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Haematologica 2016 [Epub ahead of print]

doi:10.3324/haematol.2016.142372

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Functional characterization of novel ABCB6 mutations and their clinical implications in familial pseudohyperkalemia

Immacolata Andolfo1,2*, Roberta Russo1,2, Francesco Manna1,2, Gianluca De Rosa1,2, Antonella Gambale1,2, Soha Zouwail3, Nicola Detta2, Catia Lo Pardo4, Seth L. Alper5, Carlo Brugnara6, Alok K. Sharma5, Lucia De Franceschi7 and Achille Iolascon1,2.

1. Department of Molecular Medicine and Medical Biotechnologies, “Federico II” University of Naples, Naples, Italy
2. CEINGE, Biotecnologie Avanzate, Naples, Italy
3. Department of Biochemistry and Immunology, Cardiff and Vale University Health Board, University Hospital of Wales, Cardiff, UK and Department of Medical Biochemistry, School of Medicine, Alexandria University, Alexandria, Egypt
5. Division of Nephrology and Center for Vascular Biology Research, Beth Israel Deaconess Medical Center and Department of Medicine, Harvard Medical School, Boston, MA 02215, USA.
6. Department of Laboratory Medicine, Boston Children’s Hospital And Department Of Pathology, Harvard Medical School, Boston, MA 02215, USA
7. Department of Medicine, University of Verona, Piazzale Lo Scuro 10, Verona, Italy

Keywords: ABCB6, Familial Pseudohyperkalemia, transfusions, ionic flux

Running title: ABCB6 mutations functional characterization in familial pseudohyperkalemia

Corresponding author:

* Immacolata Andolfo, PhD
Department of Molecular Medicine and Medical Biotechnologies, “Federico II” University of Naples, Naples, Italy
CEINGE, Biotecnologie Avanzate, Via Gaetano Salvatore, 486, 80145 Naples, Italy
Tel: +39-081-3737736
Fax: +39-081-3737804
e-mail: andolfo@ceinge.unina.it
Abstract

Isolated Familial Pseudohyperkalemia is a dominant red cell trait characterized by cold-induced ‘passive leak’ of red cell K⁺ into plasma. The causative gene of this condition is \( ABCB6 \), encoding an erythrocyte membrane ABC transporter protein bearing the Langereis blood group antigen system.

In this study analyzing three new families, we report the first functional characterization of \( ABCB6 \) mutants, including homozygous mutation V454A, heterozygous mutation R276W, and compound heterozygous mutations R276W and R723Q (in trans). All these mutations are annotated in public databases, suggesting that Familial Pseudohyperkalemia could be common in the general population. Indeed, we identified variant R276W in one of 327 random blood donors (0.3%). Four weeks' storage of heterozygous R276W blood cells resulted in massive K loss compared to healthy controls. Moreover, measurement of cation flux demonstrated greater loss of K⁺ or Rb⁺ from HEK-293 cells expressing \( ABCB6 \) mutants than from cells expressing \( ABCB6 \) WT. The R276W/R723Q mutations elicited greater cellular K⁺ efflux than did the other mutants tested.

In conclusion, \( ABCB6 \) missense mutations in Familial Pseudohyperkalemia erythrocytes show elevated K⁺ efflux. The patients are present at moderate frequency in the blood donor population. Storage of blood of these patients leads to significantly increased K⁺ levels, with serious clinical implications for neonates and infants receiving large-volume transfusions of whole blood. Genetic tests for Familial Pseudohyperkalemia could be added to blood donor pre-screening. Further study of \( ABCB6 \) function and trafficking could be informative for the study of other pathologies of red blood cell hydration.

1. \( ABCB6 \) mutations cause Familial Pseudohyperkalemia (FP), a red cells defect characterized by potassium passive leak. We here performed the first functional characterization of \( ABCB6 \) mutations towards understanding the pathogenic mechanism of the disease and towards population screening of blood donors for \( ABCB6 \) FP variants.

2. \( ABCB6 \) missense mutations showed increased K⁺ efflux as exhibited in erythrocytes of FP patients. FP patients are present in the blood donor population. Storage of FP blood lead to significantly increased K⁺ levels, with serious clinical implications for neonates and infants receiving large-volume transfusions of whole blood. Genetic tests for FP could be added to blood donor pre-screening.
**Supplementary Materials contain**: 1. Table 1S; 2. Table 2S; 3. Figure 1S; 4. Figure 2S; 5. Supplementary Materials and Methods; 6. Supplementary Figure legends.
Introduction

Isolated Familial Pseudohyperkalemia (FP) is a dominant red cell trait characterized by an increase of plasma [K+] upon exposure of whole blood to temperatures below 37°C. Red blood cells (RBCs) of individuals with FP exhibit increased mean corpuscular volume (MCV) and shape abnormalities. Cation leak in FP families described to date has shown several patterns of temperature-dependence1-4.

The causative gene in FP was previously mapped to 2q35-365. Functional gene mapping and sequencing analysis of the candidate genes within the 2q35-q36 critical interval identified in three multigenerational FP families two novel heterozygous missense mutations in the ABCB6 gene that cosegregated with disease phenotype6. The two genomic substitutions altered two adjacent nucleotides within codon 375 of ABCB6, a previously identified porphyrin transporter7 that in erythrocyte membranes8 bears the Langereis (Lan) blood group antigen system9. We previously showed in three separate models of erythropoiesis that ABCB6 expression increased during erythroid differentiation and localized to the plasma membrane6. The ABCB6 R375Q mutation did not alter levels of mRNA or protein, or subcellular localization in mature erythrocytes or erythroid precursor cells, but was predicted to have pathogenic consequence to protein function.

Recently, the ABCB6 substitution R723Q was found in healthy subjects from a family affected by FP, two of whom had been evaluated as regular blood donors10. The blood of both exhibited increased potassium leak upon storage at temperatures below 37°C. This interesting finding encouraged further study on the implications for neonates and infants receiving transfusion of whole blood from unknown FP subjects.

