a group of few candidate genes as found in CSNB. Similarly, in other specific IRD, like Stargardt disease (Allikmets, et al., 1997a; Allikmets, et al., 1997b) and choroideremia (Cremers, et al., 1990), showing one specific phenotype and one gene defect, candidate genomic sequencing seems to be cost and time efficient. In more progressive IRD this is unfortunately more difficult since many gene defects underlie the same phenotype. However, developing specific clinical protocols may pin-point as well to specific gene defects in the future. In the absence of a specific genotype-phenotype correlation unbiased GS seems to be the most efficient approach, which allows identifying novel gene defects as well as non-coding variants in a known gene (Carss, et al., 2017). Together these approaches will most likely soon identify the genetic defect of the 30-50% unsolved cases with IRD.

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Figures

Figure 1: **Pedigrees of patients with incomplete icCSNB harboring presumably disease-causing variants in intronic regions of *CACNA1F* or represent synonymous variants in the same gene.** Square symbols represent males and round symbols females; filled symbols represent affected and unfilled symbols unaffected individuals. Individuals screened by Sanger, targeted, exome or genome sequencing are marked with a green, yellow, orange and red arrow, respectively.

Figure 1

Figure 2: **Phenotype of index patient 132.01, II.1 of family showing icCSNB.** A)

Electroretinogram (ERG) in right and left eye of patient compared with normal response (left to right): Dark-adapted 0.01, 0.03, 0.5, and 3 ERG, and light-adapted 3.0 ERG and light adapted 3.0 30Hz-flicker (top to bottom; flash intensities in phot cd s m⁻¹, ISCEV-norm compliant). The reduced and slightly delayed dark- and light-adapted b-waves approx. match the a-waves (“electro-negative” if below isoelectric line) as is the light-adapted 3.0 flicker whose peak is broadened (often double). B) Fundus Autofluorescence images (FAF 488) at the age of 14 (upper figure) and 26 years (lower figure) were unremarkable.

Tables:

Table 1: *CACNA1F* variants in coding or known splice site regions present in a large cohort of 189 *CACNA1F*-related patients (*CACNA1F* (NM_005183.2) variants of our cohort updated from (Zeitz, et al., 2015))

CACNA1F (NM_005183.2) variants of our cohort updated from (Zeitz, et al., 2015)

Exon	Changes at DNA level	Changes at RNA or protein level	Reference	GnomAD	POPHE2	SIFT	Frequency in our cohort	Reference or origin of clinical center*
2	c.148C>T	p.Arg50*	rs886039560	0.000005707, 1 hemizygous	n.a.	n.a.	1	(Zeitz, et al., 2015)
2	c.187_193dup	p.Ala65Glyfs*55	no	no	n.a.	n.a.	1	Novel, this study Jerusalem, Israel
2	c.244C>T	p.Arg82*	rs797044676	0.000005462 never hemizygous	n.a.	n.a.	3	(Zeitz, et al., 2015) an

								d thi s st ud y 1 no ve l M on tp ell ier , Fr an ce
2	c.245G>C	p.Arg82Pro	no	no	Pr ob abl y da ma gin g (wi th a sco re of 1.0 00 (se nsi tivi ty: 0.0 0; spe cifi cit y: 1.0 0))	D el et eri ous (s co re: 0)	2	N ov el, thi s st ud y, M on tp ell ier an d Li lle , Fr an ce
2	c.245G>A	p.Arg82Gln	no	no	Pr ob abl y da ma gin g (wi th a sco re of 1.0	D el et eri ous (s co re: 0)	2	(Z eit z, et al. , 20 15) an d thi s st ud

					00 (se nsi tivi ty: 0.0 0; spe cifi cit y: 1.0 0))			y l no ve l G en t, Be lgi um
2	c.263T>A	p.Ile88Asn	no	no	Pr ob abl y da ma gin g (wi th a sco re of 0.9 89 (se nsi tivi ty: 0.7 2; spe cifi cit y: 0.9 7))	D el et eri ous (s co re: 0)	1	(Z eit z, et al. , 20 15)
3	c.299T>C	p.Leu100Pro	no	0.000 0085 23, never hemi zygo us	Pr ob abl y da ma gin g (wi th a sco re of 0.9 89 (se nsi tivi ty: 0.7	D el et eri ous (s co re: 0. 01)	1	(Z eit z, et al. , 20 15)

