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Functional characterization of *ABCC8* variants of unknown significance based on bioinformatics predictions, splicing assays and protein analyses: benefits for the accurate diagnosis of congenital hyperinsulinism

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Abstract

ABCC8 encodes the SUR1 subunit of the β-cell ATP-sensitive potassium channel whose loss of function causes congenital hyperinsulinism (CHI). Molecular diagnosis is critical for optimal management of CHI patients. Unfortunately, assessing the impact of *ABCC8* variants on RNA splicing remains very challenging as this gene is poorly expressed in leukocytes.

Here, we performed bioinformatics analysis and cell-based minigene assays to assess the impact on splicing of 13 *ABCC8* variants identified in 20 CHI patients. Next, channel properties of SUR1 proteins expected to originate from minigene-detected in-frame splicing defects were analyzed after ectopic expression in COSm6 cells. Out of the analyzed variants, 7 induced out-of-frame splicing defects and were therefore classified as recessive pathogenic, whereas 2 led to skipping of in-frame exons. Channel functional analysis of the latter demonstrated their pathogenicity. Interestingly, the common rs757110 SNP increased exon skipping in our system suggesting that it may act as a disease modifier factor. Our strategy allowed determining the pathogenicity of all selected *ABCC8* variants, and CHI-inheritance pattern for 16 out of 20 patients. This study highlights the value of combining RNA and protein functional approaches in variant interpretation and reveals the minigene splicing assay as a new tool for CHI molecular diagnostics.

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Keywords

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Introduction

Congenital hyperinsulinism (CHI; MIM# 256450), characterized by an inappropriate over-secretion of insulin from pancreatic β-cells, is the most frequent cause of persistent hypoglycemia in neonates. If not timely diagnosed and treated, CHI patients are at high risk for hypoglycemia-caused seizures, permanent brain damage or death. The management strategy of patients depends on the responsiveness to diazoxide therapy and on the histopathological form of CHI (Supp. Figure S1). The focal form consists of an adenomatoid hyperplasia of hyper functional pancreatic β-cells restricted to a limited pancreatic area and can be cured by the targeted removal of the pathological tissue. In contrast, the diffuse form affects all the β-cells and thus requires near total pancreatectomy when patients are unresponsive to an intensive medical treatment (Arnoux et al., 2014). This option is avoided as much as possible given that all patients develop diabetes subsequently to the procedure (Beltrand et al., 2012).

Severe forms of CHI are mostly associated with loss-of-function defects of the β-cell ATP-sensitive potassium (K_{ATP}) channel genes, *ABCC8* and *KCNJ11,* which encode the SUR1 and Kir6.2 channel subunits, respectively (Kapoor et al., 2013; Snider et al., 2013). These channels regulate the flux of K^+ ions and couple glucose metabolism to membrane electrical activity and insulin release. Importantly, *ABCC8* variants represent 83% of genetic K_{ATP} channel deficiency causes (De Franco et al., 2020).

CHI histological forms are determined both by the mode of inheritance and the molecular mechanisms underlying loss-of-function defects. Two consecutive molecular events characterize the focal form: (i) paternally inheritance of a K_{ATP} channel recessive variant and (ii) paternal isodisomy of the 11p15 chromosomal region, which contains the *ABCC8* and *KCNJ11* genes, with concomitant loss of the maternal allele in the ß-cells of a restricted pancreatic region. Conversely, in the diffuse form all β-cells are affected, which can be caused either by bi-allelic variants (recessive form) or by a single heterozygous variant (dominant form). The detection of a single heterozygous variant, particularly when this variation was not previously reported, represents a challenge for diagnostics: should the segregation pattern be unknown or should the inheritance be paternal, the molecular report usually cannot conclude between a focal form with a recessive variant or a diffuse form with a dominant variant.

To date, truncating variants of the *ABCC8* are always associated with recessive mechanisms whereas missense variants can be found in either dominant or recessive cases. Recent protein-dedicated *in vitro* studies focusing on missense changes facilitated discriminating between recessive and dominant pathogenic *ABCC8* variants. In these studies, recessive pathogenic variants generally resulted in K_{ATP} channels not reaching the plasma membrane, whereas channels with dominant variants typically displayed preserved trafficking but

showed impaired responses to MgADP and diazoxide (Macmullen et al., 2011). Unfortunately, these assays are solely based on cDNA constructs and do not interrogate non-coding *ABCC8* variations such as intronic or synonymous variants. Yet, these and other genetic changes, which are prominently classified as VUS, may actually be pathogenic if they affect RNA splicing signals. Indeed, variant-induced splicing alterations are now considered a major mechanism of hereditary disease (López-Bigas, Audit, Ouzounis, Parra, & Guigó, 2005; Wang & Cooper, 2007). To date about 180 VUS were reported in *ABCC8* (De Franco et al., 2020). They represent about 15% of clinically reported variants identified in CHI. Interestingly, half of the *ABCC8* VUS encountered in CHI patients are either intronic or synonymous, and these variants could be reclassified after examination of their impact on splicing.

RNA splicing is dependent on multiple signals on the pre-mRNA, including 3' and 5' splice sites (3'ss and 5'ss, respectively), polypyrimidine tract sequences (PPT) and branch sites (BS), as well as auxiliary elements that contribute to splicing regulation (Cartegni, Chew, & Krainer, 2002). The most common splicing aberrations include exon skipping and 3'ss or 5'ss shifts leading to either exonic deletions or intronic retentions (Abramowicz & Gos, 2019). Unfortunately, *ABCC8* is weakly expressed in whole blood cells (GTEx portal) and pancreatic tissue is seldom available, making it very difficult or impossible to assess the impact of VUS in patient samples notably by RT-PCR or RNA-seq. Moreover, although minigene splicing assays can be used as proxy systems in cases presenting this type of limitations (D. Baralle, Lucassen, & Buratti, 2009), no such studies have yet been performed for *ABCC8*.

The aim of this study was to investigate *ABCC8* variants suspected of inducing splicing alterations in order to determine whether they affected the normal production of SUR1 and, if so, whether they have a recessive or a dominant effect. We thus selected 13 *ABCC8* variants and performed a combination of *in silico* analyses, minigene splicing assays and protein functional tests. This strategy proved to be of great value in a diagnostic perspective, determining that nine variants were pathogenic and four were neutral. To our knowledge, this work represents the first systematic study of variant-induced splicing alterations in *ABCC8* with important implications in the molecular diagnostics of CHI.

Materials and Methods

Editorial Policies and Ethical Considerations

Ethical committee approval was not needed for this study given that all experiments were performed *in vitro* by transfection of plasmid constructs into model cell systems (minigene assays and protein functional analyses). The biological collection of the Department of Genetics of Pitié-Salpêtrière Hospital has been declared to the Minister for research and the Director of the Regional Health Agency (biobank ID #DC2009–957). Patients signed an informed consent for the molecular diagnosis of their hyperinsulinism also indicating that they approved any research project performed in relation with their disease. Results of the genetic analyses were registered in an internal restricted-access diagnosis database (CNIL certificate 16/02/2010-n°1412729).