In this report, we identify in FP families of non-dominant inheritance pattern, three novel missense mutations in ABCB6. The presence of these mutations in human variation databases confirms that the prevalence of asymptomatic FP is likely underestimated and, moreover, frequently undetected in blood donor populations. We also report results of ABCB6 screening in a blood donor population, and present the first functional study on the effects of ABCB6 FP mutations on that component of red cell K+ efflux characterized by resistance to ouabain plus bumetanide.
Methods

Patients

Three new patients from three independent pedigrees were enrolled in this study (Table 1), and blood samples were obtained from affected patients. Whenever possible, relatives were also investigated. Diagnosis was based on history, clinical findings, laboratory routine data, peripheral blood smear, and genetic testing. Information about every clinical characteristic was not available for all cases. A cohort of 327 blood donors from the blood transfusion center of the Cardarelli Hospital in Naples was enrolled to undergo genetic screening for the ABCB6 mutations found in this study.

Collection of patient data and samples was by the clinicians responsible for patient care, with informed consent according to the Declaration of Helsinki, and with approval by local university ethical committees.

Exome capture and sequencing

Blood was obtained for genetic analysis from affected and unaffected family members of the Irish family and from healthy controls, with signed informed consent according to the Declaration of Helsinki. Reads were aligned to the most recent version of human genome (GRCh37/hg19) using the BWA software package v0.5.9 as previously described

Direct sequencing analysis of the additional families was performed by direct sequencing (see supplementary material).

ABCB6 screening in donor blood subjects

Mutation screening for ABCB6 variants R276W, V454A, and R723Q in a population of 327 blood donors was performed by amplification-refractory mutation system (ARMS) analysis, using allele-specific tetra-primer ARMS-PCR primers designed by PRIMER1 (http://primer1.soton.ac.uk/primer1.html)

Bioinformatic modeling of ABCB6 protein structure

To assess the potential effects of the identified mutations on protein structure, we generated 3D structural models of dimeric human WT ABCB6 residues 231–827 and the corresponding regions of FP mutant ABCB6 polypeptides V454A, R276W, and R723Q, as described in the legends to Figures 1, 1S and 2S, and as previously described. Sequences were aligned in ClustalW2. MODELLER v9.9 was used for homology modeling in both inward- and outward-facing
conformations. The best five structural models with lowest objective function values (as implemented in MODELLER) were subjected to energy minimization in GROMACSv4.5.4\textsuperscript{14}. Structural models were converged using steepest descent energy minimization with 1,000 steps of step size 0.01 nm. Stereochemical quality of each energy-minimized structure was assessed by PROCHECK\textsuperscript{15}. The average of three models of highest stereochemical quality was chosen for ABCB6 structural models. 3D structural models were visualized and aligned using MolMol\textsuperscript{16} and PyMOL 1.5.0.4 (Schrödinger, LLC).

**Cell culture and transfection assay**

Human HEK-293 cells were maintained in DMEM medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin (all from Life Technologies), in a humidified 5% CO\textsubscript{2} atmosphere at 37°C. pcDNA3.1-ABCB6-WT and pcDNA3.1-ABCB6 mutant constructs (5\(\mu\)g) were transfected into HEK-293 cells using X-tremeGENE HP DNA Transfection Reagent (Roche, Indianapolis, IN, USA). To phenocopy the heterozygous genotypes, cells were transfected with 2.5\(\mu\)g of WT pcDNA3.1-ABCB6-WT plus 2.5\(\mu\)g of pcDNA3.1-ABCB6 mutants R375Q, p.R276W or R375W. For the compound heterozygous genotypes, cells were transfected with 2.5\(\mu\)g pcDNA3.1-ABCB6-R276W plus 2.5\(\mu\)g pcDNA3.1-ABCB6-R723Q. For the homozygous genotype, cells were transfected with 5\(\mu\)g pcDNA3.1-ABCB6-V454A. After 72 hrs, cells were harvested for analysis.

**Immunofluorescence analysis**

HEK-293 cells (2\(\times\)10\textsuperscript{6}) on coverslips were transfected with ABCB6 cDNAs as previously described\textsuperscript{6} (see supplementary methods).

**Measurements of K\textsuperscript{+} fluxes in red blood cells of blood donor carrying ABCB6 R276W variant**

Blood samples from the donor carrying the ABCB6 R276W mutation and from two controls obtained from the transfusion center of the Cardarelli Hospital (Naples) were stored four weeks at 4°C in Citrate Phosphate Dextrose Solution (CPD) as anticoagulant, under blood bank conditions. During the four weeks' storage, plasma potassium levels were measured in triplicate by atomic absorption spectroscopy (ANALYST 2000, Perkin-Elmer) as previously described\textsuperscript{17}. The RBCs were gently washed with a buffer containing (in mM) 150 choline chloride, 1mM MgCl\textsubscript{2}, 10mM Tris MOPS, then lysed for intracellular K measurement by atomic absorption spectroscopy\textsuperscript{17}. The free Hb levels were measured to evaluate the degree of hemolysis as for K.
Measurements of ouabain-plus-bumetanide-resistant Rb⁺ and K⁺ fluxes in transfected HEK-293 cells

HEK-293 cells at 72 h post-transfection were maintained for 8 h under shear stress (rotary shaking at 70 rpm 30°C) in a K⁺-free medium containing (in mM) 140 NaCl, 5 mM RbCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose, with added 10 μM ouabain and 10 μM bumetanide. At the end of the incubation, cell viability was determined by trypan blue staining and medium was removed for extracellular K determination. Cells were gently washed in buffer containing (in mM) 150 choline chloride, 1mM MgCl₂, 10mM Tris MOPS, then lysed for intracellular Rb measurement. Cell Rb content and medium K content were determined in triplicate by atomic absorption spectroscopy (ANALYST 2000, Perkin-Elmer) as previously described¹⁷.
Results

Case Reports

The Bolivian patient is a 41 year old female from a consanguineous family. Multiple outpatient blood samples indicated elevated plasma $K^+$ (8.3 mmol/L). Renal and adrenal function and EKG were normal, as were hematological indices except for macrocytosis (MCV 95-98 fL; Table 1). Past medical history included premature menopause and migraines. Physical examination was unremarkable.

