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Abbreviations: ATP, Adenosine triphosphate; ABC, ATP-binding cassette; ICP, intrahepatic cholestasis of pregnancy; LPAC, low phospholipid-associated cholelithiasis; NBD, nucleotide-binding domain; PFIC3, progressive familial intrahepatic cholestasis type 3.

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Abstract

Genetic variations of the phosphatidylcholine transporter, ABCB4 cause several biliary diseases. The large number of reported variations makes it difficult to foresee a comprehensive study of each variation. To appreciate the reliability of *in silico* prediction programs, 1) we confronted them with the assessment in cell models of two ABCB4 variations (E528D and P1161S) identified in patients with low phospholipid-associated cholelithiasis (LPAC); 2) we extended the confrontation to 19 variations that we had previously characterized *in cellulo*. Four programs (Provean, Polyphen-2, PhD-SNP and MutPred) were used to predict the degree of pathogenicity. The E528D and P1161S variants were studied in transfected HEK293 and HepG2 cells by immunofluorescence, immunoblotting and measurement of phosphatidylcholine secretion. All prediction tools qualified the P1161S variation as deleterious, but provided conflicting results for E528D. In cell models, both mutants were expressed and localized as the wild type but their activity was significantly reduced, by 48% (P1161S) and 33% (E528D). These functional defects best correlated with MutPred predictions. MutPred program also proved the most accurate to predict the pathogenicity of the 19 ABCB4 variants that we previously characterized in cell models, and the most sensitive to predict the pathogenicity of 65 additional mutations of the Human Gene Mutation Database. These results confirm the pathogenicity of E528D and P1161S variations and suggest that even a moderate decrease (by less than 50%) of phosphatidylcholine secretion can cause LPAC syndrome. They highlight the reliability of *in silico* prediction tools, most notably MutPred, as a first approach to predict the pathogenicity of ABCB4 variants.

Keywords: cholestasis, ABC transporter, MDR3, genetic disease, MutPred prediction

1. Introduction

ABCB4, also called MDR3 (multidrug resistance 3) is a lipid floppase, specialized in the secretion of phosphatidylcholine at the canalicular membrane of hepatocytes (Smit et al., 1993). ABCB4 belongs to the ATP-binding cassette (ABC) superfamily of transporters. The molecule is organized in two repeats, each containing a transmembrane domain with six transmembrane helices, and a cytoplasmic nucleotide-binding domain (NBD) (Kast et al., 1995). The transmembrane domains are involved in the specificity towards the substrate and its translocation, whereas NBDs, which are highly conserved among all ABC transporters, provide the energy for the process (Zolnerciks et al., 2011). NBDs contain several conserved sequences, notably the Walker A and B motifs found in ATPases, and the signature motif, which is a specificity of ABC transporters (Zolnerciks et al., 2011).

Variations in the *ABCB4* gene have been identified as the cause of several hepatobiliary diseases (Gonzales et al., 2009). The most severe of these diseases is progressive familial intrahepatic cholestasis type 3 (PFIC3), a rare autosomal recessive disease occurring early in childhood that may be lethal in the absence of liver transplantation (Jacquemin et al., 2001). Less severe are the low phospholipid-associated cholelithiasis (LPAC) syndrome and intrahepatic cholestasis of pregnancy (ICP), which occur in the young adult. The LPAC syndrome is a biliary gallstone disease characterized by intrahepatic stones and symptomatic cholelithiasis (Rosmorduc et al., 2001), whereas ICP is a reversible cholestatic condition, which occurs in the third trimester of pregnancy and resolves quickly after delivery (Geenes and Williamson, 2009).

A growing number of *ABCB4* disease-causing variations has been reported (Jacquemin et al., 2001; Degiorgio et al., 2007; Poupon et al., 2010a, 2013; ABCM database, http://abcmutations.hegelab.org; Human Gene Mutation Database, http://www.hgmd.cf.ac.uk). Most often, the studies were restricted to the mutational screening of the *ABCB4* gene and did not address the functional consequences of the variations. Nonsense mutations or mutations leading to a change in the reading frame will certainly result

in a loss-of-function. However, the pathophysiological consequence of missense mutations often remains uncertain. Several studies have been performed to elucidate the effect of missense variations found in PFIC3 patients, on the structure, trafficking and function of ABCB4 (Delaunay et al., 2009, 2016, 2017; Degiorgio et al., 2014; Gautherot et al., 2012; Gordo-Gilart et al., 2015, 2016). Fewer studies have addressed the effect of missense variations associated with the LPAC syndrome or ICP (Andress et al., 2014; Gautherot et al., 2014). The large number of variants identified by genome sequencing is poorly compatible with exhaustive functional analyses. A number of bioinformatic programs have been developed as a surrogate. These tools are based on different features (Thusberg and Vihinen, 2009) including the physicochemical and structural properties of amino acid residues (Ng and Henikoff, 2006) or the evolutionary conservation (Kumar et al., 2014). The relevance of these tools to predict the degree of damaging of a given substitution of ABCB4 has never been explored.

In the current study, we combined *in silico* approaches and experimental investigations based on cellular models to elucidate the impact of two ABCB4 variations, E528D and P1161S that were previously identified in patients with LPAC syndrome or ICP (Rosmorduc et al., 2003). We then extended the confrontation to nineteen additional variations that we previously experimentally characterized, and to 65 other missense mutations of the Human Gene Mutation Database.

2. Patients and Methods

2.1. Patients' data analyses

ABCB4 gene analysis was performed in patients referred to the Reference Center for Inflammatory Biliary Diseases (Hôpital Saint-Antoine, Paris, France), upon informed consent from the patients and approval by the local ethical committee. The 27 coding exons of the ABCB4 gene were amplified together with their exon/intron boundaries from genomic DNA and sequencing was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems–Life Technologies, Saint-Aubin, France), as previously reported (Rosmorduc et al., 2003; Ziol et al., 2008). Gene variations were assessed by sequence comparisons with the SeqScape Software (version 2.5; Applied Biosystems–Life Technologies).