Patients

We obtained written informed consent for all patients of the 20 families whose genetic variants were tested in this study. Clinical diagnosis of CHI was made as previously described (Bellanné-Chantelot et al., 2010). All patients were unresponsive to diazoxide therapy. The diagnosis of focal and diffuse forms was based either on ^{18}F-DOPA-positron emission tomography (PET) before surgery (Ribeiro et al., 2007) or on pancreatic histopathological analysis (de Lonlay et al., 2006).

Genetic analysis

Sequencing either by Sanger or by NGS technologies and search for genomic rearrangements of *ABCC8* (NM_001287174.1) were performed as previously described (Bellanné-Chantelot et al., 2010; Donath et al., 2019). All variants were classified, as indicated, prior to functional testing according to the ACMG guidelines (Richards et al., 2015).

Splicing-dedicated in silico analyses

In order to predict 3'ss and 5'ss alterations potentially induced by the 13 selected variants, as well as by the fortuitously detected exonic and intronic SNPs (rs757110 and rs4148644, respectively) we resorted to three bioinformatics approaches: (i) the combined MaxEntScan (MES) and SpliceSiteFinder-like (SSFL) pipeline that is based on the sequential utilization of the aforementioned algorithms (MES/SSFL) as previously recommended (Houdayer et al., 2012), both simultaneously accessed via Alamut Visual1.5.2 (Interactive Biosoftware, France), (ii) SPiCE (Leman et al., 2020) and (iii) SpliceAI 1.2.1(Jaganathan et al., 2019; Wai et al., 2020). Only concordant results between these three approaches were taken as confident forecasts. For prediction of alterations of exonic splicing regulatory elements (SRE) potentially induced by the exonic rs757110 SNP (located outside reference splice sites), we used the following SRE-predictors: QUEPASA ($\Delta tESR_{seq}$ scores) (Di Giacomo et al., 2013; Ke et al., 2011), HEXplorer (ΔHZEI scores) (Erkelenz et al., 2014), SPANR ($\Delta\psi$ scores) (Xiong et al., 2015) and HAL ($\Delta\psi$ scores) (Rosenberg, Patwardhan, Shendure, & Seelig, 2015), as recently described (Tubeuf et al., 2020).

Cell-based minigene splicing assays

Experimental assays based on the comparative analysis of the splicing pattern of wild-type (WT) and mutant pCAS2-derived reporter minigenes were performed as previously described (Soukarieh et al., 2016). Briefly, the WT and mutant genomic fragments containing the exons of interest and ~150 bp of flanking intronic sequences were amplified from patient gDNA by using primers described in Supp. Table S1. Then, the amplicons were inserted into the BamHI and MluI cloning sites of pCAS2 yielding pCAS2-ABCC8 hybrid minigenes. A subset of minigenes carrying SNPs in/near *ABCC8* exon 33, as indicated, were prepared by site-directed mutagenesis by using the two-stage overlap extension PCR method and a combination of specific primers indicated in Supp. Table S1. All constructs were sequenced to confirm the presence or absence of the variants and the integrity of the inserts. Next, WT and mutant minigenes were transfected in parallel into HeLa cells

(obtained from ATCC) and 24h later minigene transcripts were analysed by semi-quantitative RT-PCR and electrophoresis in agarose gels.

Functional analysis of mutant channels

Functional analyses of K_{ATP} channels, including immunoblotting and $^{86}Rb^+$ efflux assays as described previously (Macmullen et al., 2011; Yan et al., 2007). Briefly, for immunoblotting, COSm6 cells were co-transfected with human SUR1 with a N-terminal FLAG tag (DYKDDDDK) in pCMV6b and human Kir6.2 in pCDNA3 using FuGene6 (Roche Applied Science). SUR1 was probed with M2 anti-FLAG antibody (Sigma) followed by HRP anti-mouse secondary antibodies (GE Healthcare), and visualized by enhanced chemiluminescence (Super Signal West Femto; Pierce). For $^{86}Rb^+$ efflux assay, transfected cells were incubated overnight in medium containing $^{86}RbCl$ (1 μCi/ml). The next day, cells were pretreated with metabolic inhibitors, 1 mM 2-deoxyglucose and 2.5 μg/ml oligomycin for 30 min to activate the channels and $^{86}Rb^+$ efflux over a 40-min period as a percentage of total $^{86}Rb^+$ in cells prior to efflux measurements was calculated and normalized to WT channels.

Results

Description of the *ABCC8* variants selected for this study

In this work we focused on 13 variants identified in 20 *ABCC8* probands among 178 patients carrying an *ABCC8* variant diagnosed at the Pitié-Salpêtrière Hospital (Table 1 and Supp. Figure S2). These variants were selected based on their close position to intron-exon boundaries (Supp. Figures S3 and S4) and on uncertainties relative to their clinical classification (Table 1). Whereas for some variants there were doubts on their pathogenicity, for others it was impossible to infer the histological form (focal or diffuse) even though they were assumed as pathogenic. Among the 13 selected variants, ten are intronic and three are exonic. The latter included two synonymous variants located at exon termini and one frameshift mutation starting at an antepenultimate exonic position. Seven out of the 13 variants mapped within a 5'ss, whereas six were located close to 3'ss sequences, 12 being outside the highly conserved IVS±1 and IVS±2 positions (Supp. Figures S3 and S4) and considered as VUS. Finally, two variants were located on an atypical 3'ss not predicted by the MES algorithm (c.3992–9G>A and c.3992–3C>G, Supp. Table S2) and thus this study was an opportunity to verify if these recurrent CHI-associated variants indeed altered RNA splicing.

Of note, in contrast to the other probands, patient P8 did not carry a *bona fide* VUS but a presumed pathogenic frameshift variant (c.3754_3755del; p.Met1252fs). Albeit expected to be recessive, this maternally-inherited variant was the single *ABCC8* alteration identified in this patient. This raised the hypothesis that this variant could cause disease by a dominant mechanism possibly due to the induction of an in-frame splicing defect as already observed for truncating variants in other genes (Meulemans et al., 2020; Tuffery-Giraud, Miro, Koenig, & Claustres, 2017; Zhu et al., 2019).

In silico predictions of splicing alterations for the selected 13 *ABCC8* variants

To assess the potential impact on splicing of the 13 selected variants, we first evaluated their effect on the strength of the nearest splice sites with the help of three bioinformatics methods (MES/SSFL, SPICE and SpliceAI) (Table 1 and Supp. Table S2). These predictors provided concordant results for 6 variants (5 with predicted splicing impacts, 1 without predicted splicing defect). Among the seven remaining variants with inconsistent predictions, 3 were predicted to be spliceogenic by a single tool (either SPiCE or SpliceAI).

Detection of RNA splicing defects by using minigene splicing assays

Next, we performed minigene splicing assays to experimentally determine the consequences on RNA splicing of each selected *ABCC8* variant. As shown in Figure 1A, four out of the 13 variants had no major impact on splicing, which is in agreement with the combined *in silico* data for only one of these variants (c.1332+4del), the other three variants having disparate/inconclusive predictions (c.2041–12C>T, c.3754_3755del and c.4123–19C>T). Importantly, the remaining 9 variants led to splicing alterations: 4 induced exon skipping (Figure 1B), and 5 caused more complex splicing aberrations (Figure 1C, Supp. Figure S5).