Patient Cardiff-2 is a 35 year old female with a prior tentative diagnosis of hereditary spherocytosis with a positive family history, despite a normal red cell eosin maleimide (EMA) binding test. Outpatient values of plasma $[K^+]$ ranged between 8.0 and 8.6 mmol/L, in contrast to hospital clinical values between 5.4 and 5.9 mmol/L (Table 1). The patient had been under outpatient care for diabetes and other conditions since 1999. Her peripheral blood smear revealed polychromasia, target cells, and a few spherocytes. Past medical history included Irritable Bowel Syndrome, oophorectomy in 2007, splenectomy secondary to trauma in 2008, and diagnosis of depression in 2009. Medications included omeprazole, mebevirine and citalopram. Blood pressure was normal, as was the remainder of the physical exam. Absolute reticulocyte count was $176.0 \times 10^9$ (4.10 %) with mean reticulocytes volume of 118 fl (reference range 90-110 fl).

The large Irish family was originally described by Stewart and colleagues as autosomal dominant dehydrated hereditary stomatocytosis (DHSt) with FP. The propositus developed several thrombotic episodes following splenectomy at age 40 years. An increased passive $K^+$ leak was noted (Table 1).

The families Lille, Falkirk and East London were previously described. Affected individuals from Family Lille (of Flemish descent) presented with normal hematological indices except for a slightly elevated MCV. Affecteds from family FP Falkirk (of Pakistani origin) also presented with macrocytosis. Affected from family FP “East London” (of Bangladeshi origin) were anemic and hyperkalemic, but in the absence of reticulocytes and jaundice were considered nonhemolytic.

None of the carriers had colobomatous abnormalities of iris or retina, also associated with missense $ABCB6$ mutations. Carriers were not tested for Lan (-/-) status, as this phenotype is caused by nonsense mutations that cause complete absence of ABCB6 polypeptide in circulating red cells.
**ABCB6 mutational analysis in FP families and blood donor screening**

We sequenced the *ABCB6* gene in two patients and one family with FP. In the Bolivian patient we found the homozygous mutation c.1361T>C; p.V454A, while in patient Cardiff-2 we found compound heterozygosity for the two mutations c.826G>T; p.R276W and c.2168G>A; p.R723Q (Table 2). Parents of both patients were unavailable for genetic analysis. To analyze the allelic pattern of the two mutations in patient Cardiff-2, we cloned the genomic region encompassing both ABCB6 variants (about 6 Kb) into a plasmid vector. DNA sequencing of this cloned region demonstrated that the two mutations are in trans in patient Cardiff-2. In the Bolivian patient, the presence of heterozygous SNPs excluded the possibility of a deletion within the region of the gene harboring the mutation.

The Irish family originally diagnosed as DHSt plus FP was at first mapped to chr.16 but subsequent analysis of the *PIEZO1* gene was negative. We therefore subjected the Irish family to whole-exome analysis and identified *ABCB6* variant c.826G>T; p.R276W, with subsequent confirmation by direct sequencing. Probably, there was a misassignment in the Irish pedigree linkage analysis, however, we cannot rule out the presence of intronic mutations that could explain epistasis between PIEZO and ABCB6.

ABCB6 amino acid residues R276, R723 and V454 are conserved among all species analyzed and each have Polyphen2 scores of 1 (damaging) and SIFT scores of 0 (damaging).

Each of the three FP mutations is annotated in public databases: 1000 Genomes (URL: http://browser.1000genomes.org); NHLBI Exome Sequencing Project (URL: http://evs.gs.washington.edu/EVS); Exome Aggregation Consortium (ExAC), Cambridge, MA (URL: http://exac.broadinstitute.org). Minor allele frequency (MAF) was 0.43% for V454A variant, 0.08% for R723Q, and 1.5% for R276W (Table 2 and Table 2S).

The high frequency of the variants found in our patients prompted our genetic screening of a cohort of 327 blood donors. Of note, our analysis demonstrated the presence of variant R276W in 0.3% of this cohort (1/327) and the absence of the other two mutations V454A and R723Q, consistent with the MAF values reported to date.

**ABCB6 mutations produce conformational changes in model structures**

To analyze potential consequences of the identified mutations on protein structure, we generated three-dimensional structural models of the (putatively dimeric) human WT ABCB6 residues 231-
827 and FP mutant polypeptides V454A, R276W, and R723Q (see Methods). Figure 1 shows 3D structural models of homodimeric WT ABCB6 in inward- and outward-facing conformations, highlighting sites of the homozygous and compound heterozygous FP missense mutations studied in this paper. The structural models of ABCB6 homodimeric FP mutant V454A and heterodimeric mutant R276W (chain a)/R723Q (chain b) in both inward- and outward-facing conformations are presented in Figures 1S and 2S, respectively. Transverse views of intra-membrane bilayer regions are also presented. Comparison of WT with mutant models reveals that these mutations cause detectable conformational changes in regions on or near the missense substitution sites and at several more remote locations. In inward-facing models of homodimeric FP mutant V454A, the presence of Ala decreased by 2.3 Å the WT Cα-Cα interatomic distance between chain a residue 454 and chain b residue 454. In contrast, this change was minimal in the outward-facing conformation. The WT loop structure at aa 362-367 (packed adjacent to chain b residue 454 in the inward-facing conformation) underwent a partial loop-to-helix transition in the homodimeric FP mutant V454A. Furthermore, the WT Cα-Cα interatomic distance between chain a residue 276 and remote chain b residue 723 decreased by 3.3 Å in the heterodimeric FP mutant R276W (chain a)/R723Q (chain b). The inward-facing conformation of this heterodimeric mutant also induced a loop-to-helix transition of chain a residues 408-409 at the ecto-end of a transmembrane helix and spatially adjacent to chain a missense substitution R276W. These amino acid substitutions also modestly alter interhelical distances near the mutation sites. Structural superposition of modeled WT polypeptide with each modeled mutant polypeptide reveals larger global structural deviations of mutant polypeptides in inward-facing than in outward-facing conformations (Table 3 and Table S1). Modeled heterodimeric R276W/R723Q and homodimeric V454A mutant polypeptides exhibited greater structural deviation from WT than did homodimeric mutants R276W or R723Q (Table 3, Table S1).

