

ABCB6 Polymorphisms are not Overly Represented in Patients with Porphyria Tracking no: ADV-2021-005484R1

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Abstract:
The Mendelian inheritance pattern of acute intermittent porphyria, hereditary coproporphyria, and variegate porphyria is autosomal dominant, but the clinical phenotype is heterogeneous. Within the general population, penetrance is low, but among first-degree relatives of a symptomatic proband, penetrance is higher. These observations suggest that genetic factors, in addition to mutation of the specific enzyme of the biosynthetic pathway of heme, contribute to the clinical phenotype. Recent studies by others suggested that the genotype of the transporter protein ABCB6 contribute to the porphyria phenotype. Identifying the molecule(s) that are transported by ABCB6 has been problematic and has led to uncertainty with respect to how or if variants/mutants contribute to phenotypic heterogeneity. Knockout mouse models of Abcb6 have not provided a direction for investigation as homozygous knockout animals do not have a discrete phenotype. To address the proposed link between ABC6 genotype and porphyria phenotype, a large cohort of patients with acute hepatic porphyria and erythropoietic protoporphyria was analyzed. Our studies showed that ABCB6 genotype did not correlate with disease severity. Therefore, genotyping of ABCB6 in patients with acute hepatic porphyria and erythropoietic protoporphyria is not warranted.

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TITLE

ABCB6 Polymorphisms are not Overly Represented in Patients with Porphyria

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Short title: ABCB6 polymorphisms do not affect porphyria phenotype
Key points: #1- ABCB6 is expressed on the cell surface and by multiple organelles, but transport specificity is incompletely understood. #2 In all types of porphyria, ABCB6 polymorphisms are not overrepresented when compared to the overall population.

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ABSTRACT

The Mendelian inheritance pattern of acute intermittent porphyria, hereditary coproporphyria, and variegate porphyria is autosomal dominant, but the clinical phenotype is heterogeneous. Within the general population, penetrance is low, but among first-degree relatives of a symptomatic proband, penetrance is higher. These observations suggest that genetic factors, in addition to mutation of the specific enzyme of the biosynthetic pathway of heme, contribute to the clinical phenotype. Recent studies by others suggested that the genotype of the transporter protein ABCB6 contribute to the porphyria phenotype. Identifying the molecule(s) that are transported by ABCB6 has been problematic and has led to uncertainty with respect to how or if variants/mutants contribute to phenotypic heterogeneity. Knockout mouse models of *Abcb6* have not provided a direction for investigation as homozygous knockout animals do not have a discrete phenotype. To address the proposed link between ABC6 genotype and porphyria phenotype, a large cohort of patients with acute hepatic porphyria and erythropoietic protoporphyria was analyzed. Our studies showed that *ABCB6* genotype did not correlate with disease severity. Therefore, genotyping of *ABCB6* in patients with acute hepatic porphyria and erythropoietic protoporphyria is not warranted.

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INTRODUCTION

The following disease entities comprise the acute hepatic porphyrias (AHPs); acute intermittent porphyria (AIP); hereditary coproporphyria (HCP); variegate porphyria (VP). These are rare diseases of heme biosynthesis that are inherited in an autosomal dominant fashion with low penetrance.[1] For example, AIP, the most common of the AHPs, arises as a consequence of mutations affecting the enzyme hydroxymethylbilane synthase (HMBS) that is required to convert porphobilinogen to hydroxymethylbilane (the third of eight steps in the biosynthetic pathway of heme). In an international study of genomic/exomic databases, Chen and colleagues[2] identified 12 pathogenic sequence variants among ~92,000 alleles, a frequency of 0.00056. The estimated prevalence of symptomatic patients with heterozygous mutant HMBS is ~0.000005. [3] Based on this data, the estimated penetrance of pathogenic HMBS mutants is ~1%.[4] However, among first-degree relatives of affected patients, the penetrance is ~20%.[3] Together, these data suggest that genetic factors, in addition to mutant HMBS, determine the clinical phenotype of AIP. The basis of the low penetrance observed in AHPs is an area of active investigation, as discovery of genes that modify the disease phenotype would provide both relevant clinical information and a more a detailed understanding of heme metabolism.