In the cases of exon skipping, we observed different effects ranging from partial to total/near-total loss of the full-length minigene transcripts due to alterations affecting either 3'ss (c.3992–3C>G) or 5'ss motifs (c.1176G>A, c.1923+5G>T, and c.4611G>A). For the more complex splicing alterations, we found that two intronic variants mapping within 5'ss sequences (c.1467+6T>G and c.3991+2_3991+15del) led to completely different anomalies. Whereas c.1467+6T>G caused very minor exon 9 skipping but a prominent retention of 14 nucleotides from intron 9 (due to the activation of a pre-existing intronic cryptic 5'ss), c.3991+2_3991+15del significantly increased exon skipping and concomitantly caused the activation of a cryptic 3'ss in intron 31 (c.3871–145) and of a cryptic 5'ss within exon 32 (c.3894). This lead to the concomitant retention of 145 nucleotides from intron 31 and deletion of 97 nucleotides from exon 32 within the same RNA molecule. Interestingly, this intricate outcome had been, in part, predicted by SpliceAI (Supp. Table S2). Three intronic variants mapping near 3'ss motifs also caused complex splicing alterations (c.2924–9G>A, c.3992–9G>A and c.4415–13G>A). Indeed, the minigene assay revealed that c.2924–9G>A caused retention of 7 nucleotides from intron 24. This effect can be explained by the creation of a new 3'ss 7 nucleotides upstream of the destroyed reference 3'ss. Surprisingly, c.3992–9G>A induced a deletion of the first 20 nucleotides of exon 33 by activating a pre-existing cryptic 3'ss within the exon, an unexpected defect given the lack of *in silico* predictions. As for c.4415–13G>A, we observed that this variant induced the retention of 11 nucleotides from intron 37 by decreasing the strength of the reference splice site and simultaneously creating a new 3'ss within the intron, which is in agreement with MES/SSFL and SpliceAI predictions (Supp. Table S1 and Figure S5).

Except for the c.1176G>A and c.4611G>A exonic changes, which caused in-frame exon skipping, all the *ABCC8* variants that induced splicing alterations in the minigene assay provoked out-of-frame anomalies and are thus expected to produce frameshift transcripts in the natural physiological context (Table 1). Given that such transcripts will either be degraded by the nonsense mediated decay pathway and/or yield severely carboxy-terminal

truncated SUR1 proteins, it is very likely that 7 out of the 13 variants analyzed in this study are in fact pathogenic and recessively inherited. Overall, our results stress the importance of experimentally evaluating the impact on splicing of nucleotide changes in *ABCC8* and suggest that, although not totally accurate, a prioritization strategy based on *in silico* predictions is appropriate.

The rs757110 polymorphism modulates the splicing pattern of *ABCC8* exon 33

When analyzing the sequences of the minigene constructs obtained with the WT/mutant pairs for c.3992–9G>A (patient P16) and c.3992–3C>G (patient P10), we noticed the presence of two *ABCC8* SNPs, notably rs757110 (c.4108G>T) and rs4148644 (c.4122+93G>T) (Figure 1). More precisely, we found that P16 was homozygous for rs757110 and heterozygous for rs4148644 (*in trans* with c.3992–9G>A), whereas P10 was heterozygous for both rs757110 and rs4148644 (both *in cis* with c.3992–3C>G). Given the co-occurrence of the SNPs with the variants of interest, we wondered if they influenced the behavior of the mutant minigenes, especially in the case of c.3992–9G>A that led to an apparent increase in exon skipping and the production of abnormal transcripts lacking 20 nucleotides of the exon (Figure 1). We thus assessed the splicing pattern of minigenes carrying different combinations of the SNPs both in the WT and in the c.3992–9G>A and c.3992–3C>G mutant contexts (Figure 2A). Our results indicate that rs757110 (c.4108G>T) has a negative impact on *ABCC8* splicing by favoring exon 33 skipping in all contexts (Figure 2B and Supp. Figure S6), probably by altering splicing regulatory elements as predicted bioinformatically as opposed to a direct effect on splice site strength (Figure 2C and Supp. Table S3). In contrast, rs4148644 (c.4122+93G>T) had no major effect in the splicing pattern of exon 33 (Figure 2B and Supp. Figure S6). Altogether these data suggest that rs757110 may influence the cellular level of full-length *ABCC8* transcripts and thus can potentially act as a genetic modifying factor, whereas rs4148644 is a likely neutral variant.

Mutant channel function assessed by ^{86}Rb efflux and western-blot analysis

Because the synonymous c.1176G>A and c.4611G>A variants induced in-frame skipping of exons 7 and 38, respectively, and thus remained classified as VUS, we determined if the corresponding internally deleted proteins (SUR1 p.Gln339_Thr393del and p.Glu1517_Ala1537del, hereafter named Δexon7 and Δexon38, respectively) could be functional or, otherwise, responsible for diffuse or focal CHI cases. Indeed, c.1176G>A and c.4611G>A were inherited from the fathers of patients P1 and P20, respectively, making both diagnoses possible.

We thus ectopically expressed the two internally deleted SUR1 proteins in COSm6 cells and performed $^{86}Rb^{+}$ efflux functional assays. These experiments revealed that Δexon7 and Δexon38 were inactive when compared with WT, indicating that the deleted regions are essential for normal function (Figure 3A). Furthermore, Western blot analysis showed an absence of mature SUR1 in both cases (Figure 3B). This observation suggests that Δexon7 and Δexon38 do not reach the membrane (probably due to protein misfolding or SUR1-Kir6.2 misassembly), which in turn implies that the corresponding exon-skipping variants are likely recessive. These assumptions are based on the current biological and clinical interpretation of *ABCC8* variants showing similar SUR1 maturation defects (Boodhansingh

et al., 2019). Moreover, a recessively acting SUR1 Δexon38 protein fits the clinical phenotype of patient P20 for whom histological analysis confirmed a focal form and no other germline alterations were identified besides the paternally-inherited *ABCC8* c.4611G>A variant.

As shown in Table 1, four patients carried an *ABCC8* missense change in addition to the variants analyzed for their impact on splicing, i.e. patients P2 (p.Gly684Asp), P6 (p.Ser1576Pro), P15 (p.Ile1347Phe) and P18 (p.Gly912Arg). All were found to be compound heterozygotes, either by parental sequencing (P2, P6 and P18) or by allele-specific sequencing analysis (P15). The four missense variants were thus also selected for protein functional assays. As shown in Figure 3, whereas p.Ile1347Phe seemed to have a mild effect on SUR1 function as previously suggested (Principalli, Dupuis, Moreau, Vivaudou, & Revilloud, 2015), p.Gly684Asp, p.Gly912Arg and p.Ser1576Pro did not produce mature glycosylated proteins nor any channel activity. Moreover, the p.Ser1576Pro variant was undetectable, suggesting that this protein is unstable and rapidly degraded.

These results were consistent with the inheritance pattern of pathogenic recessive variants and allowed us to reach firm molecular diagnoses for the corresponding patients (Table 1). For instance, c.1332+4del being overruled as a pathogenic variant in the minigene assay, and c.2051G>A (p.Gly684Asp) being classified as a loss-of-function missense variant in the protein functional test, we surmised that patient P2's CHI was caused by the paternally-inherited) recessive mutation (p.Gly684Asp), a conclusion that is in agreement with the focal histology of this patient.