2.2 In silico analyses

The evolutionary conservation of E528 and P1161 was estimated using the Clustal Omega program, by alignment of the ABCB4 protein sequences of different species obtained from the NCBI database (http://www.ebi.ac.uk/Tools/msa/clustalo/). Possible topological change was analyzed via the Protter software (Omasits et al., 2014), a web-based tool which provides an integrated visual analysis of membrane proteins. The degree of pathogenicity of the two mutations was predicted using four *in silico* programs based on different approaches. Provean (Choi and Chan, 2015) bases its predictions on sequence conservation. A score measures the impact of an amino acid variation based on the amino acid residue at the position of interest and on the quality of sequence alignment derived from the neighborhood flanking sequences. PhD-SNP (Capriotti et al, 2006) is a prediction method based on support vector machines. It classifies a missense variant as pathogenic or neutral, based on the nature of the substitution and the properties of the neighboring sequence environment. Poly-Phen2 (Adzhubei et al., 2013) calculates the probability for a mutation to be damaging using eight sequence-based and three structure-based predictive features of the property of the wild type allele and the corresponding property of the mutant. MutPred (Li et al., 2009) estimates the impact of an amino-acid substitution using a large panel of attributes related to predicted protein structure

and dynamics, predicted functional properties, amino acid sequence and evolutionary information. The extent of this predicted impact is supplied in terms of probability of deleterious effect. A probability > 50% is considered as pathogenic (http://mutpred1.mutdb.org/about.html).

2.3. Homology modeling of ABCB4

Three dimensional modeling of ABCB4 protein structure was performed using the RaptorX web server as a resource for template-based tertiary structure modeling (Källberg et al., 2014). The wt ABCB4 sequence and the mutated sequences were individually submitted to this server to generate the 3D models, based on the template 4f4cA structure of the *Caenorhabditis elegans* Multi-Drug Transporter, selected as the best template. The quality of models was judged as high based on the Ramachandan plot which showed that 90% of residues are situated in a favorable area. Then, the Swiss-PDB Viewer software (V4.1) was used to visualize and to compare the generated models (Guex and Peitsch, 1997). The superposition of the two compared models ensures the calculation of the RMSD deviation, which measures the average distance between the backbone atoms of wt and mutated models. A high RMSD deviation indicates that the structural rearrangements caused by the substitution are important (Carugo and Pongor, 2001).

2.4. Mutagenesis, cells and transfection

The E528D and P1161S mutations were introduced into the ABCB4 cDNA cloned into the pcDNA3 vector (Delaunay et al., 2009), using the Quik-Change II XL mutagenesis kit from Agilent Technologies (Massy, France). DNA primers used for ABCB4 mutagenesis were from Eurogentec (Angers, France). The sequences were GACACCCTGGTTGGAGACAGAGGGGCCCAGCTGAGTGG (forward) and CCACTCAGCTGGGCGCCTCTGTCTCCAACCAGGGTGTC (reverse) for the E528D mutation and CATCGAGACGTTATCCCACAAATATGAAACAAGAGTGG (forward) and CCACTCTTGTTTCATATTTGTGGGATAAGCTCTCGATG (reverse) for the P1161S mutation. All constructs were verified by automated sequencing. HEK293 cells and HepG2

cells were transiently transfected with the plasmids using Turbofect (Fermentas France, Villebon-sur-Yvette), as previously described (Delaunay et al., 2016).

2.5. Immunofluorescence and immunoblotting

Indirect immunofluorescence was performed on cells grown on glass coverslips after fixation with methanol at -20°C, as described (Delaunay et al., 2009). In the case of HEK293 cells, coverslips were pre-coated with poly-L-lysine (Sigma-Aldrich, Saint-Quentin Fallavier, France). The monoclonal P3II-26 anti-ABCB4 was obtained from Enzo Life Sciences (Villeurbanne, France) and Alexa Fluor–labeled secondary antibodies were from Invitrogen-Life Technologies. Electrophoresis and immunoblotting were performed as described (Delaunay et al., 2009) using the monoclonal P3II-26 anti-ABCB4 antibody, and peroxidase-conjugated secondary antibodies from Rockland Immunochemicals (Gilbertsville, PA).

2.6. Measurement of Phosphatidylcholine Secretion

Phosphatidylcholine secretion was measured in the culture medium of HEK293 cells, as described (Delaunay et al., 2016, Gautherot et al., 2014). Briefly, cells grown in 6-well plates were transfected with the plasmids encoding wt or mutant ABCB4. Sixteen hours post-transfection, the medium was replaced by phenol red-free DMEM containing 0.5 mmole/L sodium taurocholate and 0.02% fatty acid–free bovine serum albumin (Sigma-Aldrich). After 24 hours, the medium was collected and lipids were extracted by chloroform/methanol/water partition. The organic phase was evaporated and resuspended in phosphate-buffered saline with 0.1% Triton X-100 (w/v). Measurement of phosphatidylcholine content was based on the amount of choline released after phospholipase D treatment, using a fluorimetric assay. Results were normalized to the expression levels of wt ABCB4 or of the mutants, which were quantified from immunoblots of the corresponding cell lysates. The Student's *t* test was used for statistical analysis**.**

3. Results

3.1. Patients and mutations

Two variations were investigated here for the first time: c.1584 G>C, which causes the amino acid change E528D, and c.3481C>T, which causes the change P1161S. These variations were previously reported with a heterozygous status in four (E528D) and two (P1161S) patients diagnosed with LPAC syndrome (Rosmorduc et al., 2001; Poupon et al., 2013). Patients' sequencing data obtained between 2010 and 2014 in our center, identified the c.1584G>C (E528D) variation in three additional patients diagnosed with LPAC syndrome. Patients' data are presented in Table 1. All patients were heterozygous for the variations. Three patients with the E528D variation were also homozygous or heterozygous for the benign variant R652G (Table 1).

3.2. In Silico predictions

The variations studied are located in the first NBD (E528D) and second NBD (P1161S), a few amino acid upstream the ABC signature motif LSGGQ, which is crucial for ATP binding. The sequence alignment of ABCB4 from different species found in the NCBI database (Fig. 1 A) showed that the proline at position 1161 is highly conserved. On the other hand, the glutamic acid at position 528 is conserved in primates and several animal species, but others displayed an aspartic acid at this position (Fig. 1 A).

The effect of the E528D and P1161S variations was examined by *in silico* tools. According to Protter (Omasits et al., 2014), these changes did not affect the overall organization of ABCB4 domains in the membrane. The pathogenicity prediction of these mutations by different programs is shown in Table 2. All programs predicted that P1161S would be deleterious, but they gave discordant predictions for E528D. Provean, which is based on homology information and PolyPhen-2, which refers to evolutionary and structural perspectives estimated that E528D was a neutral substitution. On the other hand, PhD-SNP and MutPred, which take into account structural and functional properties predicted that both E528D and P1161S were deleterious.