**ABCB6 mutations cause no alteration of expression and cellular localization**

We modeled in vitro our patients' genotypes by transient transfection of WT and mutant ABCB6 expression plasmids into HEK293 cells. No significant differences between mutant and WT mRNA accumulation were evident 72h post-transfection (Figure 2A). Similarly, immunoblot analysis of heterologous FLAG tag confirmed equivalent accumulation of WT and mutant heterologous ABCB6 polypeptides (Figure 2B).

We also tested effects of the mutations on ABCB6 membrane localization. Confocal microscopy analysis showed that all mutant polypeptides were expressed predominantly at the HEK-293 cell
peripheral membrane, as demonstrated by colocalization of ABCB6-FLAG with the membrane marker lectin WGA wheat germ agglutinin (Figure 2C).

**ABCB6 mutation R276W increases potassium efflux from red blood cells of a blood donor**

A blood sample from the blood donor heterozygous for ABCB6 variant and samples from two control donors were obtained and stored four weeks at 4°C under blood banking conditions. Extracellular and intracellular potassium levels were measured throughout the storage period. As shown in Figure 3A the potassium efflux of blood donor after 28 days of storage is about 3.5 fold higher than the controls. Correspondingly, the intracellular RBCs potassium content is about 2.5 fold lower than the controls (Figure 3B). The degree of hemolysis over time for the blood samples was evaluated during storage by measurement of free Hb levels and was the same for three samples (data not shown). The data demonstrated that the physiological consequences of the blood donor's mutation is potassium efflux higher than in controls, and similar to that observed in FP patients.

Moreover, immunoblot analysis on RBCs from the blood donor carrying R276W mutation demonstrated that the expression of ABCB6 does not differ between mutated donor and healthy controls (Figure 3A).

**ABCB6 mutations cause cation flux alterations**

We next evaluated cell potassium content in HEK-293 cells over-expressing WT ABCB6 and different ABCB6 mutant variants. Preliminary experiments comparing cells maintained for 8h at 37°C or 30°C revealed no differences (data not shown). To mimic shipping conditions (critical for the serum [K+] alteration observed in the FP patients, see Case Report section) we exposed HEK-293 cells over-expressing WT or mutant ABCB6 variants to 0.12 g rotary shaking at 30°C for 8 h. As shown in Figure 4A, levels of extracellular K in media from HEK-293 cells over-expressing mutant ABCB6 variants were significantly higher than for cells expressing WT ABCB6. Correspondingly, residual intracellular Rb content was significantly reduced in cells expressing three of the ABCB6 mutant genotypes, WT/R375Q, WT/R375W, V454A/V454A, as well as in the double mutant R276W/R723Q, compared to either WT ABCB6 or the other ABCB6 variants (WT/R273Q, WT/R276W) (Figure 4B). These data show different impacts of individual ABCB6 mutations on cellular K⁺ efflux insensitive to ouabain plus bumetanide, and an incrementally increased effect on cell K⁺ efflux of coexpression of the compound heterozygous ABCB6 mutations R276W/R723Q.
Discussion

In this study we have reported three new mutations in the FP-disease gene \textit{ABCB6}. FP had been described previously as a dominant condition, but, for the first time, we report two FP patients with homozygous or compound heterozygous mutations, both novel patterns of inheritance for FP. Of note, those patients homozygous and compound heterozygous for \textit{ABCB6} mutations showed higher plasma [K\textsuperscript{+}] than heterozygous patients. Moreover, the compound heterozygous also exhibited a value of MCV higher than in other patients. FP inheritance patterns thus constitute a crucial part of patient diagnostic evaluation.

\textit{ABCB6} variations are more common that previously predicted, as also reported for Lan- blood group carriers with \textit{ABCB6} nonsense mutations causing the \textit{ABCB6}-null red cell phenotype. Koszarska and colleagues showed that screening of erythroid \textit{ABCB6} expression reveals an unexpectedly high frequency of Lan mutations in healthy individuals\textsuperscript{21}. Indeed, in public databases (1000 Genomes, NHLBI Exome Sequencing Project, Exome Aggregation Consortium) allele frequencies are 0.43\% for V454A, 0.08\% for R723Q and 1.5\% for R276W. Moreover, the high frequency of \textit{ABCB6} variations in FP, including two FP patients found in a Cardiff blood donor cohort as recently described by Bawazir et al\textsuperscript{22} has clinical implications for blood transfusion screening and practice. Our own screening of 327 blood donors of different geographical and ethnic origin corroborates this observation, with the R276W mutation found in 0.3\% of our cohort. Our analysis of potassium efflux from blood donor RBCs under blood banking storage conditions confirmed the cation leak as shown by FP patients. Refrigerated storage of blood of FP patients causes rapid loss of potassium, and the extracellular potassium content of bags of stored cells increases during storage. This is of little consequence for the majority of transfusions, since the total amount of potassium transfused is relatively small compared to the total blood volume of the recipient. In contrast, this extracellular potassium can have serious or fatal consequences for neonates and infants receiving whole blood transfusions of large volume proportionate to body size. Several such cases of whole blood transfusion in infants leading to cardiac arrest and death have been described\textsuperscript{23-27}. The \textit{ABCB6} FP mutants overexpressed in HEK-293 cells showed no difference in accumulation of mRNA or protein, or in peripheral membrane immunolocalization as compared to WT \textit{ABCB6}, and as previously demonstrated for \textit{ABCB6} FP variant R375Q.