Variant classification and clinical considerations

Altogether the combined information from genetic testing, *in silico*, minigene and protein analyses allowed a firm conclusion on the pathogenicity of all studied variants and on the molecular diagnosis of 16 out of 20 patients (Table 1). For two out of the 4 inconclusive cases, no *ABCC8* pathogenic variant was identified (P5 and P17). Interestingly, in addition to an intronic non-spliceogenic *ABCC8* variant, patient P5 carries a paternally-inherited *KCNJ11* VUS (c.539C>T, p.Thr180Ile). Although we cannot exclude a causal role for the latter, this seems unlikely as, to our knowledge, no dominant cases of diazoxide-unresponsive CHI have yet been assigned to *KCNJ11* missense changes. As for patient P17 homozygous for c.4123–19C>T, a variant initially thought to be pathogenic (Fernández-Marmiesse et al., 2006) but now known to be a polymorphism (gnomAD database) with no effect on RNA splicing (this study), the molecular cause of CHI remains to be discovered. The two remaining unsolved cases include patients P1 and P8. Indeed, it was not possible to genetically assign a focal or diffuse CHI form for patient P1 who carries a spliceogenic variant responsible for an in-frame defect. A potential dominant-negative effect caused by the corresponding Δexon7 SUR1 protein could not be ruled out as co-expression of the mutant with the WT protein (mimicking a heterozygous state) led to a slight decline in function. This small effect was possibly due to the reduced amount of the mutant protein relative to the WT (Supp. Figure S7). For the last case (patient P8), although we could assert a diffuse CHI form, it was not possible to state on the genetic risk for this family (mode of inheritance not ascertained).

Finally, results for all patients but one were consistent with phenotype information obtained either by PET scan or histological analysis. The single inconsistent case was patient P7 who only has a paternal spliceogenic recessive variant suggestive of a focal form while PET scan results were in favor of a diffuse form. Among other potential causes, we suspect that this discrepancy is due either to a missed variant in the maternal allele or to an over-interpretation of the PET images (e.g. a giant focal lesion presenting as an apparent diffuse form).

Discussion

CHI is a severe condition where the differentiation between diffuse and focal histological forms is imperative for appropriate clinical management with distinct therapeutic solutions and genetic counseling. The 13 *ABCC8* variants analyzed in this study were identified in 20 CHI patients for whom unresolved questions remained after genetic testing. All the selected variants were located in the vicinity of natural splice sites, and thus we wondered if they could affect the normal splicing pattern of *ABCC8*.

Unfortunately, *ABCC8* is too weakly expressed in blood cells, and disease-relevant samples (pancreatic tissue) are generally not available. To overcome these limitations, we used *in silico* and cell-based approaches, both relatively easy to implement, to determine the impact on splicing of the selected *ABCC8* variants. While the minigene assay was very useful for answering this question, we also noticed that not all WT minigenes displayed equivalent splicing efficiencies. For instance, the minigene carrying *ABCC8* exons 37–38 showed very poor exon 38 inclusion even in the WT context (Figure 1B), a difficulty that may be related to the nucleotide context of the minigene construct or the type of transfected cells. The fact that the bioinformatics predictions and the experimental data from the minigene assay concurred for most variants suggests that combining MES/SSFL, SPiCE and SpliceAI *in silico* approaches helps prioritizing variants for experimental analyses. Moreover, the nature of the actual splicing defects could be predicted in most cases by SpliceAI, including the more complex alterations observed in the minigene assay with the exception of those caused by c.3992–9G>A. This exception thus illustrates the importance of performing experimental analyses for accurately assessing the impact of VUS on splicing and agrees with a recent report that highlighted a better performance for SpliceAI as compared to other splicing predictors (Wai et al., 2020).

In the case of *ABCC8*, if a VUS leads to the exclusive production of aberrantly spliced frameshift transcripts then it is possible to classify the variant as a recessive loss-of-function genetic change. However, when in-frame alterations are produced, a second line of analysis is necessary to evaluate SUR1 function. In this context, the functional protein assays that we performed for Δexon7 and Δexon38 allow classifying all variants inducing total skipping of *ABCC8* exon 7 or exon 38 as pathogenic. Exon 7 encodes Thr338-Gln392, which is part of an extracellular loop connecting transmembrane spanning helices TM6 and TM7 and almost the entirety of TM7 (Aguilar-Bryan et al., 1998; Li et al., 2017; Martin et al., 2017); deletion of exon 7 would be expected to alter the topology and folding of the protein. Exon 38 spans amino acids Glu1517-Ala1537, which is an integral part of NBD2 (nucleotide binding domain 2) and likely causes misfolding of NBD2 when missing. Consistently, Δexon7 and

Δexon38 mutant proteins fail to mature indicating they are trafficking variants which tend to manifest as recessive mutations. While the clinical pathology associated with Δexon38 (patient P20) fits this inheritance pattern, the PET-scan-based ascertainment of a diffuse form associated with a Δexon7-inducing variant goes against a similar molecular diagnosis for patient P1 therefore judged as an unsolved case. Still, given that the sensitivity of PET imaging is ~75–89% (Blomberg, Moghbel, Saboury, Stanley, & Alavi, 2013; Treglia, Mirk, Giordano, & Rufini, 2012) we cannot exclude the possibility of a missed focal form for this patient. Alternatively, a maternally-inherited variant may have escaped detection in our DNA sequencing analysis, notably a variant mapping either to transcription regulatory sequences, to 5' and 3' untranslated regions or to deep intronic regions (Flanagan et al., 2013) (regions not interrogated in our study, which focused on coding regions and flanking intronic sequences only). Another explanation could be the advent of a post-zygotic modification in the maternal allele potentially presenting as a *de novo ABCC8* mosaic variant in the pancreatic tissue (Houghton et al., 2020). It is possible that such cryptic deleterious alterations were also missed in patients P5, P8 and P17 who, together with patient P1, remain considered as unsolved cases.

Overall, the combined results from the RNA and protein functional approaches were conclusive for all analyzed variants and allowed reaching a definite diagnosis for 16 out of 20 patients. Eight patients were unambiguously assigned with diffuse forms, whereas 8 other patients were diagnosed as suffering from presumed focal forms, an inference that should be confronted with PET-scan data before considering focal surgery.

Interestingly, we found that a common *ABCC8* polymorphism (rs757110, c.4108G>T) affected the splicing efficiency of exon 33, suggesting that this SNP may play a modifier role in *ABCC8*-related disorders or drug response. The influence of SNPs in RNA splicing patterns and in genotype-phenotype correlations have already been reported in other genes and monogenetic disorders, such as in the *MCAD* and *ABCB11* genes implicated in medium-chain acyl-CoA dehydrogenase deficiency and in intrahepatic cholestasis, respectively (Byrne et al., 2009; Nielsen et al., 2007). In the minigene assay, *ABCC8* c.4108G>T (rs757110) increases the spliceogenicity of c.3992–3C>G and c.3992–9A>G, the latter being a pathogenic variant particularly known for its recurrence in Ashkenazi Jewish patients and association with a large phenotypic heterogeneity (Ismail et al., 2012; Kapoor, Flanagan, Ellard, & Hussain, 2012). The reasons for the phenotypic variability in c.3992–9A>G carriers are still unknown but it was hypothesized that it could be due either to modifier genes, epigenetic factors or environmental cues (Kapoor et al., 2012). Our results suggest that the presence of the common *ABCC8* c.4108G>T polymorphism may also contribute to differences in c.3992–9A>G expressivity and associated clinical presentations. It would be interesting to perform further genetic and clinical studies to explore this hypothesis. In addition, it would be important to analyze the impact on splicing of this variant (in the presence/absence of c.4108G>T) in a system more closely resembling the natural structure of the *ABCC8* gene, in particular because our minigene assay shows suboptimal exon 33 splicing, which precludes assessment of the eventual leakiness of c.3992–9A>G (*i.e.* the capacity to produce residual amounts of full-length transcripts). The usage of larger minigenes (containing larger intronic segments and/or additional flanking exons) may overcome this limitation as already shown for other exons/genes (M. Baralle et

