

Hereditary anemia caused by multilocus inheritance of *PIEZO1*, *SLC4A1* and *ABCB6* mutations: a diagnostic and therapeutic challenge

Red blood cell (RBC) membrane disorders encompass a vast group of hemolytic anemias that differ widely in their clinical, morphologic, laboratory, and molecular features. Subtypes include (i) RBC disorders caused by altered membrane structural organization and (ii) RBC disorders caused by altered membrane transport function.¹ The most common anemia among the first group is hereditary spherocytosis (HS), with a prevalence from 1:2000 to 1:5000 births. HS manifests clinically as hemolytic anemia with jaundice, reticulocytosis, splenomegaly, and cholelithiasis.¹ Five genes encoding cytoskeleton and transmembrane proteins are most commonly associated with HS: ankyrin-1 (*ANK1*, 8p11.21), erythrocytic α - and β -spectrin chains (*SPTA1*, 1q21; *SPTB*, 14q23.3), band 3 anion transport protein (*SLC4A1*, 17q21.31), and erythrocyte membrane protein band 4.2 (*EPB42*, 15q15-q21).² Disorders of altered membrane transport function include a wide spectrum of hemolytic disorders in which the erythrocyte membrane cation permeability is altered, with dehydrated hereditary stomatocytosis (DHS) the most frequently encountered. The prevalence of DHS remains uncertain, as this disease is often misdiagnosed.³ DHS exhibits alterations of RBC membrane permeability to monovalent cations Na^+ and K^+ , with consequent alterations of intracellular cation, water content, and cell volume. Patients present with hemolytic anemia, typically macrocytic, with increased mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), high reticulocyte count, and jaundice. Blood films may show stomatocytes, usually <10% of total erythrocytes.³ Splenectomy is contraindicated in patients affected by DHS due to the risk of severe thrombotic events.⁴ The two causative genes of DHS are *PIEZO1* (16q24.3) for DHS type 1 (DHS1) and *KCNN4* (19q13.31) for DHS type 2.^{3,5,6} DHS is also frequently associated with iron overload, which frequently leads to hemosiderosis.³ Indeed, recent findings highlight a role for *PIEZO1* in the regulation of iron metabolism.⁷ DHS patients can exhibit hyperferritinemia (and even life-threatening hemosiderosis) accompanied by very low values of plasma hepcidin. Overexpression and pharmacological activation of the R2456H and R2488Q *PIEZO1* gain-of-function (GoF) mutants in hepatoma cell lines induced decreased expression of the hepcidin-encoding gene, *HAMP*.⁷ *PIEZO1* involvement in iron metabolism was further confirmed in constitutive and in macrophage-specific transgenic *PIEZO1* GoF mice. By 1 year of age, these mice develop se-

vere hepatic hemosiderosis with elevated serum ferritin and transferrin saturation, accompanied by increased erythrophagocytosis, erythropoiesis, and erythroferrone expression.⁸

DHS may also present as a syndromic form with pseudohyperkalemia and/or perinatal edema.² Familial pseudohyperkalemia (FP) can also manifest as an isolated autosomal dominant red cell trait characterized by loss of red cell potassium at low temperatures (<37°C).⁵ It is caused by mutations in the *ABCB6* gene (2q35) encoding the *ABCB6* protein, a member of the family of ATP-binding cassette (ABC) solute transporters that translocate many types of metabolites across intra- and extracellular membranes.⁹ The prevalence of FP is probably underestimated, as reflected in the high frequency of certain *ABCB6* missense variants in population databases and in two large cohorts of blood donors.^{5,10}

We describe here a 54-year-old female proband followed for 16 years by our clinical genetics unit for severe anemia and iron overload (Figure 1A). The proband presented at age 24 with moderate anemia (hemoglobin [Hb] 9–11 g/dL), jaundice, gallstones, hepatomegaly, and severe splenomegaly (>18 cm craniocaudal length with weight up to 700 g). Hemoglobinopathies and enzymopathies were excluded. Coombs's test was negative. Blood smear revealed the presence of numerous stomatocytes, target cells, and rare ovalocytes and erythroblasts (Figure 1B). Osmotic fragility was decreased at 2 hours (h) and 24 h post-venesection. Pink and acidified glycerol lysis tests (AGLT) were positive, leading to an initial clinical diagnosis of HS treated by total splenectomy. After surgery, the proband experienced portal vein thrombosis treated with heparin. Worsening anemia to Hb values of 6–7 g/dL required multiple transfusions. Complete red cell count showed macrocytic anemia with MCV between 110 and 147 fL (Table 1). Mean red blood cell (RBC) half-life (measured by *in vitro* calcein fluorescence assay) was 21 days, with probable hepatosplenic destruction of RBC.