Consistent with these findings, \textit{in silico} modeled 3D structures of these mutant \textit{ABCB6} polypeptide dimers predicted modest structural alterations of transmembrane and cytosolic ATP binding domains in both inward- and outward-facing conformations. Prediction of the consequences of
these structural alterations to the cation leak process remain uncertain, since the relationship between the mutant cation leak and the (proposed but still debated) wild-type transport of porphyrins remains poorly understood. Future molecular dynamics simulation studies in a model lipid bilayer across microsecond time-scales will extend our understanding of the impact of these FP mutants on the structure, and possibly the function, of ABCB6.

To further characterize the role of ABCB6 mutants, we tested the hypothesis that their expression could modify K⁺ transport in HEK-293 cells in a manner similar to the altered K⁺ efflux in FP RBC. We found that cells expressing each mutant variant tested exhibited increased potassium efflux compared to WT. Co-expression in HEK-293 cells of the two mutant variants expressed by patient Cardiff in compound heterozygous form produced the highest value of potassium efflux among all tested mutants. These data demonstrated that the new mutations, whether homozygous or compound heterozygous, act at the cellular level as gain-of-function mutations.

Among ABC proteins, only the cystic fibrosis transmembrane regulator CFTR/ABCC7 itself is known to mediate ion channel function. However, several ABC proteins, in addition to CFTR, function as ion channel regulators 28-30, including the Kir6 K\textsubscript{ATP} channel regulatory subunits, sulphonylurea receptors SUR1, SUR2A, and SUR2B 31. The question remains whether ABCB6 FP mutant polypeptides generate intrinsic cation leak pathways in membranes of red cells (or experimentally in HEK-293 cells), or might secondarily dysregulate one or more endogenous membrane cation permeability pathways in red cells (or HEK-293 cells). The negative results obtained to date in our electrophysiological studies conducted in HEK-293 cells and Xenopus laevis oocytes expressing WT or mutant ABCB6 variants (not shown) encourage further consideration of dysregulated endogenous electroneutral (or low-level electrogenic) transporters as cation leak mediators in FP red cells or cell models of heterologous expression.

Our findings demonstrate that both heterozygous and homozygous missense mutations in ABCB6 lead to increased efflux of cellular K⁺ from HEK-293 cells, a property shared with RBCs of FP patients. Screening for the most frequently found ABCB6 variant, R276W, confirmed that patients with FP are relatively common in the blood donor population. Storage of FP blood can cause a significant increase in whole blood K⁺ levels, with serious clinical implications for neonates and infants receiving large-volume transfusions of whole blood. For these reasons, we endorse the proposal to conduct genetic screening for ABCB6 FP mutations among potential blood donors, especially when whole blood is needed. Finally, investigation of ABCB6 may contribute to our understanding of other pathologies of red blood cell hydration, such as sickle cell anemia.
References


Table 1. Clinical data of FP patients

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<th>Family code</th>
<th>Ethnicity</th>
<th>Hb (g/dL)</th>
<th>MCV (fL)</th>
<th>MCH (pg)</th>
<th>K+ (mmol/L) (&lt; 37°C)</th>
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§These families were previously described.6

Table 2. Mutations found in FP patients

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<td>Andolfo et al. 2013</td>
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<td>-</td>
<td>1/0</td>
<td>Andolfo et al. 2013</td>
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*Mutations in homozygous state.
§The two mutations are in trans (see results section for details).
aOverall minor allele frequencies (MAF) estimated from public databases 1000 Genomes (URL: http://browser.1000genomes.org);20 NHLBI Exome Sequencing Project (URL: http://evs.gs.washington.edu/EVS); Exome Aggregation Consortium (ExAC), Cambridge, MA (URL: http://exac.broadinstitute.org); for details see Table 2S.
### Table 3. RMSD (Å) of superposed homology-modeled structures of the indicated patient-derived mutant ABCB6 dimers with the modeled wildtype ABCB6 homodimer

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<th>Mutant ABCB6</th>
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<th>Outward-facing</th>
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<td>0.29</td>
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<td>R723Q/R723Q</td>
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<td>0.79</td>
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**Author Contributions**

I.A., R.R.; S.L.A. L.D.F and A.I. designed and conducted the study, and prepared the manuscript; I.A. performed the ionic flux assays; R.R. performed the sequencing analysis and blood donors screening; F.M. performed the ionic flux assays and real time PCR assays; G.D.R. performed the immunofluorescence assays and western blotting analysis; N.D. contributed to the critical evaluation of ionic flux assays; A.K.S. performed 3D prediction; C.B. contributed to the critical evaluation of the manuscript; A.G. and SF performed clinical evaluation of patients; CL provided samples of blood donors with relative hematological parameters.
Figure legends

Figure 1. Three-dimensional structural model of human ABCB6 mutations

A. Three-dimensional structural model of a portion of homodimeric WT human ABCB6 in an inward-facing conformation, as modeled on the aligned structure of *M. musculus* ABCB1A (PDB ID 3G5U). Monomer "a" (blue) of the homodimer represents ABCB6 aa residues 246 (N-) to 826 (-C), modeled on transmembrane helices 1-6 and NBD-1 of ABCB1A. Monomer "b" (pink) of the homodimer represents ABCB6 aa residues 237 (N'-) to 826 (-C') modeled on ABCB1A transmembrane helices 7-12 and its NBD-2. A surface model is superposed on the modeled polypeptide backbone ribbon structure. DHSt homozygous mutation site V454 (red spheres) is located between the membrane-spanning helices and the NBDs, extending into the cytoplasmic vestibule of the dimer. Locations of the compound heterozygous DHSt mutation sites R276 (magenta spheres)/R723 (olive spheres) are also shown. Arrows mark R276 of monomer A (located within the lipid bilayer) and R723 of monomer B (located within the NBD region in the cytoplasmic vestibule) of the dimer. The cavity (cyan spheres) at the intermonomeric interface outlines a postulated intra-membrane binding site for inhibitors of ABCB6-mediated porphyrin transport, corresponding to the ABCB1 binding site of inhibitor QZ59. In this and subsequent figures, each modeled ABCB6 monomer lacks its ectofacial N-terminal tail and putative transmembrane spans 1-5, but includes putative transmembrane spans 6-11 (TM) followed by the single nucleotide-binding domain (NBD).