al., 2006; Sangermano et al., 2018). Because of its considerable size, the full sequence of *ABCC8* cannot be cloned in plasmid vectors for performing cell-based splicing assays contrary to what has been recently reported for small genes (Lin et al., 2020). An alternative could be to use CRISPR-cas9 technology to modify the genome of a human cell line suitable for *ABCC8* expression studies, similar to what has been done for *BRCA, MSH2* and several other disease-implicated genes (Findlay et al., 2018; Fuster-García, García-Bohórquez, Rodríguez-Muñoz, Millán, & García-García, 2020; Kweon et al., 2020; Rath et al., 2019). Gene editing would have the advantage to allow the study of *ABCC8* in its natural genomic context, including under the control of its natural promoter.

This work highlights the importance of better characterizing molecular variants of *ABCC8* in CHI diagnosis practice. We believe that our strategy could be applied to most K_{ATP} variants and propose a flowchart aiming to help the interpretation of CHI-associated variants based on RNA and protein data (Figure 4). Not only the increasing number of identified *ABCC8* VUS requires means to re-classify the variants either as pathogenic or as benign variants, but the specifics of this condition add another level of complexity requiring further knowledge on the inheritance pattern. We showed that combining data from clinical examination, genetic testing, bioinformatics analyses and functional assays, including RNA splicing and protein studies, is beneficial for CHI diagnosis. The combination of these approaches is therefore helpful for optimizing the clinical management of hypoglycemic neonates/infants who are at risk of developing severe seizures and brain damage.

In a broader note, we believe that the implementation of multi-approach strategies such as the one described in our work (combination of *in silico* analysis, RNA splicing assays and protein functional tests) will facilitate the interpretation of variants of uncertain significance identified not only in *ABCC8* but also in other disease-implicated genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflicts of interest/competing interests

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Data Availability Statement

All data generated or analyzed during this study are included in this published article and its supporting information files. Novel nucleotide variants reported in this study were be submitted into LOVD (Leiden Open Variation database; www.lovd.nl/ABCC8).

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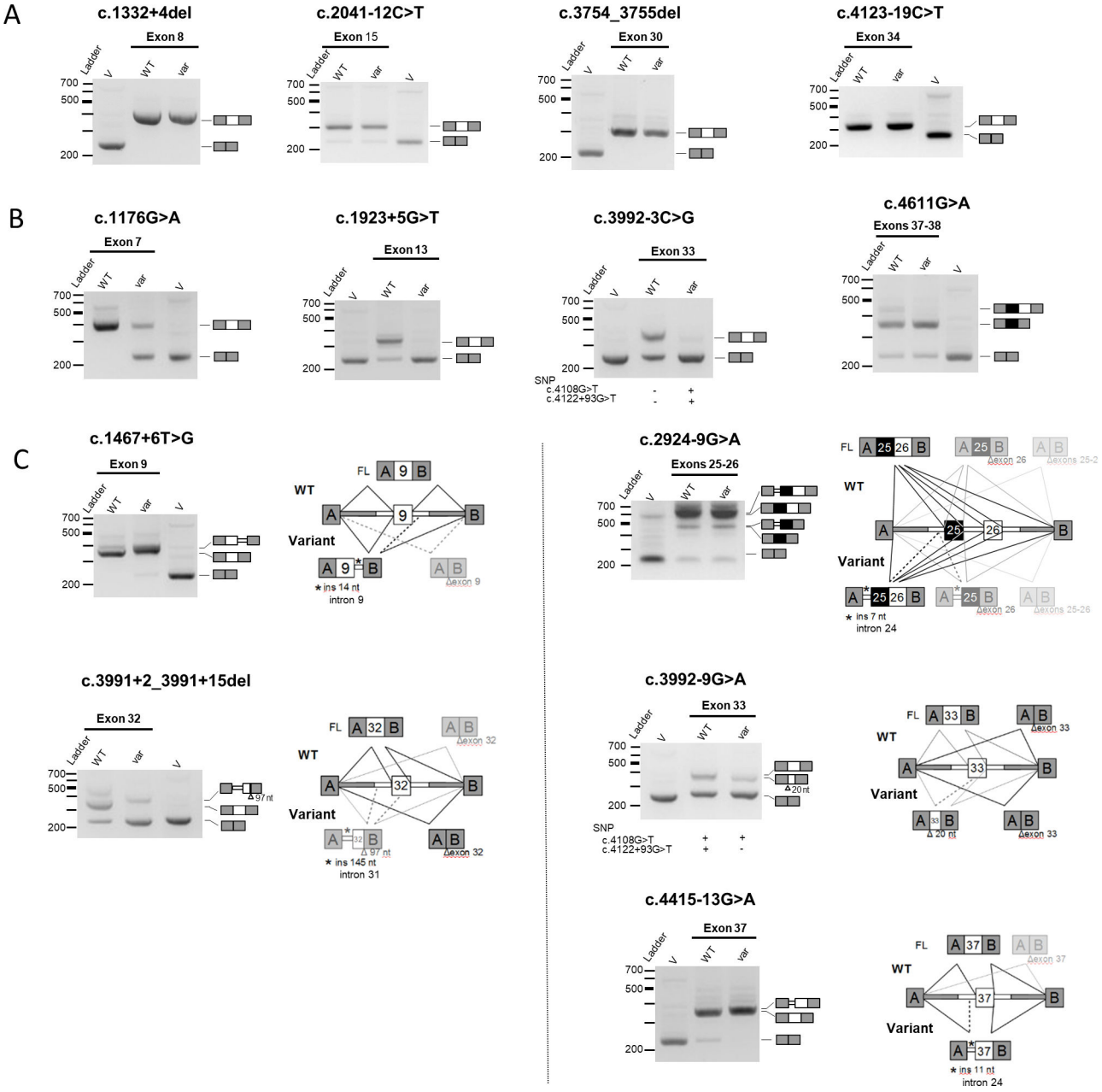
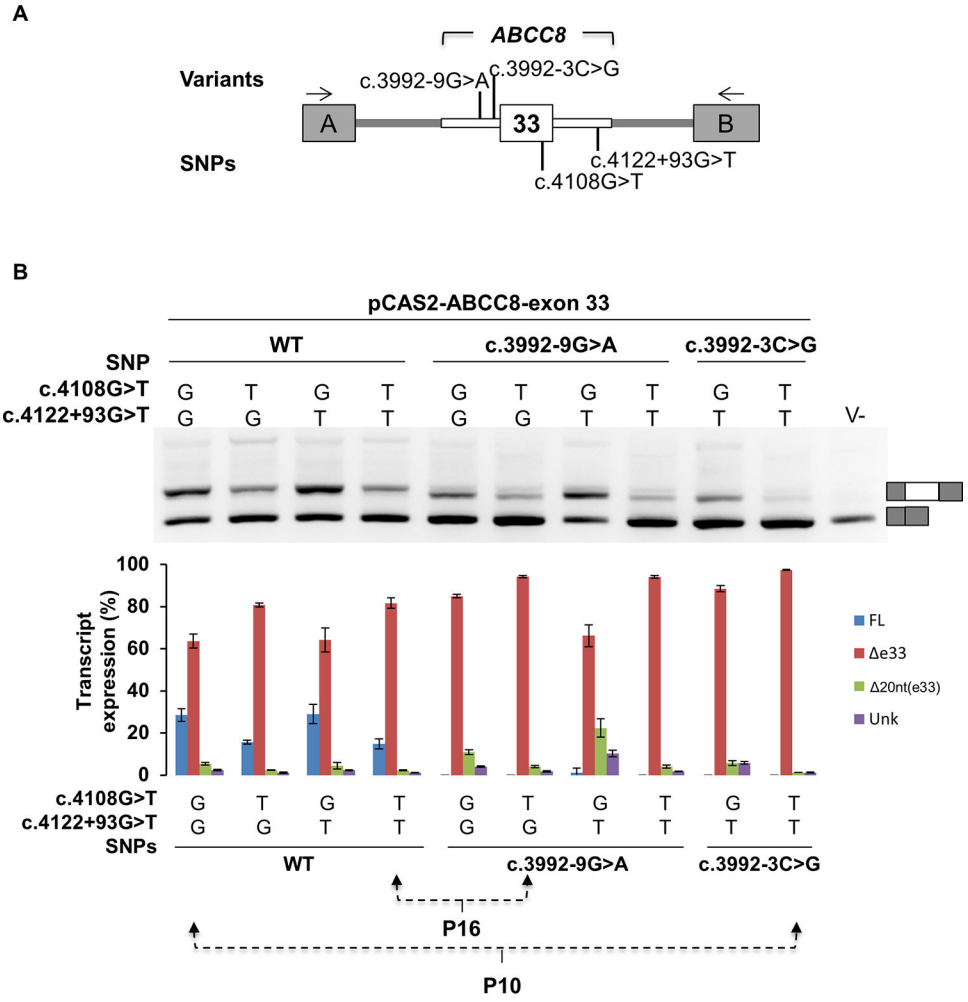