					2; specificity: 0.97))			
3	c.299T>G	p.Leu100Arg	no	no	Probably damaging (with a score of 0.966 (sensitivity: 0.78; specificity: 0.95))	Deleterious (score: 0.01)	1	(Zeitz, et al., 2015)
3	c.371dup	p.Asn124Lysfs*175	no	no	n.a.	n.a.	1	(Zeitz, et al., 2015)
4	c.396C>A	p.Tyr132*	no	no	n.a.	n.a.	1	(Zeitz, et al., 2015)
4	c.396C>G	p.Tyr132*	no	no	n.a.	n.a.	1	Novel this study, Li

								lle , Fr an ce
4	c.431T>C	p.Leu144Pro	no	no	Pr ob abl y da ma gin g (wi th a sco re of 0.9 97 (se nsi tivi ty: 0.4 1; spe cifi cit y: 0.9 8))	D el et eri ous (s co re: 0. 02)	1	N ov el, thi s st ud y, Pa ris , Fr an ce
4	c.448G>A	p.Gly150Arg	no	no	Pr ob abl y da ma gin g (wi th a sco re of 1.0 00 (se nsi tivi ty: 0.0 0; spe cifi cit y: 1.0 0))	D el et eri ous (s co re: 0)	2	(Z ho u, et al. , 20 18) an d thi s st ud y, Pa ris an d M on tp ell ier , Fr an ce

4	c.495_496insTACCTA	p.Leu165_Leu166insTyr Leu	no	no	n.a. .	n. a.	1	N ov el, thi s st ud y, M on tp ell ier , an d Pa ris , Fr an ce
5	c.584del	p.Gly195Glufs*8	no	no	n.a. .	n. a.	1	N ov el, thi s st ud y, N an tes , Fr an ce
5	c.611C>T	p.Ala204Val	no	no	Pr ob abl y da ma gin g (wi th a sco re of 0.9 99 (se nsi tivi ty: 0.1 4; spe cifi cit	D el et eri ou s (s co re: 0)	1	N ov el, thi s st ud y, Pa ris , Fr an ce

					y: 0.9 9))			
5	c.647T>G	p.Leu216Arg	no	no	Pr ob abl y da ma gin g (wi th a sco re of 0.9 99 (se nsi tivi ty: 0.1 4; spe cifi cit y: 0.9 9))	D el et eri ou s (s co re: 0)	1	(Z eit z, et al. , 20 15)
I V S 5	c.664+1G>T	p.?	no	no	n.a .	n. a.	1	N ov el, thi s st ud y, St ra sb ou rg, Fr an ce
6	c.731T>G	p.Val244Gly	no	no	Pr ob abl y da ma gin g (wi th a sco re of 0.9	D el et eri ou s (s co re: 0)	1	N ov el, thi s st ud y, G he nt, Be lgi um

					99 (se nsi tivi ty: 0.1 4; spe cifi cit y: 0.9 9))			
6	c.784C>T	p.Arg262*	no	no	n.a .	n. a.	1	(C ar ss, et al. , 20 17) , Th is st ud y, Le ic est er, U nit ed Ki ng do m
6	c.764G>A	p.Gly255Glu	no	no	Pr ob abl y da ma gin g (wi th a sco re of 1.0 00 (se nsi tivi ty: 0.0 0; spe cifi	Del et eri ous (s co re: 0)	1	(Z eit z, et al. , 20 15)

					cit y: 1.0 0))			
6	c.796_797delinsGAACTT	p.Thr266Glu fs*34	no	no	n.a .	n. a.	1	(Z eit z, et al. , 20 15)
6	c.804C>A	p.Tyr268*	no	no	n.a .	n. a.	1	N ov el, thi s st ud y, G he nt, Be lgi u m
6	c.816dup	p.Asp273Arg fs*26	no	no	n.a .	n. a.	1	N ov el, thi s st ud y, N an tes , Fr an ce
7	c.868C>T	p.Arg290Cys	no	no	Pr ob abl y da ma gin g (wi th a sco re of 1.0 00 (se nsi tivi	D el et eri ou s (s co re: 0)	2	(T ay lor , et al. , 20 17) , thi s st ud y Gi es se n,

					ty: 0.00; specificity: 1.00))			Germany and Leicester, United Kingdom
7	c.896G>A	p.Cys299Tyr	no	no	Probably damaging (with a score of 1.000 (sensitivity: 0.00; specificity: 1.00))	Deleterious (score: 0)	1	Novel, this study, Ghent, Belgium
7	c.909G>T	p.Trp303Cys	rs782020175	0.00005575, never homozygous	Probably damaging (with a score of 1.000	Deleterious (score: 0)	1	Novel, this study, Montpellier, France