Steps in the heme biosynthetic pathway take place in both the mitochondria and the cytosol. Therefore, genes that encode proteins that transport heme metabolites between

cellular components are logical targets to investigate for sequence variants that contribute to the disease heterogeneity characteristic of porphyria. Krishnamurthy and colleagues reported that ATP-binding cassette B6 (ABCB6) is a mammalian mitochondrial porphyrin transporter.[5] In unrelated studies, ABCB6 was shown to specify the Langereis (Lan) blood group system.[6] Fukuda et al. hypothesized that sequence variants affecting ABCB6 modulate the clinical phenotype of porphyria by altering the transport of toxic metabolic intermediates.[7] Those investigators reported data suggesting that sequence variants in *ABCB6* were causally related to disease severity both in AHP patients and in patient with the cutaneous porphyria, erythropoietic protoporphyria (EPP).[7] The cohort of patients examined by Fukuda et al., however, was small (total of 36 patients). To investigate further the relationship between ABCB6 and the severity of the AHPs and EPP, we studied the ABCB6 gene sequence in 562 patients (1124 alleles). Our findings demonstrate that, with two exceptions, ABCB6 variants are present at the same frequency in patients with EPP and each type of AHP as in the general population, and when EPP and AHPs are analyzed together, there is no correlation between ABCB6 genotype and porphyria phenotype.

Materials

A retrospective study was performed on DNA samples obtained from patients with AIP, HCP, VP, EPP, and X-linked protoporphyria (XLP). The samples were collected under studies approved by the local Institutional Review Boards of the 6 academic medical centers of the Porphyrias Consortium or by the French Porphyria Group. All research was performed in accordance with the World Medical Association Declaration of Helsinki ethical principles for medical research involving human subjects and its subsequent amendments (R162-16-7 and 145-15-4 French ethical agreement). All family members provided written informed consent to participate in genetics studies. The European Union Institutional Review Board at INSERM approved the study protocols.

Methods

Genomic DNA (gDNA) was isolated from peripheral blood mononuclear cells using an automated magnetic bead-based system (PerkinElmer Chemagic 360). Briefly, whole blood was transferred to 50 mL conical tubes containing 50 µL of protease and 900 µL magnetic beads that bind DNA. Lysis buffer, binding buffer, and wash buffers were sequentially dispensed by the machine during the extraction run. Samples of DNA were removed from beads with 1 mL of elution buffer (10 mM Tris HCL, pH = 8.0), and the sample was incubated at 65° C for 1 hour. DNA was quantified using a Nanodrop One spectrophotometer (ThermoFisher), and the concentration was adjusted to 200 ng/µL. Based on agarose gel electrophoresis, samples used for sequencing consisted of high molecular weight DNA.

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Sequencing of the French cohort for ABCB6 was performed using primers shown in Supplemental Table 1. All patient sequences were compared to the NCBI Reference Sequence, NG_032110.1 based on the ABCB6 sequence that was initially reported by Hilias and colleagues.[6] Allele typing of the American cohort was performed using an ABCB6 SNP detection protocol developed by the Molecular Diagnostic Laboratory at the Icahn School of Medicine at Mount Sinai. ABCB6 exons were amplified in 3 groups: Ex1-4 (3445 bp); Ex 5-13 (3426 bp); and Ex13-19 (3435 bp). All PCR primers and sequencing primers (Supplemental Table 2) were designed in-house using the MacVector software and checked for common SNPs by the SNP-check program (http://www/ngrl.org.uk/Manchester/projects/snpcheck). PCR amplification was performed with the Takara PrimeStar GXL DNA Polymerase kit from Clontech Laboratories using the Bio-Rad C1000 Touch Thermal Cycler. ABCB6 exons were sequenced bi-directionally. Sequencher software (Gene Codes Corporation) was used for alignment and analysis of traces and SNPs identification.