Figure 1. Analysis of the impact on splicing of *ABCC8* variants based on minigene splicing assays.

(A) Variants with no effect on splicing. (B) Variants that induce exon skipping. (C) Variants causing complex splicing defects. The images show RT-PCR products separated on agarose gels as described under Materials and Methods. The identities of the RT-PCR products are indicated on the right. The images are representative of three independent experiments, including the results obtained with c.4611G>A for which the pCAS2.ABCC8 ex37–38 minigene consistently showed poor inclusion of exon 38 in the WT context and increased/total exon skipping in the presence of c.4611G>A (shown in 1B). Ladder, size marker (bp); WT, wild-type; var, variant; v, empty pCAS2 vector; FL, full-length; Δ, deletion (Del); ins, insertion, nt, nucleotides.



C

c.4108G>T SRE predictors (*in silico* scores) and splicing predictions			
QUEPASA (ΔtESRseq)	**HEXplorer** (ΔHzei)	**SPANR** (ΔΨ)	**HAL** (ΔΨ)
-1.6	-70.02	-1.73	-24
exon skipping	exon skipping	exon skipping	exon skipping

Figure 2. Effect of single-nucleotide polymorphisms in the splicing pattern of *ABCC8* exon 33.

(A) Distribution of variants and SNPs detected in or near *ABCC8* exon 33**. (B)** Splicing patterns of pCAS2-*ABCC8* exon 33 minigenes carrying the variants and SNPs of interest. The upper panel shows RT-PCR products separated on an agarose gel and the lower panel indicates their quantification after migrating the fluorescent RT-PCR products on an automated sequencer as illustrated in Supp. Figure S6. P10 and P16 refer to minigene constructs prepared with gDNA amplified from patients P10 and P16, respectively. The SNPs on the other minigenes were introduced by site-directed mutagenesis. **(C)** SRE-

dedicated *in silico* predictions for the exonic SNP (c.4108G>T). V, empty minigene vector, FL, full-length; Δ33, skipping of exon 33; Δ20nt(e33), deletion of 20 nucleotides from the beginning of exon 33; Unk, unknown, SRE, splicing regulatory elements.