					(sensitivity: 0.00; specificity: 1.00))			ce
7	c.926G>A	p.Gly309Asp	no	no	Probability of damaging (with a score of 1.000 (sensitivity: 0.00; specificity: 1.00))	Deleterious (score: 0)	1	(Zeitz, et al., 2015)
7	c.935del	p.Asn312Thrfs*10	no	no	n.a.	n.a.	1	(Zeitz, et al., 2015)
7	c.943_945del	p.Asn315del	no	no	n.a.	n.a.	1	(Zeitz, et al., 2015)
7	c.952_954del	p.Phe318del	no	no	n.a.	n.a.	1	(Zeitz, et

								al. , 20 15) an d thi s st ud y, N an tes , Fr an ce
7	c.973C>T	p.Gln325*	no	no	n.a. .	n. a.	1	(Z eit z, et al. , 20 15)
7	c.985_986insC	p.Met329Thrfs*18	no	no	n.a. .	n. a.	1	(Z eit z, et al. , 20 15)
7	c.994T>C	p.Trp332Arg	no	no	Pr ob abl y da ma gin g (wi th a sco re of 1.0 00 (se nsi tivi ty: 0.0 0; spe cifi cit	D el et eri ous (s co re: 0)	1	N ov el, thi s st ud y, M on tp ell ier , Fr an ce

					y: 1.0 0))			
7	c.1004_1009delTGCTCT	p.Val335_Tyr337delinsA sp	no	no	n.a .	n. a.	1	(Z eit z, et al. , 20 15)
7	c.1014G>A	p.Trp338*	no	no	n.a .	n. a.	1	N ov el, thi s st ud y, Tü bi ng en , G er m an y
I V S 7	c.1014+1G>A	p.?	no	no	n.a .	n. a.	1	N ov el, thi s st ud y, N an tes , Fr an ce
8	c.1046G>A	p.Trp349*	no	no	n.a .	n. a.	1	(Z eit z, et al. , 20 15)
8	c.1106G>A	p.Gly369Asp	no	no	Pr ob abl y da ma gin g	D el et eri ou s (s co	1	(Z eit z, et al. , 20 15

					(with a score of 1.000 (sensitivity: 0.00; specificity: 1.00))	re: 0)		)
I V S 8	c.1119-2_1119-1del	p.?	no	no	n.a.	n.a.	1	Novel, this study, Paris, France
9	c.1141A>T	p.Lys381*	no	no	n.a.	n.a.	1	Novel, this study, Lille, France
I V S 1 1	c.1496+1G>A	p.?	no	no	n.a.	n.a.	1	Novel, this study, Paris, France

13	c.1537C>T	p.Arg513*	no	0.000007059, 1 hemizygous	n.a.	n.a.	2	(Zeitz, et al., 2015) and this study 1 novel Mottipellier, France
13	c.1556G>C	p.Arg519Pro	no	no	Probably damaging (with a score of 0.998 (sensitivity: 0.27; specificity: 0.99))	Deleterious (score: 0.01)	1	Novel, this study, Nantes, France
13	c.1599G>A	p.Trp533*	no	no	n.a.	n.a.	1	Novel, this study

								ud y, Le ic est er, U nit ed Ki ng do m
I V S 1 3	c.1684+1G>A	p.?	no	no	n.a .	n. a.	1	N ov el, thi s st ud y, N an tes , Fr an ce
1 4	c.1794C>A	p.Cys598*	no	no	n.a .	n. a.	2	(Z eit z, et al. , 20 15)a nd thi s thi s st ud y, G he nt, Be lgi u m
1 4	c.1739del	p.Leu580Cysfs*27	no	no	n.a .	n. a.	1	N ov el, thi s st ud y, Le ed

								s, U nit ed Ki ng do m
1 4	c.1849C>T	p.Gln617*	no	no	n.a. .	n. a.	1	(Z eit z, et al. , 20 15)
1 4	c.1873C>T	p.Arg625*	rs88 6039 559	0.000 0134 4, never hemi zygo us	no	no	2	(Z eit z, et al. , 20 15)
I V S 1 4	c.1910+2T>G	p.?	no	no	n.a. .	n. a.	1	(Z eit z, et al. , 20 15)
1 5	c.2071C>T	p.Arg691*	rs78 2074 040	no	n.a. .	n. a.	4	(Z eit z, et al. , 20 15) and thi s st ud y: Gi es se n, G er m an y and