At the age of 34 years the proband was diagnosed with severe iron overload and hemosiderosis, and deferoxamine treatment was initiated. At age 38 pulmonary emboli were diagnosed, and she was treated with warfarin. At this time the proband also had hyperkalemia (5.0–6.8 mM). Studies of RBC cation content revealed elevated intracellular [Na] and low intracellular [K], with increased isotopic fluxes at 37°C (Table 2). Temperature-dependence studies

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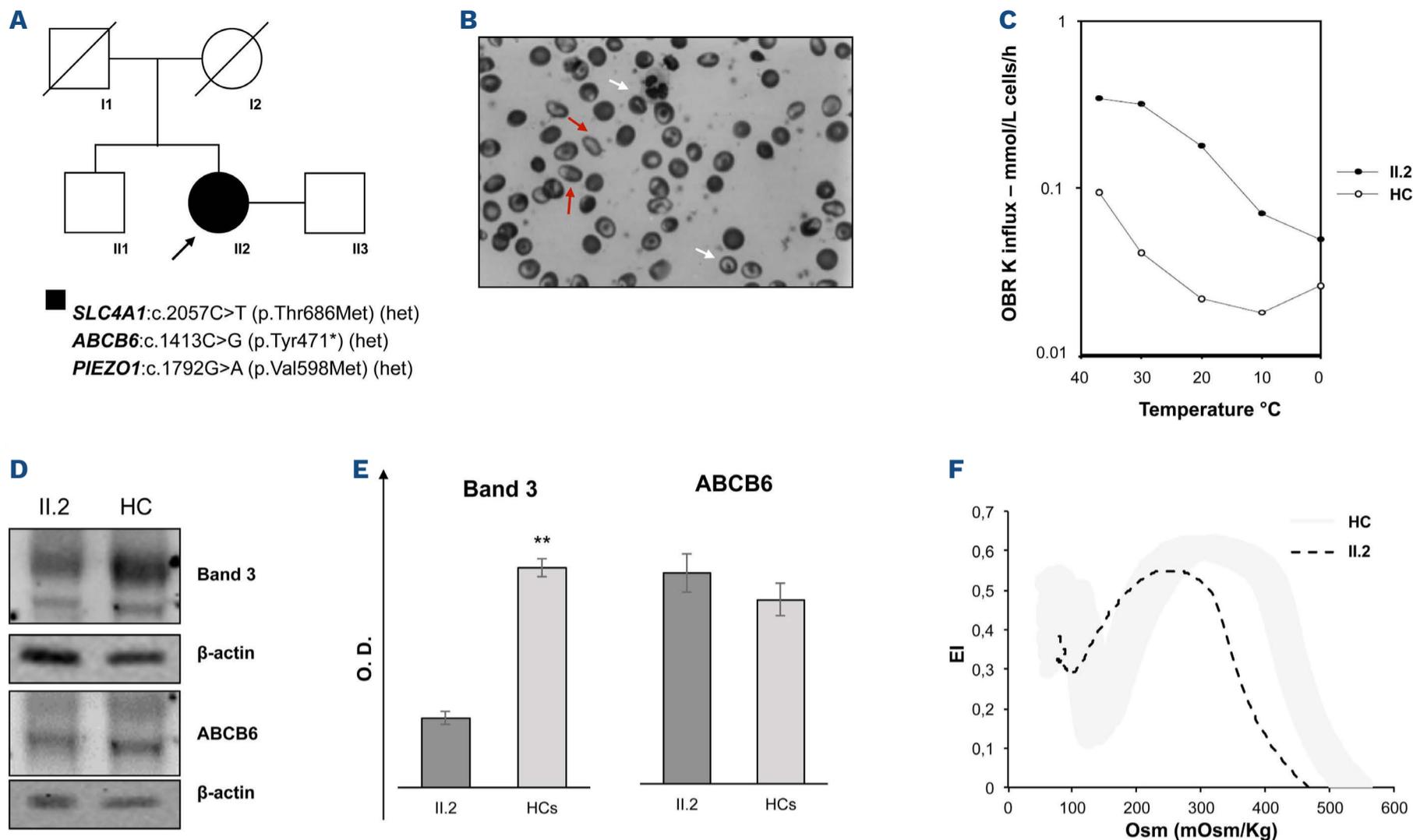