The effect of the two variations was also studied at the structural level by homology modeling of wt ABCB4 and of the mutants. First, the overall structural effect was estimated by the calculation of the root mean square deviation (RMSD) and showed that the mutation P1161S leads to a greater deviation in the structure (RMSD = 8.51 Å) than the E528D mutation, which causes minor structural modifications (RMSD = 3.56 Å). Then, we modeled two regions of the molecule, one encompassing amino acids 526-539 and the other one encompassing amino acids 1159-1181, in order to include the sequence LSGGQ of the signature, which is involved in ATP binding in each NBD. The E528D variation led to the addition of a new hydrogen bond within the signature motif between S534 and Q537 (Fig. 1 B). The mutated residue S1161 shared a new hydrogen bond with its neighbor residue H1162 due to the presence of a hydroxyl functional group in the side chain of the serine. This new link replaced the hydrogen connection between D1170 and K1171, which disappeared after replacement of the proline by a serine (Fig. 1 C).

Taken together, the *in silico* analyses suggested that the E528D variation would bring moderate changes to the ABCB4 molecule while P1161S would be more deleterious.

3.3. Expression of the E528D and P1161S ABCB4 mutants in cell models

Computational results were confronted with those of experimental investigations performed in cell models. Plasmids encoding wt ABCB4 or ABCB4 mutants were transfected in HepG2 and HEK293 cells. The localization of both mutants, indicated by immunofluorescence, was similar to the wt. In HepG2 cells which polarize in culture, wt ABCB4 and the mutants localized at the membrane of bile canaliculi (Fig. 2 A). In HEK293 cells, which do not polarize, all constructs were essentially detected over the whole plasma membrane (Fig. 2 B). By immunoblotting, the pattern of migration of the mutants was similar to that of the wt protein (Fig. 2 C). All three recombinant proteins migrated under a major form corresponding to the fully glycosylated molecule and a minor immature form, as previouly described (Gautherot et al., 2014). These results indicated that the mutations did not affect the maturation process of ABCB4 or its targeting to the plasma membrane.

3.4. Measurement of phosphatidylcholine secretion by the E528D and P1161S mutants

Then, we examined whether the phosphatidylcholine secretion activity of ABCB4 mutants was impaired. Phosphatidylcholine secretion was measured in the culture medium of transiently transfected HEK293 cells as previously described (Gautherot et al., 2014). The amount of phosphatidylcholine released over 24 hours was corrected for the level of the mature protein expressed in the corresponding cell culture well. Then the ratio of phosphatidylcholine released between the mutant and wt transporters was calculated. Cells transfected with the E528D or P1161S mutants released respectively 33% and 48% less phosphatidylcholine than the wt protein (Fig. 3). These results show that both mutations caused a defect in phosphatidylcholine secretion, which was moderate in the case of the E528D mutant and more pronounced for the P1161S mutant.

3.5. Correlation between in silico prediction and experimental analysis of ABCB4 variations

The results presented above showed that MutPred was the best tool to predict the pathogenicity of two *ABCB4* variants found in LPAC patients, especially to predict the mild pathogenicity of the E528D variant. We extended the comparison of *in silico* vs *in cellulo* data to all the variants previously studied in our laboratory (Gautherot et al. 2014; Delaunay et al., 2016, 2017). Table 3 shows that MutPred predictions closely correlated with the biological effects that we previously demonstrated. The highest probability of a damaging effect (>90%) was attributed to seven variations including the I541F and L556R variations, which are trafficking-defective variations and have been shown to cause the most severe phenotype in patients (Delaunay et al., 2016), and the G535D, G536R, S1076C, S1176L, and G1178S variation that affect amino acids directly involved in ABCB4 activity (Delaunay et al., 2017). All four programs agreed on the deleterious effect of these latter variations. Eleven other variations with MutPred scores ranging from 52 to 88% were not all recognized as deleterious by the three other programs. Finally, the MutPred score of three variations was below 50%. This was the case of the R652G variation (23%), previously established as benign, and to the T175A variation (42%), which caused no apparent defect in cell models. In spite of the apparent safety of these variants, they were predicted to be pathogenic by certain programs (Table 3). The only contradiction to MutPred prediction was the T34M variation

which was given a low score (30%), although it has been shown to cause activity defect (Gautherot et al., 2014). This variation was also recognized as benign by all other prediction programs. This discrepancy may be linked to the particular effect of the T34M mutation on ABCB4 phosphorylation (Gautherot et al, 2014) that may not be well taken into account by prediction tools. Table 4 scores the accuracy of the predictions given by the four programs. The prediction of MutPred was in accordance with results obtained in cell models in 20 cases out of 21, whereas all other programs were less performant.

3.6. Confrontation of MutPred, PhD-SNP, Polyphen2 and Provean to predict the pathogeniciy of disease-associated ABCB4 variants

In order to further investigate the capacity of MutPred to accurately predict the pathogenicity of ABCB4 variants, we confronted the prediction of the four programs for 65 additional missense mutations reported in the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk). Although reported as disease-associated, most of these mutations have not been characterized in cell models. Results are detailed in Table 5. MutPred predicted that all mutations would be pathogenic, except the G384R mutation considered as pathogenic by all other programs, and the T667I mutation, also predicted as benign by the other programs. A summary is presented in Table 6. Only 80% of the mutations were predicted to be pathogenic by PhD-SNP, and 83% by Polyphen2 and Provean, when the predictions of MutPred were 97% .

4. Discussion

In this study, we analyzed the effect of two missense variations in the *ABCB4* gene that were identified in LPAC patients, both by using *in silico* prediction tools and by expressing the mutants in cell models. We show that these variations decreased ABCB4 phosphatidylcholine transport activity, and thus can be considered as responsible for the disease. The P1161S mutation decreased phosphatidylcholine secretion to lower level than the E528D, in accordance with MutPred predictions. The high performance of this program was confirmed with previously characterized ABCB4 variants.