								Mon tpellier, France
15	c.2094_2095del	p.(Gln699Glyfs*12)	no	no	n.a.	n.a.	1	Novel, this study, Ghent, Belgium
15	c.2098G>C	p.Ala700Pro	no	no	Probably damaging (with a score of 0.999 (sensitivity: 0.14; specificity: 0.99))	Deleterious (score: 0.03)	1	Novel, this study, Ghent, Belgium
IVS15	c.2118+1G>A	p.?	no	no	n.a.	n.a.	1	Novel, this study, Ghent

								nt, Belgi um
I V S 1 5	c.2119-2A>G	p.?	no	no	n.a. .	n. a.	1	N ov el, thi s st ud y, Tü bi ng en , G er m an y
1 6	c.2123T>C	p.Leu708Pro	no	no	Pr ob abl y da ma gin g (wi th a sco re of 1.0 00 (se nsi tivi ty: 0.0 0; spe cifi cit y: 1.0 0))	D el et eri ou s (s co re: 0)	1	(Z eit z, et al. , 20 15)
1 7	c.2258T>G	p.Phe753Cys	no	no	Pr ob abl y da ma gin g (wi th a	D el et eri ou s (s co re: 0)	1	(Z eit z, et al. , 20 15) an d

					score of 0.983 (sensitivity: 0.74; specificity: 0.96))			this study, Jerusalem, Israel
IVS17	c.2321+1G>T	p.?	no	no	n.a.	n.a.	2	(Zeit, et al., 2015) and this study: Lille, France
IVS17	c.2321+5G>T	p.?	no	no	n.a.	n.a.	1	(Zeit, et al., 2015)
18-48	Del ex18_48	p.?,p?	no	no	n.a.	n.a.	1	Novel, this study, Nantes, France

								ce
19	c.2378dup	p.Glu794Glyfs*54	no	no	n.a.	n.a.	1	Novel this study, Freiburg, Germany
19	c.2387_2388del	p.Glu796Glyfs*51	no	no	n.a.	n.a.	1	Novel, this study, Leicester, United Kingdom
20	c.2449G>T	p.Glu817*	no	no	n.a.	n.a.	2	Novel, this study, Montpellier, France and Giesen, Germany

								m an y
2 0	c.2503G>T	p.Glu835*	no	no	n.a .	n. a.	1	N ov el, thi s st ud y, Je ru sal e m, Isr ae l
2 0	c.2534T>A	p.Ile845Asn	no	no	Pr ob abl y da ma gin g (wi th a sco re of 1.0 00 (se nsi tivi ty: 0.0 0; spe cifi cit y: 1.0 0))	D el et eri ous (s co re: 0)	1	N ov el, thi s st ud y, Pa ris , Fr an ce
I V S 2 0	c.2576+1G>A	p.?	no	no	n.a .	n. a.	1	N ov el, thi s st ud y, M on tre al, Ca na da

I V S 2 0	c.2577-1G>A	p.?	rs78 2553 358	0.000 0057 87, 1 hemi zygo us	n.a .	n. a.	2	(Z eit z, et al. , 20 15)
I V S 2 0	c. 2577-2A>T	p.?	no	no	n.a .	n. a.	1	(Z eit z, et al. , 20 15)
2 1	c.2627T>G	p.Leu876Arg	no	no	Pr ob abl y da ma gin g (wi th a sco re of 0.9 98 (se nsi tivi ty: 0.2 7; spe cifi cit y: 0.9 9))	T ol er at ed (s co re: 0. 05)	1	N ov el, thi s st ud y, M on tp ell ier , Fr an ce
2 1	c.2637_2638dup	p.Phe880Cysfs*43	no	no	n.a .	n. a.	1	N ov el, thi s st ud y, Li lle , Fr an ce
2 1	c.2683C>T	p.Arg895*	rs12 2456	no	n.a .	n. a.	4	(Z eit