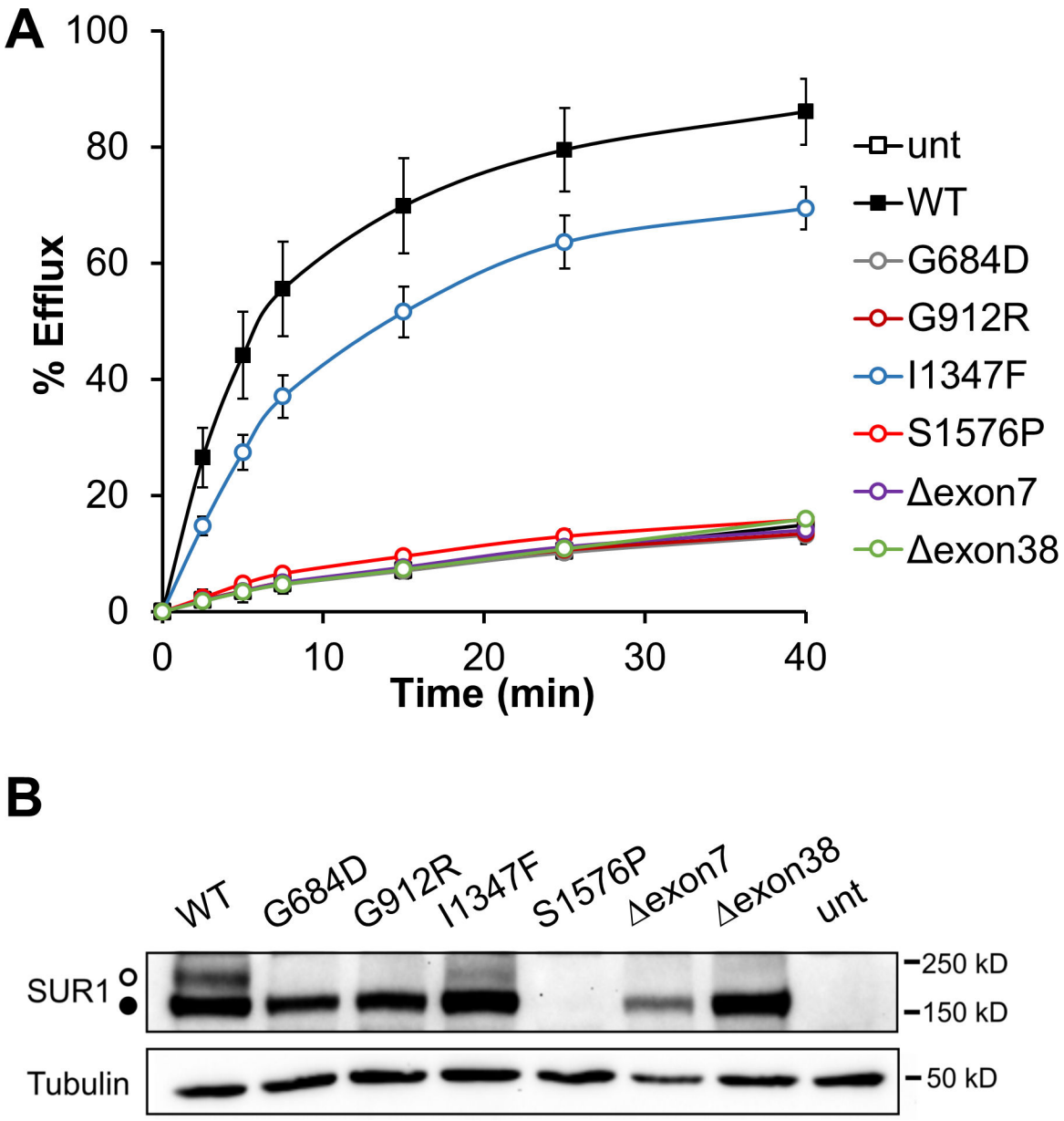


Figure 3. Protein functional analysis shows that all tested SUR1 variants reduce or abolish K_{ATP} channel activity.

(A) $^{86}Rb^{+}$ efflux results showing that all mutant channels are inactive, with the exception of I1347F that retains residual activity. For simplicity, one-letter amino acid nomenclature was used to designate full-length mutant proteins. G684D, G912R, I1347F and S1576P stand for p.Gly684Asp, p.Gly912Arg, p.Ile1347Phe and p.Ser1576Pro, respectively. Δexon7 and Δexon38 indicate internally deleted SUR1 proteins corresponding to p.Gln339_Thr393del and p.Glu1517_Ala1537del, respectively. The graph shows the average of three independent experiments. Error bars represent the standard error of the mean. WT, wild-type; unt, untransfected cells. **(B)** Representative Western blot experiment showing that complex-glycosylated mature SUR1 (open circle) is undetectable in most mutant protein preparations,

with a slight detection for I1347F. Closed circle, core-glycosylated immature SUR1 protein band.

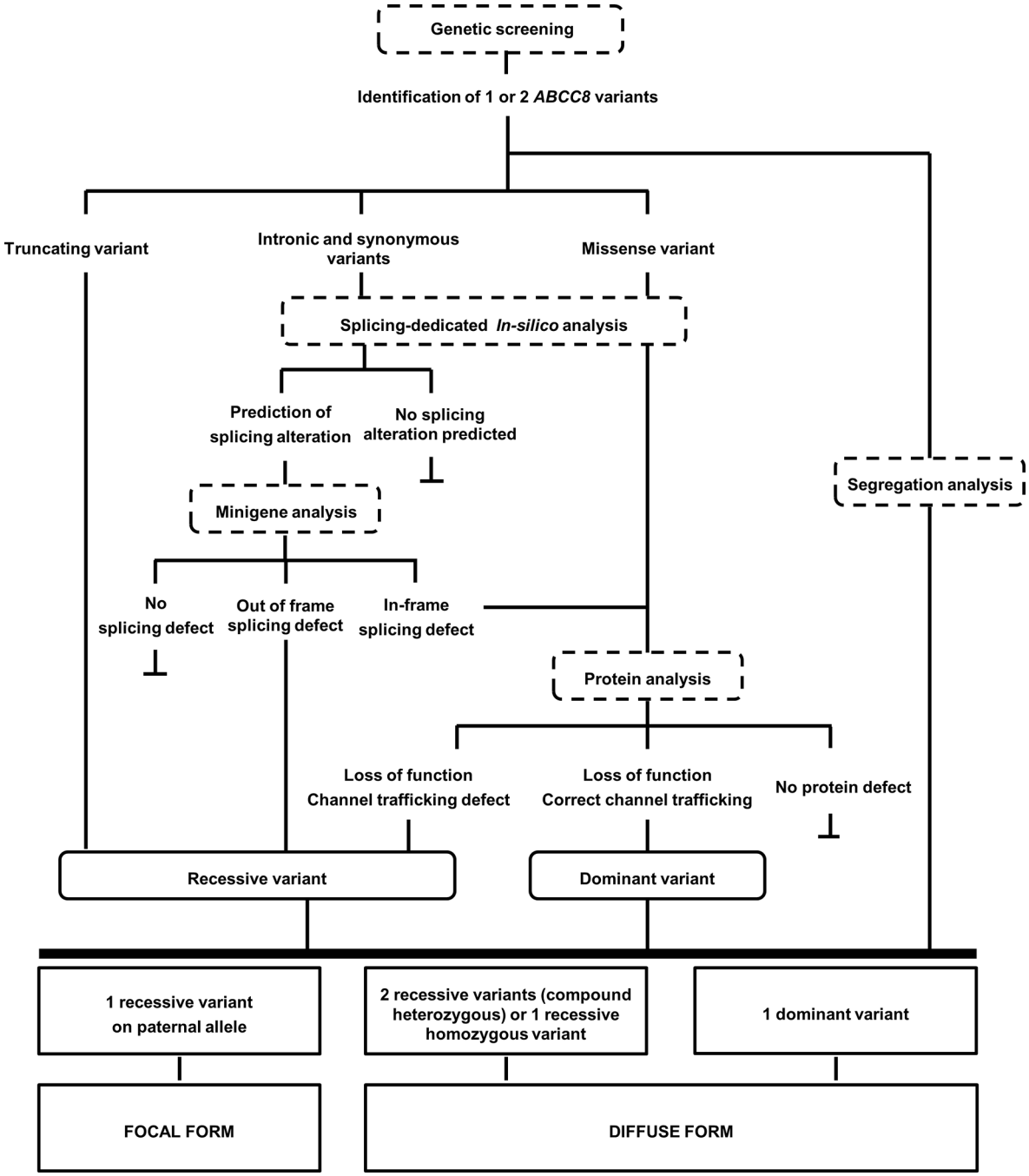


Figure 4. Flowchart to assist in the interpretation of functional data for classification of CHI-associated K_{ATP} channel variants.

Functional analyses can contribute to pathogenicity assessment of *ABCC8* variants currently considered as of unknown significance (VUS) or associated with partially explained phenotypes. These may include all types of variants as shown. In this context, splicing-dedicated *in silico* analysis represents a first evaluation step that helps stratifying variants for further testing. Predictions of RNA splicing defects should be verified by performing experimental analyses such as cell-based minigene splicing assays. RNA splicing analyses of truncating variants (nonsense and frameshift variants), which are typically classified as

pathogenic, are not required in a systematic fashion. However, they may be helpful in exceptional situations such as in cases with intriguing genotype-phenotype correlations as this type of variants can also alter splicing (Meulemans et al., 2020; Tuffery-Giraud et al., 2017; Zhu et al., 2019) (not shown). In addition, functional protein analysis are recommended for variants preserving *ABCC8* reading frame (*bona fide* missense variants and variants leading to in-frame indels). Besides functional data, pathogenicity assessment also benefits from results of familial segregation analysis. This flowchart highlights the importance of combining clinical, segregation, and functional data in the molecular diagnosis of focal and diffuse forms of CHI.

Table 1.