The two variations, E528D and P1161S are located in the NBDs near the ABC signature motif, in well-conserved regions. Studies in cell models showed that these amino acid changes did not affect the processing or targeting of the mutants to the plasma membrane, but reduced the phosphatidylcholine secretion activity. A previous report has shown that two variations located in strategic NBD motifs (L481R and Y403H) also reduced phosphatidylcholine efflux without affecting ABCB4 expression (Degiorgio et al., 2014). Recently, we reported that variations that affect amino acids directly involved in ATP binding within the ABC signature (G535D and G536R in the first NBD, or S1076C, S1176L and G1178S in the second NBD), have a dramatic effect on ABCB4 function (Delaunay et al., 2017). The E528D and P1161S variations are not directly involved in linking ATP, but they may influence its binding or hydrolysis. Indeed, modeling of these regions showed changes in the number and position of hydrogen bonds at the level of the ABC signature motif. Since hydrogen bonds are engaged in maintaining the appropriate folding needed for function, their reorganization is expected to cause activity defect. The E528D mutation induced little changes at the structural level, and accordingly ABCB4 activity was only slightly decreased. The P1161S mutation caused more changes in the ABCB4 structure, and also affected ABCB4 activity more severely.

The E528D and P1161S variations were first identified with a mono allelic status, in patients diagnosed with LPAC syndrome (Rosmorduc et al., 2003). The E528D variation has also been reported in patients with ICP (Pauli-Magnus et al., 2004; Floreani et al., 2006). Our

finding regarding the fact that the E528D and P1161S variations affect the phosphatidylcholine secretion activity of ABCB4 can explain the pathogenic phenotype. It must be noted that the decrease observed in the case of the E528D mutant was only 33%, so that the decrease of total ABCB4 activity in heterozygous patients would be less than 17% presumably. This result suggests that even a moderate defect in ABCB4 function may at least partly contribute to cholestatic symptoms. However, we cannot exclude that other genetic or epigenetic factors in patients with minor ABCB4 defect, may contribute to the occurrence of the disease. Additional parameters, in particular the hormonal status may play a role. It has already been noted that the LPAC syndrome is more prevalent in female patients and frequently associated with ICP (Poupon et al., 2013). The balance diet or the nature of the bile salt pool may also play a role. *Abcb4* knock-out mice are more severely injured when fed cholic acid, the major bile acid in humans, and are almost unaffected when fed ursodeoxycholic acid (van Nieuwkerk et al., 1996). It is interesting to note that certain animal species can tolerate an aspartic acid at position 528 (Fig. 1 A). This is the case of the mouse and the rat, in which the major bile acid is muricholic acid, and ursidae in which the major bile acid is ursodeoxycholic acid, two relatively hydrophilic bile acids.

The bioinformatics investigation was performed in our study in order to assess the value of tools that have been developed to predict whether non-synonymous variants would be neutral or disease-causing. Four computational tools based on different criteria were used to take advantage of their potential complementarity. All four programs predicted that the P1161S variation would be disease-causing whereas they yielded discordant results for the E528D. These discrepancies can be explained in part by the fact that the performance of the prediction may be affected in the case of acidic residues (Thusberg et al., 2011), and probably also because the E528D variation was not very deleterious. The prediction, which best fitted with our studies in cell models was given by MutPred, which attributed a probability of 52.2% for E528D and of 88.2% for P1161S indicative of a moderate and a more severe defect, respectively. MutPred takes into account a large panel of attributes related to protein structure, function and evolution, whereas other programs are based on less parameters. The

MutPred program has already been recognized as one of the best effective methods for the assessment of the effect of missense variants (Frousios et al., 2013). The accuracy of MutPred to predict the pathogenicity of ABCB4 missense variations is attested by the good correlation with the biological effect of the twenty-one ABCB4 variants analyzed in our laboratory, compared with other programs (Table 4). Furthermore MutPred predicted that 97% of the mutations reported in the Human Mutation Database would be pathogenic when the other programs predicted only 80-83%, suggesting that it is more sensitive (Table 6).

5. Conclusion

In conclusion, our results show that the E528D mutation has mild effect while the P1161S is more damaging, and that among four different *in silico* tools the prediction of MutPred best fitted these results. MutPred most accurately predicted the pathogenic or benign character of previously characterized ABCB4 variants in good agreement with the experimental data. MutPred was also the most sensitive in the prediction of the pathogenicity of a large cohort of variations reported in databases. We therefore suggest that MutPred may be chosen as a first computational approach to predict the degree of pathogenicity of ABCB4 variants.

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Legend to figures

Fig. 1. *In silico* analysis of the ABCB4 sequences encompassing the E528D and P1161S variations. (A) Alignment of ABCB4 sequences from different species using the Clustal Omega program. The sequences include E528 or P1161 and the signature sequences. (B and C) Three-dimensional structure of the peptide 526-539 with E (Glu) or D (Asp) at position 528 (B), and of the peptide 1159-1181 with P (Pro) or S (Ser) at position 1161 (C). The models were built by homology with the structure of *C. elegans*, and visualized using Swiss-PDB viewer. The wt residue is colored in yellow, the mutated one in green, and the signature in purple. Hydrogen bonds are indicated by green dotted lines. The mutation E528D adds a novel hydrogen bond between S534 and Q535 in the signature (indicated by a red arrow in B, right panel). The mutation P1161S introduces a hydrogen bond between P1161 and H1162 (red arrow in C, right panel), and removes the hydrogen bond between D1170 and K1171 (red arrow in C, left panel).

Fig. 2. Expression of ABCB4 mutants in transfected cells. HepG2 cells (A) or HEK293 cells (B) transiently expressing wt ABCB4 or the indicated mutants were fixed, permeabilized and stained with the P3II-26 monoclonal antibody against ABCB4, followed by Alexa-Fluor 488-conjugated secondary antibody. Nuclei were stained with Draq5 (blue). Images were obtained by confocal microscopy. Bile canaliculi are indicated by arrows. Bars, 10 µm. (C) Control HEK293 cells (Ctrl) or HEK293 cells transiently expressing wt ABCB4 or the mutants were lyzed and analyzed by electrophoresis and immunoblotting using the P3II-26 anti-ABCB4 antiboby. The same amount of protein was loaded in each lane. Standard molecular mass is indicated in kDa.

Fig. 3. Phosphatidylcholine secretion by ABCB4 mutants. Phosphatidylcholine (PC) secretion was measured in HEK293 cells transfected with plasmids encoding wt ABCB4 or the

indicated mutants. Cells were incubated in serum-free media containing sodium taurocholate and BSA for 24 hours, and the amount of secreted phosphatidylcholine was measured. Results are expressed as the percentage of phosphatidylcholine secretion relative to wt ABCB4 expressing cells, after normalization to ABCB4 mature protein levels. Results are means (± SEM) of five independent experiments. *P<0.01 , **P<0.001.