			135					z, et al. , 20 15)
2 2	c.2747C>G	p.Thr916Ser	no	no	Pr obl y da ma gin g	T ol er at ed	1	N ov el, thi s st ud y, Pa ris , Fr an ce
2 3	c.2771_2772del	p.Thr924Serfs*14	no	no	n.a .	n. a.	1	N ov el, thi s st ud y, St ra sb ou rg, Fr an ce
2 3	c.2796_2797insT	p.Gly933Trpfs*6	no	no	n.a .	n. a.	1	N ov el, thi s st ud y, Li lle , Fr an ce
2 3	c.2805del	p.Cys936Alafs*30	no	no	n.a .	n. a.	1	N ov el, thi s st ud y, Je ru sal

								e m, Isr ae l
2 3	c.2812A>G	p.Ser938Gly	no	no	Po ssi bly da ma gin g (wi th a sco re of 0.9 49 (se nsi tivi ty: 0.7 9; spe cifi cit y: 0.9 5))	T ol er at ed (s co re: 0. 09)	1	(Z eit z, et al. , 20 15)
2 3	c.2822A>C	p.Asn941Thr	no	no	Po ssi bly da ma gin g (wi th a sco re of 1.0 00 (se nsi tivi ty: 0.0 0; spe cifi cit y: 1.0 0))	D el et eri ou s (s co re: 0)	1	(Z eit z, et al. , 20 15)
2 3	c.2829_2830delinsCT	p.Leu943_Asp944delinsP heTyr	no	no	n.a .	n. a.	1	(Z eit z,

								et al., 2015)
23	c.2830G>T	p.Asp944Tyr	no	no	Probably damaging (with a score of 0.995 (sensitivity: 0.68; specificity: 0.97))	Deleterious (score: 0)	1	(Zeitz, et al., 2015)
23	c.2854dup	p.Leu952Profs*115	no	no	n.a.	n.a.	1	(Zeitz, et al., 2015)
24	c.2905C>T	p.Arg969*	rs122456134	no	n.a.	n.a.	3	(Zeitz, et al., 2015)
	c.2932C>T	p.Arg978*	no	0.000007313, never hemizygous	n.a.	n.a.	6	(Zeitz, et al., 2015)
I	c.2961+1G>A	p.?	no	no	n.a.	n.	1	(Z

V S 2 4					.	a.		et z, et al. , 20 15)
2 5	c.2992C>T	p.Arg998Trp	no	no	Pr ob abl y da ma gin g (wi th a sco re of 1.0 00 (se nsi tivi ty: 0.0 0; spe cifi cit y: 1.0 0))	D el et eri ous (s co re: 0)	1	N ov el, thi s st ud y, Pa ris , Fr an ce
2 5	c.3009_3011del	p.Ile1003del	no	no	n.a .	n. a.	1	(Z eit z, et al. , 20 15) , thi s st ud y Li lle , Fr an ce
2 5	c.3052G>A	p.Gly1018Arg	no	no	Pr ob abl y da ma	D el et eri ous s	3	(Z eit z, et al. ,

					g g (wi th a sco re of 1.0 00 (se nsi tivi ty: 0.0 0; spe cifi cit y: 1.0 0))	(s co re: 0)		20 15)
2 6	c.30121_3122+2del	p.Lys1041Glyfs*25	no	no	n.a. .	n. a.	2	(Z eit z, et al. , 20 15) an d thi s st ud y, Pa ris , Fr an ce
2 7	c.3166dup	p.Leu1056Profs*11	no	no	n.a. .	n. a.	2	(Z eit z, et al. , 20 15)
I V S 2 7	c.3269+1G>A	p.?	no	no	n.a. .	n. a.	1	(H ov e, et al. , 20 16 ;