The ExAC database of January 3, 2019 was the source of allele frequency data. Fisher's Exact Test was used to compare allele frequencies among groups.[8] Differences were considered significant if the *p*-value was equal to or less than 0.05. In cases where all 11 ABCB6 polymorphisms were examined across the genome, the significance of the result is compounded by the number of tests performed. Therefore the Bonferroni correction method was applied to the data (0.05/11), resulting in a significant *p*-value of less than or equal to 0.0045.

Results

Sequencing of ABCB6 Allele Variants

We sequenced the gene encoding ABCB6 in 557 subjects with various forms of porphyria (1124 alleles analyzed). Subjects with a confirmed molecular diagnosis were divided into two groups, those with severe disease (216 patients) and those with mild/asymptomatic disease (341 patients), (Table 1). AIP subjects (allele variant in *HMBS*) were divided into those with severe disease (67 patients experiencing > 4 acute attacks per year) and those with mild/asymptomatic disease (155 patients experiencing <2 acute attacks per year). The mild/asymptomatic cohort included 105 individuals identified by sequencing *HMBS* in first degree relatives of probands.

The study group included 49 subjects with HCP, 15 presenting with a severe clinical phenotype and 34 with mild/moderate symptoms. (Table 1) The study group also included 125 subjects with VP, 46 with severe disease and 79 with a mild/moderate symptoms. (Table 1)

There were 30 subjects with EPP, of those, half were classified as having mild disease based on the frequency and severity of symptoms. (Table 1) Five subjects were included in the study that had XLP, the X-linked form of EPP. (Table 1) XLP is the result of gain of function

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mutations in the X-linked gene *ALAS2*, which increase enzymatic activity by 2-3 fold[9], leading to increased erythrocyte PPIX and Zn-PPIX. Also included in the analysis were five subjects that had biochemically confirmed increases of PPIX and Zn-PPIX consistent with EPP but with no sequence variants involving genes known to cause EPP (Table 2).

Eleven sequence variants were identified in *ABCB6* in the 1124 alleles examined, 10 are classified as missense mutations, and one is a synonymous variant. This single nucleotide polymorphism leads to a synonymous change in the codon used for arginine (R589R, c.1767 T>C) and was identified in the heterozygous state in a patient with AIP. Of the 1124 alleles sequenced, 86 missense variants were identified in the ten known *ABCB6* polymorphisms. (Table 2).

ABCB6 Variants in Acute Intermittent Porphyria

There were 222 subjects with a diagnosis of AIP, 67 were classified as severe and 155 were classified as mild (Table 1). In the severe AIP group, 134 alleles were sequenced, and 11 *ABCB6* variants were identified in six of the 10 missense alleles (Table 2). In the mild AIP group, the 310 alleles sequenced had 29 *ABCB6* variants in nine of the missense alleles. None, of the identified *ABCB6* variants were associated with disease severity (range of *p* values=0.21-1.00).

Two of the known *ABC6* missense variants, one, involving the codon for arginine at position 192 (R192W), and the second, involving the glycine 588 codon (G588S), were significantly overrepresented in the total of AIP subjects (the severe and mild/moderate groups combined) when compared to the allele frequencies reported in the ExAC database (p=0.0038, p=0.0042, respectively) (Table 2). The presence of missense variants did not correlate with the urine concentration of ALA and/or PBG, and the frequency of these missense variants was not significantly different compared to those patients with mild vs. severe disease. Missense alleles were identified in nine other positions in *ABCB6*; however, none were overrepresented when compared to the ~120,000 ABCB6 alleles in the ExAC database (Table 2).