Table 1. Characteristics of patients with the E528D and P1161S ABCB4 variations

Patient n°/gender	Nucleotide change	Amino acid change	Status	Diagnosis	Reference
1 / F	c.1584G>C c.1954A>G	p.E528D p.R652G	HTZ HTZ	LPAC	Rosmorduc et al., 2003
2 / F	c.1584G>C	p.E528D	HTZ	ICP	Rosmorduc et al., 2003
3*/ F	c.1584G>C	p.E528D	HTZ	LPAC	Poupon et al., 2013
4 / F	c.1584G>C	p.E528D	HTZ	LPAC	Poupon et al., 2013
5 / F	c.1584G>C	p.E528D	HTZ	LPAC	this study
6 / F	c.1584G>C c.1954A>G	p.E528D p.R652G	HTZ HMZ	LPAC	this study
7 / F	c.1584G>C c.1954A>G	p.E528D p.R652G	HTZ HMZ	LPAC	this study
8 /M	c.3481C>T	p.P1161S	HTZ	LPAC	Rosmorduc et al., 2001
9**/ F	c.3481C>T	p.P1161S	HTZ	LPAC	Poupon et al., 2013

Numbering of the nucleotides corresponds to NM_000443.3 (ABCB4, transcript variant A, mRNA).

Abbreviations: LPAC, low phospholipid-associated cholelithiasis; ICP, intrahepatic cholestasis of pregnancy; HTZ, heterozygous; HMZ, homozygous.

* Daughter of patient 2.

**Daughter of patient 8.

Table 2. Prediction scores for the E528D and P1161S ABCB4 variants

	E528D	**P1161S**
Provean	- 1.32 benign	- 6.78 deleterious
Polyphen-2	0.001 benign	0.932 deleterious
PhD-SNP	disease-related	disease-related
MutPred	0.52 deleterious (P_{dele} 0.52)	0.88 deleterious (P_{dele} 0.88)

Provean: a score equal or below -2.5 qualifies the substitution as deleterious.

Polyphen-2: values range from 0 to 1. The substitution is predicted to be damaging if >0.5 and benign if <0.5.

PhD-SNP: a binary classification is provided : disease-related or polymorphism.

MutPred: a probability of deleterious effect is attributed to each substitution.

Table 3. *In silico* predictions of ABCB4 missense variants with a known biological effect

	MutPred[a]	PhD-SNP	Poly-phen 2	Provean	*In cellulo* effect	Reference
G535D	96.0%	Disease	Probably damaging	Deleterious	Activity defect	Delaunay et al., 2017
G536R	93.9%	Disease	Probably damaging	Deleterious	Activity defect	Delaunay et al., 2017
I541F	92.8%	Disease	Probably damaging	Deleterious	Maturation defect	Delaunay et al., 2009
L556R	96.1%	Disease	Probably damaging	Deleterious	Maturation defect	Delaunay et al., 2016
S1076C	95.5%	Disease	Probably damaging	Deleterious	Activity defect	Delaunay et al., 2017
S1176L	94.2%	Disease	Probably damaging	Deleterious	Activity defect	Delaunay et al., 2017
G1178S	98.1%	Disease	Probably damaging	Deleterious	Activity defect	Delaunay et al., 2017
R47G	59.3%	Disease	Probably damaging	Deleterious	Activity defect	Gautherot et al., 2014
S346I	71.2%	Disease	**Benign**	Deleterious	Activity defect	Delaunay et al., 2016
F357L	88.1%	**Neutral**	**Benign**	Deleterious	Activity defect	Delaunay et al., 2016
T424A	85.1%	**Neutral**	Probably damaging	Deleterious	Stability defect	Delaunay et al., 2016
N510S	68.8%	Disease	Probably damaging	Deleterious	Stability defect	Delaunay et al., 2016
E528D	52.2%	Disease	**Benign**	**Neutral**	Activity defect	This study
P726L	83.8%	Disease	Probably damaging	Deleterious	Activity defect	Delaunay et al., 2016
T775M	74.1%	Disease	Probably damaging	**Neutral**	Activity defect	Delaunay et al., 2016
Q855L	72.2%	**Neutral**	Probably damaging	Deleterious	Maturation defect	Delaunay et al., 2016
G954S	82.2%	**Neutral**	Probably damaging	**Neutral**	Activity defect	Delaunay et al., 2016
P1161S	88.2%	Disease	Probably damaging	Deleterious	Activity defect	This study

T34M	**30%**	**Neutral**	**Benign**	**Neutral**	Activity defect	Gautherot et al., 2014
T175A	40.5%	Neutral	**Probably damaging**	**Deleterious**	No detectable defect	Delaunay et al., 2016
R652G	23.5%	**Disease**	Benign	Neutral	No detectable defect	Delaunay et al., 2016

[a]A value >50% is considered as deleterious.

Predictions noted in bold characters are in contradiction with the biological effect.

Table 4. Accuracy of the prediction programs in accordance with *in cellulo* effect of ABCB4 mutations

Prediction[a]	**MutPred**	**PhD-SNP**	**Poly-Phen2**	**Provean**
True pathogenic	18	14	15	15
True benign	2	1	1	1
False pathogenic	0	1	1	1
False benign	1	5	4	4
Correct prediction	20/21 (95%)	15/21 (71%)	17/21 (80%)	17/21 (80%)

[a]Predictions in accordance with the *in cellulo* phenotype are classified as "true" and predictions in contradiction are classified as "false"