								Taylor, et al., 2017), this study Leuven, Belgium
IVS27	c.3270-3C>G	p.?	no	no	n.a.	n.a.	1	Novel, this study, Montpellier, France
28	c.3433G>A	p.Glu1145Lys	rs138447882	0.0005399, 3 hemizygous	Probability of 1.000 (sensitivity: 0.00; specificity: 0.00)	Deleterious (score: 0.02)	1	(Zeit, et al., 2015)

					cificity: 1.00))			
28	c.3440del	p.Glu1147Glyfs*41	no	no	n.a.	n.a.	1	(Zeitz, et al., 2015)
28	c.3448_3457dup	p.Leu1153Glnfs*3	no	no	n.a.	n.a.	1	Novel, this study, Nantes, France
28	c.3469C>T	p.Gln1157*	no	no	n.a.	n.a.	1	Novel, this study, Lausanne, Switzerland
28	c.3470A>G	p.Gln1157Arg	no	no	Possibly damaging (with a score of 0.9)	Deleterious (score: 0)	1	Novel, this study, Ghent, Belgium

					42 (sensitivity: 0.80; specificity: 0.94))			m
I V S 2 8	c.3471+2T>G	p.?	no	no	n.a.	n.a.	1	Novel this study, Lille, France
2 9	c.3482T>G	p.Val1161Gly	no	no	Probably damaging (with a score of 0.974 (sensitivity: 0.76; specificity: 0.96))	Deleterious (score: 0)	1	Novel, this study, Paris, France
2 9	c.3491C>A	p.Alal164Asp	no	no	Probably damaging	Deleterious (s	1	(Zeit, et al., 20

					g (with a score of 1.000 (sensitivity: 0.00; specificity: 1.00))	core: 0)		15)
29	c.3504_3505del	p.Gln1168Hisfs*25	no	no	n.a.	n.a.	1	(Zeit, et al., 2015)
29	c.3502C>T	p.Gln1168*	no	no	n.a.	n.a.	1	Novel, this study, Paris, France
29	c.3551G>A	p.Trp1184*	no	no	n.a.	n.a.	1	Novel, this study, Strasbourg, France
29	c.3525dup	p.Lys1176Glnfs*18	no	no	n.a.	n.a.	1	Nov

								el, thi s st ud y, Le ic est er, U nit ed Ki ng do m
3 0	c.3695T>G	p.Leu1232Arg	no	no	Pr ob abl y da ma gin g (wi th a sco re of 1.0 00 (se nsi tivi ty: 0.0 0; spe cifi cit y: 1.0 0))	D el et eri ous (s co re: 0. 01)	1	(Z eit z, et al. , 20 15)
3 0	c.3721del	p.Ile1241Serfs*26	no	no	n.a .	n. a.	1	N ov el, thi s st ud y, A ng er s, Fr an ce
3 1	c.3751_3825+54del	p.Thr1251_Asn1275del	no	no	n.a .	n. a.	2	(Z eit

								z, et al. , 20 15) and this study, Jerusalem, Israel
I V S 3 2	c.3847-1G>A	p.?	no	no	n.a. .	n. a.	1	N ovel, this study, Paris , France
3 3	c.3935- 3948delinsAGCCACATAGGGCAAGGCCT ATAGGGATGGGGGAGGGGGCAGAG AATAGATGCACAGGCTCAGGCAAAGA ACTATAAGCCCCAGAATGCACTGCTTT CCCTGGCCTGGGCCTACTGGGAGATG CAGTCCATCTCCAAAGTGCCAAGGA or c.3935_3948delins149	p.(Gly1312_Pro1328delins16*)	no	no	n.a. .	n. a.	1	N ovel, this study, Tübingen , Germany
3 3	c.3886C>T	p.Arg1296Cys	no	0.000 0055 67, never hemi zygo us	Pr ob abl y da ma gin g	D el et eri ous (s co	1	(Z eit z, et al. , 20 15

					(with a score of 1.000 (sensitivity: 0.00; specificity: 1.00))	re: 0)		)
33	c.3895C>T	p.Arg1299*	no	no	n.a.	n.a.	2	(Zeit, et al., 2015) and this study, Cermon t-Ferrand, France
33	c.3954G>A	p.Trp1318*	no	no	n.a.	n.a.	1	Novel, this study, Jerusalem, Israel