ABCB6 Variants in HCP and VP

In HCP 49 subjects were evaluated, 15 classified as severe and 34 classified as mild (Table 2). A total of 9 *ABCB6* allele variants were identified in this group. In the VP population DNA from 125 patients were evaluated, 46 with a severe classification and 79 with a classification of mild. A total of 14 allele variants in *ABCB6* were identified in the VP population. When the frequency of *ABCB6* variant alleles was compared between the severe and mild groups, for HCP and VP independently, variants were not associated with disease severity in either type of porphyria (Table 2). There was no increase in variants in *ABCB6* when compared to the ExAC database.

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Allele frequency in EPP

The study included 166 subjects with EPP, 85 with the severe phenotype. Three had XLP. There were 78 subjects with biochemically confirmed EPP that presented with mild symptoms (less severe sun sensitivity) with 73 having genetic validation of EPP (mutations in *FECH*, *ALAS*2, *CLPX*). *ABCB6* missense alleles were identified in 23 of the 332 alleles sequenced, with no variant overrepresented compared to the allele frequencies in the ExAC database.

RBCs from EPP and XLP patients have a marked increase in fluoresce due to the increased PPIX content (Figure 1). These cells are referred to as fluorocytes and have PPIX concentrations in the ~450 µM range when analyzed by HPLC. In comparison, the level of PPIX in RBCs from normal individuals is in the low nM range. The concentration of PPIX in the plasma of patients with EPP is approximately 0.350 nM (+/- 73 nM) and 0.423 nM (+/- 149 nM) in patients with XLP, with a normal range of 0-10 nM.[10] The majority of EPP patients are wild type at both *ABCB6* loci and none are homozygous or compound heterozygous for any of the variants in *ABCB6* (Table 2). In the EPP/XLP cohort, the cellular concentration of PPIX is approximately 0.5 mM and the plasma PPIX is 0.5 nM.

Erythrocytes from a patient with EPP and wild-type at ABCB6 retain PPIX as evidenced by autofluorescence observed by exciting a peripheral blood sample at 405 nm. Erythrocyte PPIX was identified based on fluorescence emitted at 600-650 nm (Figure 1).

Discussion

Our data show that the frequency of sequence variants that produce missense mutations in ABCB6 is not different among patients with severe porphyria compared to those with mild and asymptomatic disease, and with two exceptions (R192W and G588S in AIP patients), the frequency of each sequence variant among patients with porphyria types was not different from that of the general population (Table 1 & Table 2). We hypothesize that neither R192W nor G588S is clinically relevant as the prevalence of neither genotype is statistically different when ABCB6 genotypes from all patients with porphyria are compared with the general population, and these ABCB6 genotypes do not correlate with clinical severity of EPP. (Table 2). ABCB6 specifies the LAN blood group. Therefore, if ABCB6 genotype were a clinically relevant porphyrin transporter, EPP phenotype would likely be influenced by sequence variants that decrease expression or function of the transporter.[7] In our study of 158 patients with classic EPP, none of the sequence variants reported by Fukuda and colleagues [7] impacted disease severity. Our observations suggest that in patients with AHP and EPP, the ABCB6 genotype is clinically irrelevant and therefore does not warrant analysis in patients with porphyria.

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The unique pathology of each porphyria type is due to the particular pattern of accumulation of heme intermediates when one of the enzymes in the heme biosynthetic pathway is mutated. In AIP, an autosomal dominant disease, acute attacks are characterized by increased levels of ALA and PBG in the urine. ALA is produced in the mitochondria and then transported into the cytosol to form PBG. During an acute attack, levels of PBG are generally in the range of 20-300 mg/g creatinine (normal=0-2 mg/g creatinine) with ALA approximately half the value of PBG. Therefore, values during an attack are 10-150 times above normal.[11] The levels decrease between attacks, however, values often remain elevated above normal for months to years.[12] During an acute attack, there can also be a modest increase in urine uroporphyrin in the range of 20-200 µg/g creatinine (normal <10 µg/g creatinine). Upon successful treatment of the acute attack, uroporphyrin values typically return to normal, and levels of urine uroporphyrin in asymptomatic carriers are generally in the normal range.[13]