B. Transverse intra-membrane profile of the modeled inward-facing conformation of dimeric WT ABCB6 (as in panel A), with transmembrane helices rotated 90° around the axis shown. The view (lacking NBDs) looks outward from the ICL region, near the site of separated mutation site V454 (red) and farther from mutation site R276. The colored M1 domain helices are numbered 6-11 for ABCB6 monomer "a", and 6'-11' for the monomer "b" of the ABCB6 dimer. The arrows between helices 9 and 11 on one side, and helices 9' and 11' on the other side of the dimer mark the locations of side apertures proposed in mouse ABCB1 to mediate hydrophobic drug uptake from the inner leaflet of the lipid bilayer for subsequent efflux from the cell, or for flippase-like transfer to the outer leaflet.

C. Three-dimensional structural model of homodimeric WT human ABCB6 in an outward-facing conformation, as modeled on the aligned structure of *S. aureus* Sav1866 (PDB ID 2HYD). The black oval encloses a central cavity at the inter-monomeric interface, hypothesized to be an intra-membrane substrate binding site (as predicted for homodimeric Sav1866 of *S. aureus*). Sites of homozygous and compound heterozygous mutations are shown using similar colored spheres as in panel A.

D. Transverse intramembranous profile of the modeled outward-facing conformation of dimeric ABCB6 (as in panel C), with the transmembrane helices rotated 90° around the axis shown. The view (lacking
NBDs) looks inward from the extracellular edge of the outer leaflet of the membrane bilayer towards the approximated mutation sites V454 (at the level of the ICL region) and R276 (farther from the ICL region); color scheme as in panel C. Helices are labeled at ends closest to reader. The figure was prepared in PyMOL.

**Figure 2. Expression and localization of ABCB6 mutants.**

A. ABCB6 mRNA levels in HEK-293 cells transfected with ABCB6 WT and mutants and empty vector as control. Values are means +/- s.e.m. of three independent experiments. *p < 0.001 WT, WT/R375Q, WT/R375W, WT/R276W, V454A/V454A, WT/R723Q, R276W/R723Q vs empty vector.

B. Immunoblot showing ABCB6 Flag protein expression in HEK-293 cells transfected with FLAG-tagged WT or mutant ABCB6 variants, or with empty vector as control and GAPDH as loading control. One of two similar experiments. C. Laser-scanning confocal microscopy images of HEK-293 cells transfected with WT or mutant ABCB6 variants, or with empty vector as control, analyzed by immunofluorescence with rabbit polyclonal anti-ABCB6 antibody (green) and WGA (membrane marker, staining both the nuclear envelope and the plasma membrane, red), with merged signal showing regions of colocalization in yellow (white arrows indicate the yellow regions in the merge). Cells were imaged with a Zeiss LSM 510 meta confocal microscope equipped with a 1.4 NA oil immersion plan Apochromat 100× objective. Intensity and contrast were adjusted with Axiovision software. Representative of three independent experiments.

**Figure 3. ABCB6 protein expression and potassium efflux in red blood cells of blood donor carrying ABCB6 R276W variant.**

A. Plasma K content (expressed as mmol/L of whole blood) of blood from donor heterozygous for ABCB6 mutation R276W and from two healthy controls after 0D, 7D, 14D and 28D cold storage under blood banking conditions. * p < 0.01 donor vs two healthy controls. B. Intracellular K content (expressed as mmol/10^13 cells) of blood from donor heterozygous for R276W and from two healthy controls after 0D, 7D, 14D and 28D cold storage as in A. Ion contents in A and B were measured by atomic absorption spectrometry, and represent means +/- s.e.m. of 3 independent experiments. * p < 0.01 donor vs two healthy controls . C. Immunoblot showing ABCB6 protein expression in RBC membranes from blood donor heterozygous for mutation R276W and pooled membranes from two healthy controls. β-actin was used as loading control. One of three similar experiments.
Figure 4. Analysis of potassium efflux of ABCB6 mutants.

A. K content of extracellular medium sampled from cultures of cells overexpressing ABCB6 WT or ABCB6 FP mutants. Ion contents measured by atomic absorption spectrometry were expressed as mmol/mg protein. **p < 0.001 R276W/R723Q vs empty vector and WT; *p < 0.05 for WT/R375Q, WT/R375W, WT/R276W, V454A/V454A, WT/R723Q vs WT. B. Rb content of cells overexpressing ABCB6 WT and ABCB6 FP mutants, expressed as mmol/mg protein. Values in A and B are means +/- s.e.m. of four independent experiments.
Supplementary data of "Functional characterization of novel ABCB6 mutations and their clinical implications in familial pseudohyperkalemia" by Andolfo et al.

Supplementary Table S1: RMSD values of modeled mutant ABCB6 dimers superposed on modeled WT ABCB6 homodimers in both inward- and outward-facing conformations. The heterodimeric (compound heterozygous) mutant polypeptides are considered in both possible conformations, since the productive alignments for modeling of monomer "a" and monomer "b" within the dimer were of different length, as explained in Methods and Figure 1 legend.

Supplementary Table S2: Minor allele frequency of the variants identified in this study by the analysis of the public databases 1000 genomes, NHLBI Exome Sequencing Project and Exome Aggregation Consortium.