I V S 3 3	c.3976-1G>C	p.?	no	no	n.a. .	n. a.	1	l N ov el, thi s st ud y, La us an ne , S wi tz erl an d
I V S 3 4	c.4041+1G>A	p.?	rs78 2557 600	0.000 0055 80, 1 hemi zygo us	n.a. .	n. a.	1	N ov el, thi s st ud y, G he nt
I V S 3 4	c.4041+5G>A	p.?	no	no	n.a. .	n. a.	1	N ov el, thi s st ud y, Pa ris , Fr an ce
3 5	c.4049G>T	p.Gly1350Val	no	no	Pr ob abl y da ma gin g (wi th a sco re of 1.0 00 (se	Del et eri ous (s co re: 0)	1	(Z eit z, et al. , 20 15)

					nsi tivi ty: 0.0 0; spe cifi cit y: 1.0 0))			
3 5	c.4084C>T	p.Arg1362*	no	no	n.a .	n. a.	3	(Z eit z, et al. , 20 15) an d thi s st ud y 2 ca se s fr o m Je ru sal e m, Isr ae l
3 5	c.4124T>A	p.Leu1375His	no	no	Pos sib ly da ma gin g (wi th a sco re of 0.8 78 (se nsi tivi ty: 0.8	Del et eri ous (s co re: 0)	1	(Z eit z, et al. , 20 15)

					2; specificity: 0.94))			
I V S 3 5	c.4134-1G>C	p.?	no	no	n.a.	n.a.	2	(Zeitz, et al., 2015) and this study, Montpellier, France
3 6	c.4259A>G	p.Tyr1420Cys	no	no	Probably damaging (with a score of 0.995 (sensitivity: 0.68; specificity: 0.97))	Deleterious (score: 0)	2	(Zeitz, et al., 2015) and this study, Montpellier, France
3 7	c.4376T>C	p.Leu1459Pro	no	no	Prob	Del	1	Nov

					ably damaging (with a score of 1.000 (sensitivity: 0.00; specificity: 1.00))	et erious (score: 0)		el, this study, Paris, France
37	c.4397G>T	p.Trp1466Leu	no	no	Probably damaging (with a score of 1.000 (sensitivity: 0.00; specificity: 1.00))	Deleterious (score: 0)	2	(Zeitz, et al., 2015) and this study, Tübingen, Germany
IVS37	c.4422-1G>A	p.?	no	no	n.a.	n.a.	1	Novel, this study,

								Mon tp ell ier , Fr an ce
3 8	c.4457T>C	p.Leu1486Pro	no	no	Pr ob abl y da ma gin g (wi th a sco re of 1.0 00 (se nsi tivi ty: 0.0 0; spe cifi cit y: 1.0 0))	D el et eri ous (s co re: 0)	1	(Z eit z, et al. , 20 15)
3 8	c.4466del	p.Ile1489Thrfs*23	no	no	n.a .	n. a.	1	(Z eit z, et al. , 20 15)
3 8	c.4487G>A	p.Gly1496Glu	no	no	Pr ob abl y da ma gin g (wi th a sco re of 1.0 00	D el et eri ous (s co re: 0)	1	(Z eit z, et al. , 20 15) an d thi s stud y

					(se nsi tivi ty: 0.0 0; spe cifi cit y: 1.0 0))			Je ru sal e m, Isr ae l
3 8	c.4504C>T	p.Arg1502*	rs78 2581 701	no	n.a .	n. a.	1	N ov el, thi s st ud y, Li lle , Fr an ce
3 8	c.4474C>G	p.Pro1492Ala	no	no	Pr ob abl y da ma gin g (wi th a sco re of 1.0 00 (se nsi tivi ty: 0.0 0; spe cifi cit y: 1.0 0))	D el et eri ous (s co re: 0)	5	(Z eit z, et al. , 20 15) an d thi s st ud y Li lle , Fr an ce an d G he nt, Be lgi um
3 8	c.4480G>A	p.Gly1494Arg	no	no	Pr ob abl y da	D el et eri ou	2	(Z eit z, et al.

					mag in g (wi th a sco re of 1.0 00 (se nsi tivi ty: 0.0 0; spe cifi cit y: 1.0 0))	s (s co re: 0)		, 20 15) an d thi s st ud y N an tes , Fr an ce
I V S 3 8	c.4518+2T>A	p.?	no	no	n.a. .	n. a.	1	(Z eit z, et al. , 20 15)
3 9	c.4523T>C	p.Leu1508Pro	no	no	Pr ob abl y da ma gin g (wi th a sco re of 1.0 00 (se nsi tivi ty: 0.0 0; spe cifi cit y: 1.0 0))	D el et eri ou s (s co re: 0)	1	(Z eit z, et al. , 20 15)
3 9	c.4577C>A	p.Thr1526Lys	no	no	Pr ob	D el	1	N ov