HCP is characterized by significant increases in fecal coproporphyrin III. Urinary increases in coproporphyin III are also present. The clinical presentation of patients with HCP is similar to that of patients with AIP, however, an associated cutaneous photosensitivity may be observed in patients with HCP due to an increase in circulating plasma porphyrins.[1] In VP PPIX accumulates in the plasma, and acute attacks are similar to those of AIP. Accumulation of protoporphyrin may result in photosensitivity that is similar to HCP.[1]

EPP is a consequence of accumulation of PPIX in RBCs that is released into the plasma. On exposure of skin to sunlight, the increased plasma PPIX produces a painful, non-blistering photosensitivity response.[14,15] Symptoms gradually decrease in 48-72 hours following elimination of sun exposure. [11] Multiple genetic causes have been linked to PPIX accumulation including mutations in the gene encoding ferrochelatase (FECH), the erythroid form of ALA-synthase (ALAS2), and the mitochondrial protein CLPX, an AAA-ATPase family member that is needed to insert the pyridoxal phosphate cofactor (PLP) into ALAS and then ultimately degrade the ALAS protein.[16] Mutations in FECH account for 90-95% of all cases of EPP. The X-linked form of EPP, XLP, accounts for 5-10% of cases, and in the remaining 1-2% of cases, the genetic basis is unknown.[17]

When FECH is rate-limiting due to mutation as in EPP, PPIX accumulates in erythrocytes because enzyme activity is insufficient to meet the demand for insertion of ferrous iron into available PPIX to form heme. In XLP, nonsense mutations in *ALAS2* result in truncation of the last 18-25 residues of the ALAS2 protein.[9] These are gain of function mutations that lead to an approximate 2-3 fold increase in enzymatic activity.[18 19] The RBCs of these subjects accumulate PPIX because FECH becomes rate-limiting as a consequence of increased flux of intermediates through the heme biosynthetic pathway. In XLP, erythrocyte Zn-PPIX concentration is higher compared with that of EPP patients because FECH activity is preserved.

The compound(s) that is transported by ABCB6 has not been identified. The location of the protein varies in different cell types, and loss of ABCB6 in mouse and man have minimal

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phenotypic consequences.[6] Specific identification of a defect in cells or animals lacking ABCB6 suggests that the compound(s) transported are either essential, and as such, other transporters accommodate the transport need in situations of in which ABC6 function is lost or that the ABCB6 transporter is needed for a specific metabolite that is non-essential for viability in cells or organisms.[20,21] Once a true biologic substrate has been identified, it may be possible to define a stress situation that uncovers the physiological relevance of ABCB6. Several studies that link ABCB6 to heme biosynthesis, however, ABCCB6 has not been consistently localized to the mitochondria.[5,20-24] Identifying the cellular location of ABCB6 is important because the initial step and the final 3 steps in heme biosynthesis take place in the mitochondria with enzymatic steps 2-5 being catalyzed in the cytosol.[25] Many of the enzymes in heme biosynthesis are reported to be expressed with the same temporal pattern as *ABCB6.*[7] Recent studies show that heme biosynthesis requires a large shift in metabolic patterns within the developing erythron. Feeding appropriate abundant intermediates, such as α-ketoglutarate, into the tri-carboxylic acid (TCA) cycle for the formation of succinyl-CoA, a required metabolic building block of δ-aminolevulinic acid, has been shown.[26] This metabolic shift requires that a different set of host proteins be produced. Conceivably, ABCB6 is required for amino acid balancing rather than for transporting intermediates of heme biosynthesis.[26] More recent studies have shown that ABCB6 is necessary for proper melanin production.[24] These studies, however, did not identify the ABCB6 substrate. ABCB6 was localized to the endosomal compartment in this system, suggesting that ABCB6 is not involved in heme biosynthesis as none of the 8 reactions of the heme pathway take place in the endosomal compartment.