Table 5. *In silico* predictions of disease-associated ABCB4 missense variants reported in the Human Gene Mutation Database

mutation	Clinical phenotype	MutPred[a]	PhD-SNP	Poly-phen2	Provean	Reference
R47Q	LPAC	60.7%	**Neutral**	Probably Damaging	Deleterious	Davit-Spraul et al. 2010
L73V	PFIC3	53.4%	Disease	Probably damaging	**Neutral**	Colombo et al., 2011
G126E	PFIC3	94.7%	Disease	Probably Damaging	Deleterious	Degiorgio et al., 2007
W138R	PFIC3	92.2%	**Neutral**	Probably Damaging	Deleterious	Jacquemin et al., 2001
R150K	ICP	96.9%	Disease	Probably Damaging	**Neutral**	Müllenbach et al., 2003
F165I	LPAC	94.6%	Disease	Probably damaging	Deleterious	Rosmorduc et al., 2003
N168D	ICP	63.3%	Disease	**Benign**	**Neutral**	Bacq et al., 2009
R176W	PFIC3	86.2%	Disease	Probably damaging	Deleterious	Davit-Spraul et al. 2010
A250P	PFIC3	93.3%	Disease	Probably Damaging	Deleterious	Degiorgio et al., 2007
A286V	PFIC3	87.4%	Disease	Probably damaging	deleterious	Degiorgio et al., 2007
M301T	LPAC	68.7%	**Neutral**	**Benign**	Deleterious	Rosmorduc et al., 2003
G319E	LPAC	64.8%	Disease	Probably Damaging	Deleterious	Hopf et al., 2011
S320F	LPAC	73.8%	Disease	Probably damaging	Deleterious	Rosmorduc et al., 2001
A358P	PFIC3	62.1%	Disease	**Benign**	Deleterious	Davit-Spraul et al. 2010
A364V	PFIC3	89.7%	Disease	Probably damaging	Deleterious	Degiorgio et al., 2007
G384R	LPAC	**45.8%**	Disease	Probably Damaging	Deleterious	Poupon et al., 2010b
E395G	PFIC3	81.9%	Disease	Probably Damaging	Deleterious	Jacquemin et al., 2001
Y403H	PFIC3	97.8%	Disease	Probably Damaging	Deleterious	Degiorgio et al., 2007
R406Q	LPAC	78%	Disease	Probably Damaging	Deleterious	Poupon et al., 2010b
V425M	PFIC3	84.4%	**Neutral**	Probably Damaging	**Neutral**	Jacquemin et al., 2001
L445V	LPAC	82.3%	Disease	Probably Damaging	**Neutral**	Davit-Spraul et al. 2010
D459G	PFIC3	76.9%	Disease	Probably Damaging	Deleterious	Fang et al., 2012
V475A	PFIC3	95.6%	Disease	Probably Damaging	Deleterious	Degiorgio et al., 2007
P479L	PFIC3	88.4%	Disease	Probably	Deleterious	Davit-Spraul et al.

				Damaging		2010
L481R	PFIC3	88.4%	Disease	Probably damaging	Deleterious	Degiorgio et al., 2014
A511T	PFIC3	91.1%	Disease	Probably Damaging	Deleterious	Degiorgio et al., 2007
E513K	LPAC	63%	Disease	**Benign**	Deleterious	Davit-Spraul et al. 2010
V526F	Anicteric cholestasis	96.3%	Disease	Probably Damaging	Deleterious	Ziol et al, 2008
R545G	Anicteric cholestasis	99.2%	Disease	Probably Damaging	Deleterious	Ziol et al, 2008
A546D	ICP	93.5%	Disease	Probably Damaging	Deleterious	Dixon et al., 2000
R549H	ICP	80.5%	Disease	Probably Damaging	Deleterious	Floreani et al., 2006
E558K	PFIC3	97.6%	Disease	Probably Damaging	Deleterious	Degiorgio et al., 2007
D564G	PFIC3	95.7%	Disease	**Benign**	Deleterious	Jacquemin et al., 2001
R582Q	PFIC3	74.1%	Disease	Probably Damaging	Deleterious	Fang et al., 2012
R590Q	PFIC3	76.2%	Disease	Probably Damaging	Deleterious	Degiorgio et al., 2007
L591Q	LPAC	96%	Disease	Probably Damaging	Deleterious	Rosmorduc et al., 2003
T593A	PFIC3	85%	**Neutral**	Probably Damaging	Deleterious	Degiorgio et al., 2007
M630V	PFIC3	78%	**Neutral**	**Benign**	**Neutral**	Degiorgio et al., 2007
T667I	ICP	**34%**	**Neutral**	**Benign**	**Neutral**	Floreani et al., 2008
L701P	PFIC3	94.9%	Disease	Probably Damaging	Deleterious	Degiorgio et al., 2007
F711S	PFIC3	81%	**Neutral**	Probably Damaging	Deleterious	Jacquemin et al., 2001
T715I	PFIC3	81.9%	**Neutral**	**Benign**	**Neutral**	Degiorgio et al., 2007
G723E	PFIC3	86.4%	Disease	Probably Damaging	Deleterious	Degiorgio et al., 2007
P726T	PFIC3	91.5%	Disease	Probably Damaging	Deleterious	Degiorgio et al., 2007
A737V	PFIC3	83.6%	Disease	**Benign**	**Neutral**	Degiorgio et al., 2007
I738L	Anicteric cholestasis	78.2%	**Neutral**	**Benign**	**Neutral**	Ziol et al, 2008
G762E	ICP, PFIC3	93.6%	Disease	Probably Damaging	Deleterious	Pauli-Magnus et al., 2004 Degiorgio et al., 2007
I764L	Drug-induced cholestasis	83.8%	Disease	**Benign**	**Neutral**	Lang et al., 2007
G773R	LPAC	85.6%	Disease	Probably Damaging	Deleterious	Davit-Spraul et al. 2010
R788E	LPAC	84.2%	Disease	Probably Damaging	Deleterious	Rosmorduc et al., 2003
R788W	Cholestasis, ICP	95.7%	Disease	Probably Damaging	Deleterious	Gotthardt et al, 2008
A840D	PFIC3	94.2%	Disease	Probably Damaging	Deleterious	Degiorgio et al., 2007
L859W	LPAC	64.6%	Disease	Probably	Deleterious	Davit-Spraul et al.

				Damaging		2010
A934T	LPAC	87.7%	**Neutral**	Probably Damaging	Deleterious	Rosmorduc et al., 2003
A946T	LPAC	55.4%	**Neutral**	Probably Damaging	Deleterious	Poupon et al., 2010b
A953D	PFIC3	94.9%	Disease	Probably Damaging	Deleterious	Keitel et al., 2005
G983S	PFIC3	85.1%	**Neutral**	Probably Damaging	Deleterious	Jacquemin et al., 2001
V1068E	LPAC	90.8%	Disease	Probably Damaging	Deleterious	Denk et al., 2010
L1082Q	Hepatocellular injury	95.6%	Disease	Probably Damaging	Deleterious	Lang et al., 2007
A1110P	PFIC3	58.1%	Disease	Probably Damaging	Deleterious	Davit-Spraul et al. 2010
Q1181E	PFIC3	95.2%	Disease	Probably Damaging	Deleterious	Kubitz et al., 2011
A1186T	PFIC3	99.1%	Disease	Probably Damaging	Deleterious	Degiorgio et al., 2007
D1199N	ICP	92%	Disease	Probably Damaging	Deleterious	Davit-Spraul et al. 2010
E1200A	LPAC	96.4%	Disease	Probably Damaging	Deleterious	Davit-Spraul et al. 2010
H1231Y	PFIC	92.9%	Disease	Probably Damaging	Deleterious	Dzagania et al., 2012

[a]A value >50% is considered as deleterious.