					ably da ma gin g (with a score of 0.9 99 (sensitivity: 0.1 4; specificity: 0.9 9))	et eri ous (score: 0)		el, this stud y, Le icester, United King dom
3 9	c.4580_4582delinsCC	p.Leu1527Profs*9	no	no	n.a. .	n. a.	1	(Zeit z, et al. , 20 15)
3 9	c.4594dup	p.Arg1532Profs*37	no	no	n.a. .	n. a.	1	
3 9	c.4594C>T	p.Arg1532Trp	no	no	n.a. .	n. a.	1	(Hov e, et al. , 20 16) and this stud y, Nan tes, France
4 1	c.4768C>T	p.Gln1590*	no	no	n.a. .	n. a.	1	(Zeit

								z, et al. , 20 15)
4 1	c.4818del	p.Asn1607Thrfs*153	no	no	n.a .	n. a.	1	N ov el, thi s st ud y, Pa ris , Fr an ce
4 1	c.4845del	p.Ala1616Profs*144	no	no	n.a .	n. a.	1	(Z eit z, et al. , 20 15)
4 2	c.4900del	p.Ala1634Profs*126	no	no	n.a .	n. a.	1	N ov el, thi s st ud y, St ra sb ou rg, Fr an ce
4 6	c.5479C>T	p.Arg1827*	rs78 2034 481	0.000 0054 59, 1 hemi zygo us	n.a .	n. a.	1	(Z eit z, et al. , 20 15)
4 7	c.5641C>G	p.Pro1881Ala	rs78 2033 301	0.000 0159 9, 1 hemi zygo us	Be nig n (wi th a sco	De let eri ous	1	N ov el, thi s st ud

					re of 0.0 02 (se nsi tivi ty: 0.9 9; spe cifi cit y: 0.3 0))			y, M on tp ell ier , Fr an ce
4 7	c.5665del	p.His1889Metfs*43	no	no	n.a .	n. a.	1	(Z eit z, et al. , 20 15) an d thi s st ud y, Pa ris , Fr an ce
SUM							1 8 2	

n.a. not applicable; *the original center of the variants can be found in (Zeitz, et al., 2015)

Table 2: *CACNA1F* variants in intronic regions or representing synonymous variants present in a large cohort of 189 *CACNA1F*-related patients

Family	Genomic region	Exon/Intron	Nucleotide exchange	rs	Amino acid exchange	Frequency	MaxEnt	NNSPLICE	HSF	Summary of splice site effect	Segregation
A	ChrX:49082866	IVS11	c.1496+5G>C	-	p.?	no	-100%	-99.7%	-14.8%	-71.5%	Hemizygous in 132.01 heterozygous in 132.02 and 132.03
B	ChrX:49067561	IVS36	c.4294-9G>A	-	p.?	no	-65.5%	-7.9%	-0.1%	-24.5%	Hemizygous in 8789, heterozygous in 8800, 8801, Ref in 8799
C	ChrX:49087087	IVS4	c.522-16G>A	-	p.?	no	-30.5%	6.6%	0.0%	-8.0%	Hemizygous in CIC04152, CIC05614 and heterozygous in CIC07368
D	ChrX:49087784	IVS2	c.276-15C>A	-	p.?	no	-	-75.5%	0%	-37.7%	Only 5875 available
E	ChrX:49078976	IVS17	c.2321+5G>T	-	p.?	no	-82.5%	-97.5%	-13.4%	-64.5%	Only CH880 available
F	ChrX:49086947	Exon 5	c.646C>T	-	p.(=)	no	See picture new	-	-	-	Only CIC05358

							splice donor				available
G	ChrX: 49081414	Exon 14	c.1719G> A	-	p.(=)	-	See picture new splice acceptor	-	-	-	Only 1100_1 available

Splice predictions programs were used to evaluate the putative effect on splicing due to the identified variants. MaxEntSca is a method based on the Maximum Entropy principle (Yeo and Burge, 2004). NNPLICE (available at the Berkeley Drosophila Genome Project web site: http://www.fruitfly.org/seq_tools/splice.html) is a prediction method based on neural networks (Reese, et al., 1997). HSF, Human Splicing Finder is based on position weight matrices with some position-dependent logic (Desmet, et al., 2009).