Improvements in the molecular diagnosis of 20 CHI patients based on the combined analysis of genotyping results, minigene splicing assays, protein functional tests and clinical data.

The upper section of the Table synthesizes the genotypes of 20 patients (each carrying at least one of the 13 variants investigated in this study), the impact on RNA splicing of these 13 variants (bioinformatics predictions and minigene splicing assay data), the impact on SUR1 steady-state protein levels and function, and variant classification. The bottom section of the Table synthesizes our attempt to reach a final diagnostic for each case and provides a comparison with clinical data.

	CHI patient																					**Impact on RNA splicing**		**Impact on protein**	**Variant classification** ††
***ABCC8* variant** †	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		*In silico* predictions ‡ (SD/NE/NP)	Minigene assay §	SUR1 assay ¶	
c.1176G>A, p.Gln392=	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Splicing defect (3/0/0)	In-F exon 7 skipping	LoF (**Δexon7**, p.Thr338_Gln392del), DN?	3 ▸ 4
c.1332+4del^{N}	-	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	No effect (0/3/0)	No splicing defect	-	3 ▸2
c.1467+6T>G^{N}	-	-	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Splicing defect (2/1/0)	Out-F alteration	-	3 ▸4
c.1536C>G, p.Tyr512*	-	-	-	-	-	-	-	-	-	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5
c.1923+5G>T^{N}	-	-	-	n	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Splicing defect (3/0/0)	Out-F alteration	-	3 ▸4
c.2041-12C>T^{N}	-	-	-	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*Ambiguous* (1/0/2)	No splicing defect	-	3 ▸2
c.2051G>A^{N}, p.Gly684Asp	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	LoF (p.Gly684Asp)	3 ▸4
c.2588_2591del^{N}, p.His863fs	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	5
c.2734G>C^{N}, p.Gly912Arg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	-	-	-	-	-	LoF (p.Gly912Arg)	3 ▸4
c.2800C>T, p.Arg934*	-	-	-	-	-	-	-	-	-	-	-	-	-	M	-	-	-	-	-	-	-	-	-	-	5

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c.2924-9G>A[N]	-	-	-	-	-	P	P	-	-	-	-	-	-	-	-	-	-	-	-	-	Splicing defect (3/0/0)	Out-F alteration	-	3 ▸4
c.3754_3755del[N], **p.Met1252fs**	-	-	-	-	-	-	-	M	-	-	-	-	-	-	-	-	-	-	-	-	*Ambiguous* (0/1/2)	No splicing defect	-	5[##]
c.3991+2_3991+15del[N]	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-	-	-	-	Splicing defect (3/0/0)	Out-F alteration	-	3 ▸4
c.3992-9G>A	-	-	-	-	-	-	-	-	-	-	-	-	P	P	#	?	-	-	-	-	*Ambiguous* (1/1/1)	Out-F alteration	-	4 ▸ 5
c.3992-3C>G	-	-	-	-	-	-	-	-	-	P	M	P	-	-	-	-	-	-	-	-	*Ambiguous* (1/1/1)	Out-F alteration	-	3 ▸ 4
c.4039A>T, p.Ile1347Phe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	#	-	-	-	-	-	-	-	ReF (p.Ile1347Phe)	4
c.4123-19C>T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P M	-	-	-	*Ambiguous* (0/1/2)	No splicing defect	-	3 ▸2
c.4163_4165del, p.Phe1388del	-	-	-	-	-	-	-	-	-	-	-	-	M	-	-	-	-	-	-	-	-	-	-	4
c.4415-13G>A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	?	-	Splicing defect (2/0/1)	Out-F alteration	-	3 ▸4
c.4611G>A[N], **p.Ala1537=**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	Splicing defect (3/0/0)	In-F exon 38 skipping	LoF (**Δexon38**, p.Glu1517_Ala1537del)	3 ▸3
c.4726T>C[N], p.Ser1576Pro	-	-	-	-	-	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	LoF (p.Ser1576Pro)	3 ▸4
c.4734_4737del[N], p.Arg1579fs	-	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
Interpretation based on molecular data *##*																								
Recessive (somatic LOH) >> [Fo]		•		•			•		•			•				•			•	•				
Dominant >> [Di]																								
Recessive (c-htz or hmz) >> [Di]			•			•				•	•		•	•	•			•						
Interpretation based on PET/histology *§§*																								

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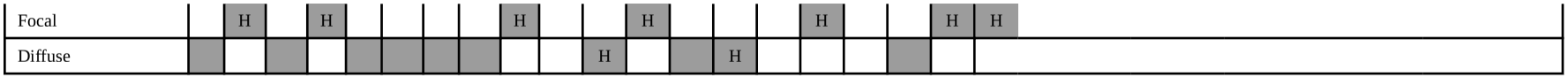

†Variants selected for minigene splicing assays are indicated in bold. The p.Arg1579fs, p.Met1252fs and p.His863fs mutant protein descriptions were abbreviated from p.(Arg1579Glnfs*31), p.(Met1252Glyfs*154), and p.(His863Argfs*5), respectively. N, novel variant (newly reported); P, paternal allele; M, maternal allele; n, *de novo*; #, compound heterozygosity ascertained by allele-specific DNA sequencing but parents' genotype not available; ?, undetermined mode of inheritance.

≠RNA splicing-dedicated *in silico* predictions are detailed in Supp. Table S2. Predictions were considered unambiguous (splicing defect versus no effect) if at least 2 out of the 3 methods provided a similar output; otherwise they were considered ambiguous. The number of methods yielding the following outputs are indicated between parenthesis: SD (splicing defect)/NE (no effect) /NP (no prediction, *i.e.* no score was obtained).

‡Cell-based minigene splicing assay data are shown in Figure 1. In-F, in-frame; Out-F, out-of-frame.

§Cell-based SUR1 assay data are shown in Figure 3. Protein nomenclature is indicated between parentheses. Δ, exon skipping; DN?, potential dominant-negative effect; LoF, loss-of-function (absence of mature protein and inactive channel); ReF reduced-function (reduced expression of the mature protein and diminished channel activity).

¶Variant classification based on a 5-tier system (1, neutral; 2, probably neutral; 3, unknown signification; 4, probably pathogenic; 5, pathogenic). Revised classifications (highlighted in grey) are preceded by a triangle and reflect the results obtained in this study (from RNA splicing and/or SUR1 assays). The triangles further indicate the direction of the upgraded classifications either towards pathogenicity (▸) or neutrality (▹). ##, variant typically interpreted as Class 5 but associated with an unexpected phenotype in Patient P8.

#Case interpretation based on molecular data (DNA sequencing results and functional data). The hypotheses formulated at the beginning of this study are shown in grey, and our final conclusions are highlighted by the bullets (•). Cases without a bullet could not be unequivocally assigned and thus remain considered as unsolved. LOH, loss of heterozygosity; c-htz, compound heterozygous; hmz, homozygous; >> [Fo], expected focal pancreatic lesion; >> [Di], expected diffuse pancreatic lesion.

‖Interpretation of pancreatic lesions based on ^{18}F-DOPA-PET scan analysis and/or histology-based diagnosis of focal and diffuse CHI forms (grey background). H, post-surgery histological confirmation of focal or diffuse forms, as shown. The white background indicates patients for whom no ^{18}F-DOPA-PET imaging data nor histology diagnosis were available.