Supplementary Figure legends

Figure 1S. A. Three-dimensional structural model of a portion of homodimeric human ABCB6 DHSt mutant p.V454A in an inward-facing conformation, as modeled on the aligned structure of *M. musculus* ABCB1A (PDB ID 3G5U). Monomer "a" (blue) of the homodimer represents ABCB6 aa residues 246 (N-) to 826 (-C), modeled on transmembrane helices 1-6 and NBD-1 of ABCB1A. Monomer "b" of the homodimer (pink) represents ABCB6 aa residues 237 (N')- to 826 (-C') modeled on ABCB1A transmembrane helices 7-12 and its NBD-2. A surface model is superposed on the modeled polypeptide backbone ribbon structure. Residue A454 (red spheres) is located between the membrane-spanning helices and the NBDs, extending into the cytoplasmic vestibule of the dimer. The cavity (cyan spheres) at the intermonomeric interface outlines a postulated intra-membrane binding site for inhibitors of ABCB6-mediated porphyrin transport, corresponding to the ABCB1 binding site of inhibitor QZ59. Note that in this and subsequent figures, each modeled ABCB6 monomer lacks its ectofacial N-terminal tail and putative transmembrane spans 1-5, but includes putative transmembrane spans 6-11 (TM) followed by the single nucleotide-binding domain (NBD). B. Transverse intra-membrane profile of the modeled inward-facing conformation of homodimeric ABCB6 mutant V454A (as in panel A), with transmembrane helices rotated 90° around the axis shown. The view (lacking NBDs) looks outward from the ICL region, near the separated mutant A454 residues (red). The colored M1 domain helices are numbered 6-11 for ABCB6 monomer "a", and 6'-11' for the monomer "b" of the ABCB6 dimer. The arrows between helices 9 and 11 on one side, and helices 9' and 11' on the other side of the dimer mark the locations of side apertures proposed in mouse ABCB1 to mediate hydrophobic drug uptake from the inner
leaflet of the lipid bilayer for subsequent efflux from the cell, or for flippase-like transfer to the outer leaflet. C. Three-dimensional structural model of homodimeric human ABCB6 mutant p.V454A in an outward-facing conformation, as modeled on the aligned structure of S. aureus Sav1866 (PDB ID 2HYD). The black oval encloses a central cavity at the inter-monomeric interface, hypothesized to be an intra-membrane substrate binding site (as predicted for homodimeric Sav1866 of S. aureus). D. Transverse intramembranous profile of the modeled outward-facing conformation of dimeric ABCB6 (as in panel C), with the transmembrane helices rotated 90° around the axis shown. The view (lacking NBDs) looks inward from the extracellular edge of the outer leaflet of the membrane bilayer towards the approximated mutant A454 residues (red) at the level of the ICL region. Helices are labeled at ends closest to reader. The figure was prepared in PyMOL.

Figure 2S. A. Three-dimensional structural model of a portion of the heterodimeric human ABCB6 compound heterozygous DHSt mutant p.R276W/p.R723Q in an inward-facing conformation (please Fig. 1 legend for details). Mutant residue W276 (magenta spheres) of monomer A is located within the lipid bilayer. Mutant residue Q723 (olive spheres) of monomer B is located within the NBD region in the cytoplasmic vestibule of the dimer. The cavity (cyan spheres) at the intermonomeric interface outlines a postulated intramembranous inhibitor binding site. B. Transverse intra-membrane profile of the modeled inward-facing conformation of heterodimeric ABCB6 mutant p.R276W/p.R723Q (as in panel A) with transmembrane helices rotated 90° around the axis shown. The view, lacking NBDs, looks outward from the cytoplasmic edge of the inner leaflet, and includes mutant residue W276 (magenta) of monomer A, but not mutant residue Q723 of monomer B. Helical numbering and arrows are as defined in Fig. 1B. C. Three-dimensional structural model of a portion of the heterodimeric human ABCB6 compound heterozygous DHSt mutant p.R276W (magenta)/p.R723Q (olive) in an outward-facing conformation, as modeled on the aligned structure of S. aureus Sav1866 (PDB ID 2HYD). The black oval encloses a central cavity at the inter-monomeric interface (as in Fig. 1). D. Transverse intra-membrane profile of the modeled outward-facing conformation of heterodimeric ABCB6 p.R276W/p.R723Q (as in panel C), with the transmembrane helices rotated 90° around the axis shown. The view, lacking NBDs, looks inward from the extracellular edge of the outer leaflet, highlighting mutant residue W276 protruding for the periphery of the transmembrane helical bundle. Helices are labeled at ends closest to reader. The figure was prepared in PyMOL.
Supplementary Methods

Sequencing analysis

The search for mutations was performed by direct sequencing, using 75 ng genomic DNA. All exons and flanking splice junctions of the \textit{ABCB6} gene were amplified by PCR in a 25 μl volume with Master Mix 2X (Promega). Oligonucleotide primers were designed by the program Primer3 v.0.4.0. Primer sequences are available on request (e mail: achille.iolascon@unina.it). Integrity of PCR products was checked by agarose gel electrophoresis. Direct sequencing was performed using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Branchberg, NJ, USA) and a 3730 DNA Analyzer (Applied Biosystems). Missense substitution mutations in \textit{ABCB6} (Q9NP58) were evaluated by Poly-Phen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml) and SIFT (http://sift.jcvi.org/) online tools.

Immunofluorescence analysis

HEK-293 cells (2x10^6) on coverslips were transfected with ABCB6 cDNAs. After 72 hrs, cells were fixed, and immunostained with anti-FLAG (1:200; F3165, Sigma) and anti-WGA (Wheat Germ Agglutinin, Alexa Fluor 555 Conjugate, Life Technologies). Secondary antibodies for FLAG (Alexa Fluor 488 goat anti-mouse; Life Technologies) were incubated at 1:200 dilution in PBS for 30 min at room temperature. Nuclei were stained with 1 μg/ml DAPI in PBS for 15 min at room temperature. The coverslips were mounted in 50% glycerol (v/v) in PBS and imaged by Zeiss LSM 510 Meta confocal microscope equipped with an oil immersion plan Apochromat 63× objective 1.4 NA, Green channel excitation of Alexa488 by the argon laser 488 nm line was detected with the 505-550 nm emission bandpass filter. Red channel excitation of Alexa546 by the Helium/Neon laser 543 nm line was detected with the 560-700 nm emission bandpass filter (using the Meta monochromator). Blue channel excitation of DAPI by the blue diode laser 405 nm line was detected with the 420-480 nm and emission bandpass filter.