Prediction of benign mutations is noted in bold characters.

Table 6. Scoring of *in silico* prediction programs for disease-associated ABCB4 mutations of the Human Gene Mutation Database.

Prediction	MutPred	PhD-SNP	Poly-Phen2	Provean
Pathogenic	63	52	54	54
Benign	2	13	11	11
Score	97%	80%	83%	83%

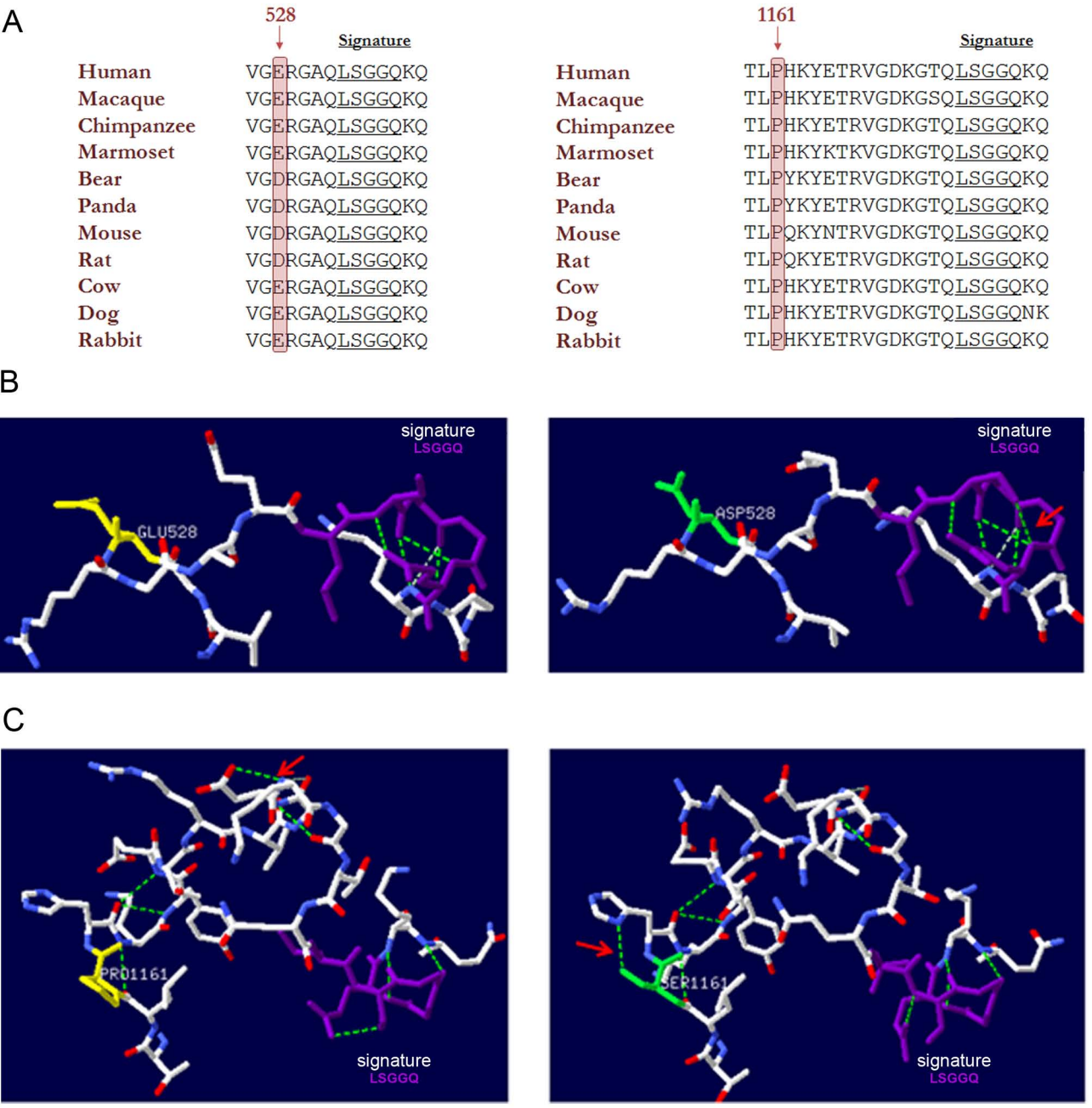
A
528
Signature
Human VGERGAQLSGGQKQ
Macaque VGERGAQLSGGQKQ
Chimpanzee VGERGAQLSGGQKQ
Marmoset VGERGAQLSGGQKQ
Bear VGDRGAQLSGGQKQ
Panda VGDRGAQLSGGQKQ
Mouse VGDRGAQLSGGQKQ
Rat VGDRGAQLSGGQKQ
Cow VGERGAQLSGGQKQ
Dog VGERGAQLSGGQKQ
Rabbit VGERGAQLSGGQKQ
1161
Signature
Human TLPHKYETRVGDKGTQLSGGQKQ
Macaque TLPHKYETRVGDKGSQLSGGQKQ
Chimpanzee TLPHKYETRVGDKGTQLSGGQKQ
Marmoset TLPHKYKTKVGDKGTQLSGGQKQ
Bear TLPYKYETRVGDKGTQLSGGQKQ
Panda TLPYKYETRVGDKGTQLSGGQKQ
Mouse TLPQKYNTRVGDKGTQLSGGQKQ
Rat TLPQKYETRVGDKGTQLSGGQKQ
Cow TLPHKYETRVGDKGTQLSGGQKQ
Dog TLPHKYETRVGDKGTQLSGGQNK
Rabbit TLPHKYETRVGDKGTQLSGGQKQ
B
signature
LSGGQ
GLU528
signature
LSGGQ
ASP528
C
PRO1161
signature
LSGGQ
SER1161
signature
LSGGQ