ABCB6 is the gene encoding the Langereis (Lan) blood group antigen.[6] Lan negative individuals have no clinical phenotype, suggesting that ABCB6 is not essential for erythropoiesis. Further, the absence of signs and symptoms of porphyria (particularly an erythropoietic type of porphyria such as EPP or congenital erythropoietic porphyria) in Lan negative individuals argues against ABCB6 as an essential transporter of heme metabolites. This interpretation is supported by our data showing that the presence of wild-type ABCB6 does not prevent accumulation of PPIX in the erythrocytes of a patient with EPP (Figure 1).

Murine studies have demonstrated that the localization pattern and quantity of PPIX accumulation in the liver varies among strains, indicating that genetic factors influence PPIX homeostasis.[27] Intercrossing mice with different PPIX hepatic deposition phenotypes did not identify allelic segregation patterns in the F1 or F2 hybrid generations that mapped to the *Abcb6* locus in these progeny (data not shown). However, several genetic loci were highly ranked, suggesting that hepatic uptake and processing of PPIX from the plasma into the biliary system is modulated by genetic factors.

Fukuda et al. showed that some of the ABCB6 polymorphisms that they identified affect the stability of the protein. However, without knowing the transported substrate, the pathophysiological consequences of the polymorphisms that affect stability of ABCB6 is speculative.[7] Our study and the study by Fukuda et al., do not account for the contribution of

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familial patterns to overrepresentation of any single polymorphism (Supplemental Table 3).[7] Although the role of ABCB6 in erythrocyte function has not been delineated, it does not appear to involve transport of intermediates of the heme biosynthetic pathway. This conclusion is further supported by the phenotypic characterization of the *Abcb6-/-* mouse that showed no defect in red blood cell development or tetrapyrrole homeostasis.[28]

Data Sharing Statement:
Alleles of ABCB6 identified in the porphyria population, in this study, have been curated with dbSNP, entries can be found in Supplemental Table 4.

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Table 1. Cohort of 562 Porphyria patients from the USA (American Porphyria Consortium) and Europe [French Porphyria Center (FCP)]

	Cohort Composition	AIP		HCP		VP		EPP		XLP	
	Total (USA / France)	USA	FPC	USA	FPC	USA	FPC	USA	FPC	USA	FPC
All (n alleles)	1124 (390/734)	250	194	0	98	4	246	130	196	6	0
Severe (n alleles)	432 (90/342)	22	112	0	30	2	90	60	110	6	0
Mild/ Asymptom. (n alleles)	472 (80/392)	18	82	0	68	2	156	60	86	0	0
Unknown severity* (n alleles)	220 (220/0)	210	0	0	0	0	0	10	0	0	0

AIP: acute intermittent porphyria; HCP: hereditary coproporphyria; VP: variegate porphyria; EPP: classical erythropoietic protoporphyria; XLP: X-linked protoporphyria + EPP variant form (CLPX, others).

* Known allele carriers from family study with no documentation of clinical history

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Table 2. Allele frequency of ABCB6 polymorphisms by porphyria subtype comparing severity.

	AIP		HCP		VP		EPP		EPP*	XLP		
	S†	M§	S	M	S	M	S	M			Total	MAF**
Patients	67	155	15	34	46	79	85	73	5	3	562	
Alleles∞	134	310	30	68	92	158	170	146	10	6	1124	
R192Q	2	5				1	3			1	12	0.00342
R192W	2	1	1	2	1	2	3	2			14	0.00183
R247C		1					1				2	0.00646
R276W	1	9	1		1	1	2	1			16	0.01895
R343Q		1							1		2	0.01911
A492T	2	2		1	1	1	1	2			10	0.00716
T521S	2	2	1	1	1	1	2	1			11	0.00385
G588S	2	7		2	1	3	2	1			18	0.00467
A681T											0	0.00027
G729S		1									1	0.00005
R589R	1										1	0
total VUS#	12	29	3	6	5	9	14	7	1	1	87	

* Protoporphyrin accumulation without mutation in FECH, ALAS2, CLPX. A681T was not identified in this population.