Figure 1. Genetic analysis and functional studies to assess the pathogenicity of the identified variants in the patient here analyzed. (A) Family pedigree of the proband. Squares, males; circles, females; solid symbols, affected patient; arrow indicates the proband. The proband shows three heterozygous variants in three different genes: *SLC4A1*:c.2057C>T (p.Thr686Met), *ABCB6*:c.1413C>G (p.Tyr471*), *PIEZO1*:c.1792G>A (p.Val598Met). (B) Peripheral blood smear (May-Grünwald Giemsa stain 40x) examination of the proband II.2 showing marked anisopoikilocytosis. White arrows indicate stomatocytes; red arrows indicate ovalocytes. (C) Temperature dependence of ‘leak’ K⁺ influx in red blood cells (RBC) of patient II.2 (solid symbols) and control (open symbols). (D) Representative immunoblot showing proband and healthy controls (HC) RBC membrane expression of band 3 (upper) and ABCB6 (lower), normalized to β -actin. (E) Densitometric analysis of immunoblotting shown in panel (D). Data are means \pm standard deviation (SD) of three independent experiments. (***P*-value < 0.001, Student’s *t*-test). O.D.: optical density. (F) The RBC deformability index of RBC from proband II.2 (dashed black curve) and internal healthy controls (light grey lines) was measured as a function of increasing osmolarity. Values are means \pm standard error of the mean of 2 independent experiments. EI: elongation index (EI).

performed by flame photometry confirmed the high cation leak flux at 37°C. This cation flux markedly diminished upon cooling of RBC below ~30°C (Figure 1C).¹⁰ The findings were consistent with pseudohyperkalemia.

The 38-year-old proband was referred to our unit and underwent initial genetic testing for HS using a gene-by-gene approach. Sanger sequencing analysis of the *SLC4A1* gene revealed the presence of the heterozygous missense variant NM_000342.4:c.2057C>T (p.Thr686Met) [rs143131877, AF A=0.000088 (12/135994, GnomAD); HGMD ID: CM204624]. This variant in the first nucleotide of exon 17 is predicted to impair exon 16-17 splicing (<https://hsf.genomnis.com/>). Accordingly, it was predicted to be a likely pathogenic variant per current guidelines of the American College of Medical Genetics and Genomics (ACMG). In order to determine the effect of this variant on protein expression, we isolated RBC membrane proteins from the peripheral blood of the patient. Band 3 polypeptide expression in patient RBC mem-

branes was reduced compared to that of healthy controls (HC) by western blotting, confirming the pathogenicity of the identified variant and the diagnosis of HS (Figure 1D and E).

At the age of 54 years, increasing severity of anemia and continued post-splenectomy thrombotic events prompted a re-evaluation of the diagnosis of HS. We extended the molecular analyses by applying a previously described custom gene panel for hereditary anemias.¹¹ Genomic DNA preparation, genetic testing by targeted next-generation sequencing (NGS) for hereditary anemias, and validation of the variant by Sanger sequencing were performed as previously described.^{11,12} We found a novel heterozygous nonsense variant in the *ABCB6* gene (NM_005689.4): c.1413C>G (p.Tyr471*) [rs141029409, AF C=0.00000 (0/14046, ALFA)], predicted as likely pathogenic per ACMG guidelines. We also found the heterozygous missense variant (NM_001142864.4): c.1792G>A (p.Val598Met) [HGMD ID:

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Table 1. Clinical features of the proband pre- and post-splenectomy.