Figure 1

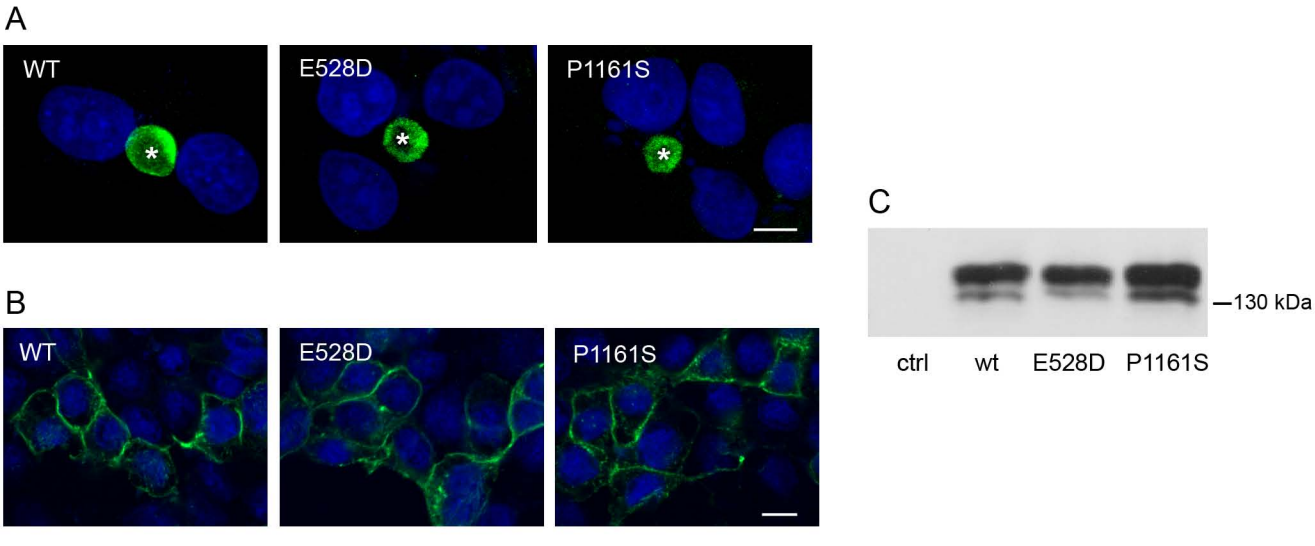
A
WT
E528D
P1161S
B
WT
E528D
P1161S
C
130 kDa
ctrl
wt
E528D
P1161S

Figure 2

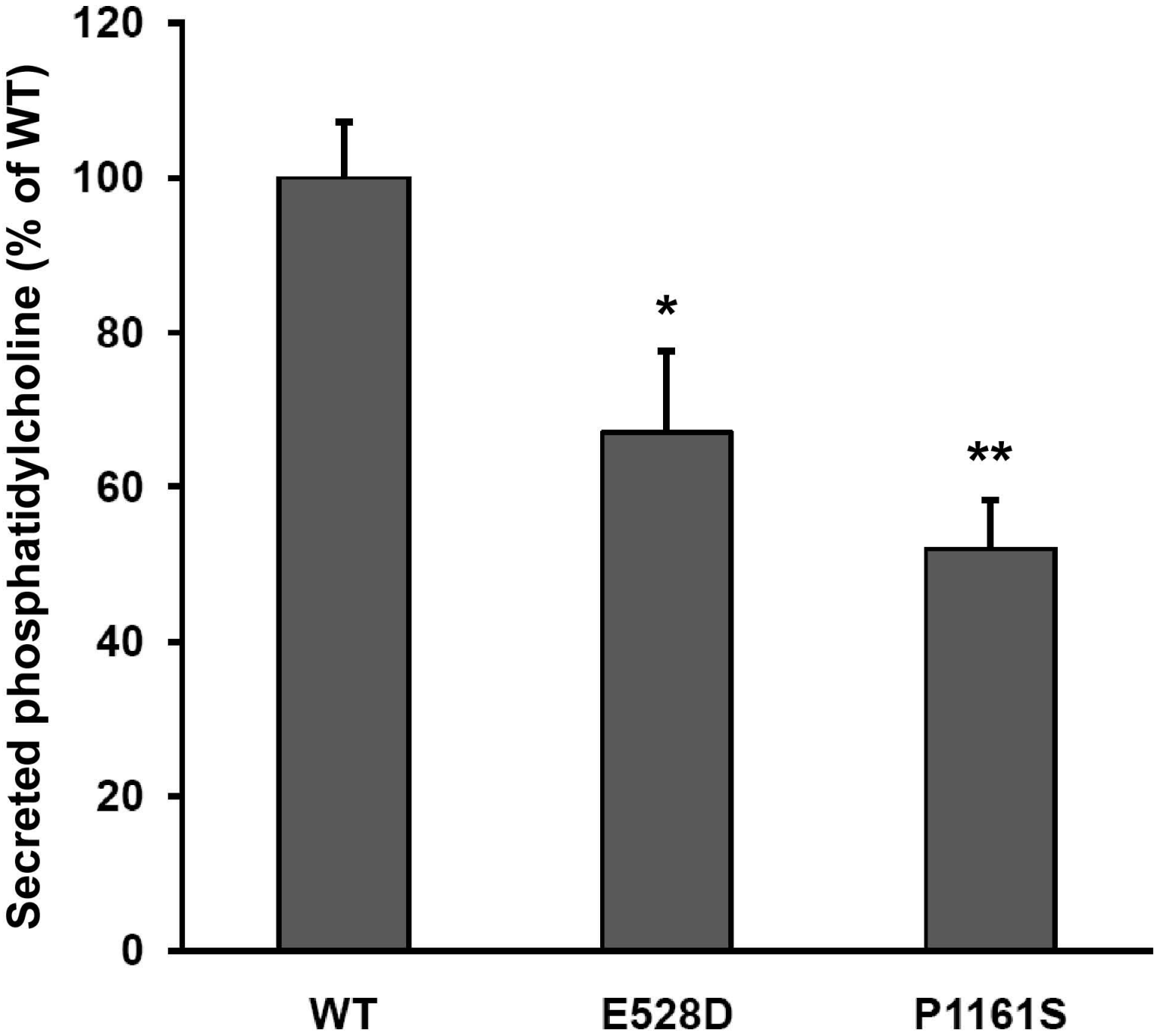
Secreted phosphatidylcholine (% of WT)
120
100
80
60
40
20
0
*
**
WT
E528D
P1161S

Figure 3