† S, severe phenotype

§ M, mild/asymptomatic phenotype

∞ All allele variants have been deposited with dbSNP and accession are listed in Supplemental Table 4

** MAF, minor allele frequency

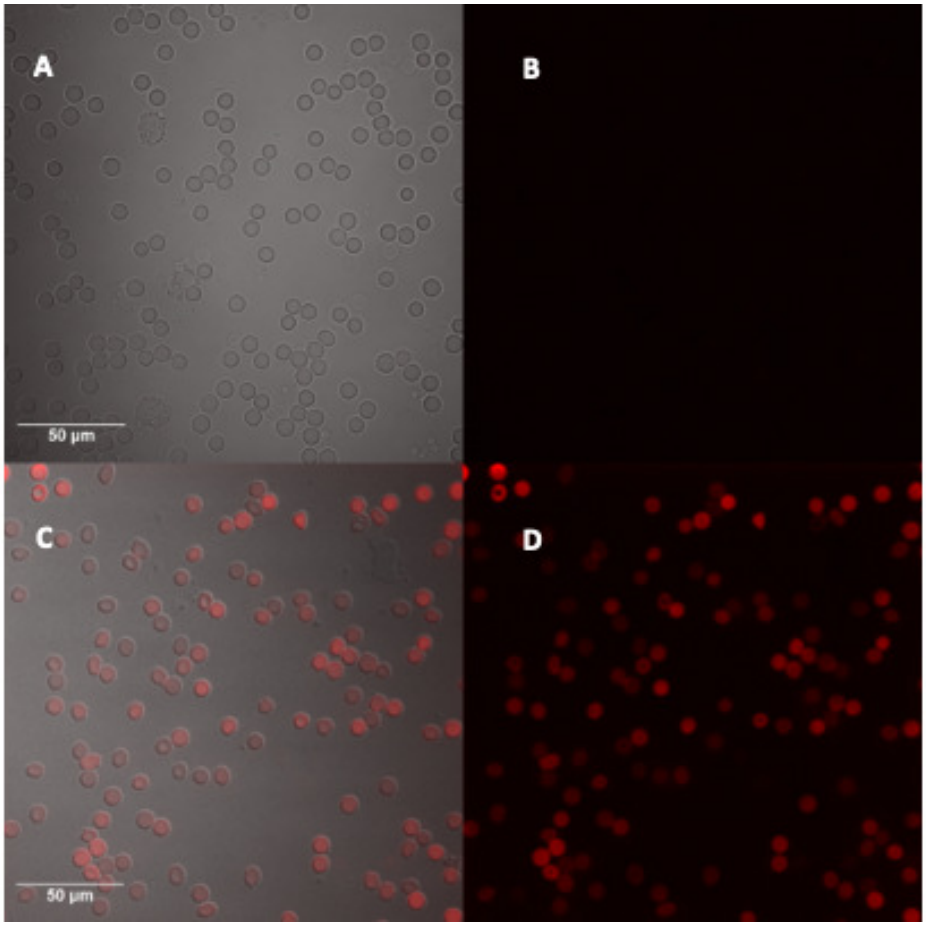
VUS, variant of unknown significance

Figure Legend

Figure 1. Peripheral blood film from a control subject (A, B); and a patient with erythropoietic protoporphyria (EPP) (C, D), all are wild-type at ABCB6. In panels A and C, the differential interference contrast (DIC) and fluorescence image are shown as an overlay; in panels B and D, only fluorescence is shown. Fluorescent protoporphyrin is visualized by excitation at 405 nm with emission peak at 600-650 nm. Protoporphyrin is present in RBCs, but not in lymphocytes. PPIX accumulates in EPP erythrocytes, suggesting that ABCB6 does not act as a PPIX exporter.

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Figure 1



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