Cell culture and transfection assay

Human HEK-293 cells were maintained in DMEM medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin (all from Life Technologies), in a humidified 5% CO\textsubscript{2} atmosphere at 37°C. pcDNA3.1-ABCB6-WT and pcDNA3.1-ABCB6 mutant constructs (5μg) were transfected into HEK-293 cells using X-tremeGENE HP DNA Transfection Reagent (Roche, Indianapolis, IN, USA). To phenocopy the heterozygous genotypes, cells were transfected with 2.5μg of WT
pcDNA3.1-ABCB6-WT plus 2.5μg of pcDNA3.1-ABCB6 mutants R375Q, p.R276W or R375W. For the compound heterozygous genotypes, cells were transfected with 2.5μg pcDNA3.1-ABCB6-R276W plus 2.5μg pcDNA3.1-ABCB6-R723Q. For the homozygous genotype, cells were transfected with 5μg pcDNA3.1-ABCB6-V454A. After 72 hrs, cells were harvested for analysis.

**Exome capture and sequencing**

Blood was obtained for genetic analysis from affected and unaffected family members of the Irish family and from healthy controls, with signed informed consent according to the Declaration of Helsinki. Blood collection was according to protocols approved by local university ethics committees. Genomic DNA was prepared from peripheral blood with the Wizard Genomic DNA purification kit (Promega, Milano, Italy). 5μg of DNA from two affected and two unaffected members of DHSt family Edinburgh was diluted in 700 μl of Nebulization buffer (Illumina, San Diego, CA) and sheared by nebulizers (Invitrogen, Carlsbad, CA) into fragments of 200–400 bp in length (Bioanalyzer 2100, Agilent, Santa Clara, CA). Sheared samples purified by QIAquick spin columns (Qiagen, Hilden, Germany) were processed for library preparation (Illumina protocol), omitting size-selection of adapter-ligated fragments prior to capture. After several cycles of PCR amplification, 500 ng of DNA from the resulting libraries was hybridized to the bait set from the SureSelect Human All Exon Kit (Agilent, Santa Clara, CA, USA) at 65°C for 24 h. Hybrid capture with streptavidin-coated Dynal magnetic beads (Invitrogen, Carlsbad, CA) was performed as manufacturer’s protocol. Captured samples were further purified through Agencourt AMPure XP beads and subjected to PCR amplification. All samples at each step of library preparation were quantified by Bioanalyzer 2100 (Agilent). Individual sample libraries were NaOH-denatured and loaded onto one lane of an Illumina Flowcell v4. DNA clusters were generated through a one-step workflow on the Cluster Station using TruSeq PE Cluster Kit v5 (Illumina, San Diego, CA). A PhiX control library added to each sample at 1% volume served as internal control. Sequencing was performed on the Illumina Genome Analyzer IIx platform as paired-end 100-bp reads according to the manufacturer’s protocol. An exome capture was considered successful if >80% of the target regions were covered with a high quality genotype.

Reads were aligned to the most recent version of human genome (GRCh37/hg19) using the BWA software package v0.5.9.
Molecular cloning of ABCB6 and Site-directed mutagenesis

To detect and determine the inheritance pattern of the two mutations in patient Cardiff 2, the DNA fragment encompassing the two mutations R276W and R723Q, of ABCB6 was PCR-amplified and cloned into PCR Cloning Vector pSC-A-amp/kan (StrataClone PCR Cloning Kit, Agilent). Point mutations c.1123 C>T, p.R375Q; c.1361T>C; p.V454A; c.826G>T; p. R276W; c.2168G>A; p.R723Q; c.1124 G>A; p.R375W were introduced into pcDNA3.1-ABCB6 with the QuickChange kit (Stratagene, La Jolla, CA). The integrity of the complete ABCB6 coding region was confirmed by sequencing after mutagenesis. Primers sequences are available upon request.

RNA isolation, cDNA preparation and quantitative qRT-PCR

Total RNA was extracted from cell lines and peripheral blood samples from patients and healthy controls using Trizol reagent (Life Technologies). Synthesis of cDNA from total RNA (2 μg) was performed using Super Script II First Strand kits (Life Technologies). Quantitative RT-PCR (qRT-PCR) was by the SYBR-green method using the ABI PRISM 7900HT Sequence Detection system. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method, where $\Delta C_t$ indicates the differences in the mean Ct between selected genes and the internal control (GAPDH or $\beta$-actin). Sequences of the qRT-PCR primers designed for each gene using Primer Express 2.0 (Life Technologies) are available upon request.

Immunoblotting

Total cell lysates (80 μg protein) electrophoresed on SDS-polyacrylamide gels were electroblotted onto polyvinylidene difluoride membranes (BioRad, Milan, Italy), incubated with the following antibodies: anti-FLAG (1:500; F3165, Sigma), anti-$\beta$-actin antibody (1:1000; Sigma, used as loading control), then imaged with HRP-conjugated anti-rabbit Ig (1:5000) (GE Healthcare, UK) and enhanced chemiluminescence substrate (Supersignal West Pico Chemiluminescent Substrate Kit, ThermoScientific, Miami USA). Labeled bands were visualized and densitometric analysis performed with the BioRad Chemidoc using Quantity One software (BioRad).

Statistical analysis

Data are presented as means ± standard error of the mean (s.e.m.). Statistical significance was calculated using the Mann-Whitney test and Student’s t test. Correlation analysis was calculated using Spearman’s rank correlation coefficient and Pearson’s correlation coefficient. P < 0.05 was considered as statistically significant.
Table S1. RMSD values (Å) of homology-modeled structures of WT and mutant ABCB6 homodimers superposed on modeled WT and other mutant ABCB6 dimer structures

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Figure 2S. Andolfo et al. 2015