	Pre-splenectomy	Post-splenectomy		Reference ranges*
		Before iron chelation treatment	After iron chelation treatment	
Blood count				
Age, years	24	25	54	-
RBC x10 ⁶ /mL	3.5	1.5	1.9	3.9-5.6
Hb, g/dL	10.5	6.5	8.8	11.0-16.0
Hct, %	29.0	18.0	25.4	33.0-45.0
MCV, fL	129.0	125.7	128.0	70.0-91.0
MCH, pg	36.0	43.0	44.4	23.0-33.0
Plt x10 ³ /mL	268.0	1,126.0	481.6	150.0-450.0
Retics count x10 ³ /μL	250.0	17.9	11.85	-
Retics, %	7.1	259.6	224.0	0.5-2.0
Hemolytic indices and iron balance				
Total bilirubin, mg/dL	1.6	1.8	1.6	0.2-1.2
Indirect bilirubin, mg/dL	1.3	1.5	1.3	-
LDH, U/L	358.0	380.0	369.0	125.0 – 243.0
Haptoglobin, g/L	0.03	0.02	0.01	0.3-2.0
Iron, g/dL	92.0	130.0	178.0	60.0-180.0
Ferritin, ng/mL	210.00	1,020.0	355.0	22.0-275.0
Transferrin, saturation %	28.0	65.0	-	<45
Hepcidin, nM	-	0.6	-	4.1-8.5

*Reference ranges from AOU Federico II, University of Naples, Italy. RBC: red blood cells; Hb: hemoglobin; Hct: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; Plt: platelets; Retics: reticulocytes; LDH: lactate dehydrogenase.

Table 2. Intracellular erythrocyte [Na⁺] and [K⁺]. Isotopic fluxes at 37°C.

	Storage time/temperature hours/°C	Intracellular [Na ⁺] (mmol/L cells)	Intracellular [K ⁺] (mmol/L cells)
Patient II.2	on arrival	31.98	71.25
Control	on arrival	21.57	86.09
Patient II.2	O.N./ 4°C	35.00	63.58
Control	O.N./ 4°C	24.29	81.01
Reference range	on arrival	5-11	85-105
K⁺ influx at 5 mM external [K⁺] (86Rb⁺ tracer)			
	NaK pump mmol/(L cells.h)	NaK²Cl cotransport mmol/(L cells.h)	Leak mmol/(L cells.h)
	ouabain-sensitive	bumetanide-sensitive	ouabain- + bumetanide-insensitive
Patient II.2	9.695	0.353	0.293
Control	2.163	0.099	0.049
Reference range	1-2	0-1	0.05-0.10

O.N.: over night.

CM1713992] of the *PIEZO1* gene previously described and functionally validated as pathogenic of DHS with hemosiderosis.¹³ We did not perform an inheritance analysis of

all the identified variants, as the proband's parents had already died (for causes not related to anemia).

We further analyzed ABCB6 protein expression in the pa-

tient's erythrocyte membrane proteins to assess if the novel identified variant might cause FP (GoF variants associated with no impairment of the protein expression) or Lan- blood group (loss-of-function variants associated with downregulation or absence of the protein expression).⁵ We demonstrated no alterations of protein expression in the patient compared to healthy controls (Figure 1D and E). Thus, the occurrence of this *ABCB6* pathogenic variant suggested an explanation for the patient's increased serum K values, and functional studies of temperature dependence confirmed the diagnosis of FP.

The presence of the *PIEZO1* variant suggested the diagnosis of DHS.¹⁴ This finding further explained the altered permeability of the RBC, the hemosiderosis, and the post-splenectomy exacerbation of phenotype. The diagnosis of DHS was further confirmed through ektacytometry analysis. Deformability of RBC of the patient and of control subjects was evaluated by osmotic gradient ektacytometry using the laser-assisted optical rotational cell analyzer (LORCA), as previously described.¹⁵ Ektacytometry revealed a left shift of the osmotic curve and a slightly decreased DiMax in the proband compared to healthy controls (Figure 1F). This peculiar curve is typical of multi-locus inheritance caused by the combined presence of HS and DHS, as recently demonstrated.¹⁵

The proband was thus diagnosed, at 54 years of age, with anemia caused by multilocus inheritance (HS, DHS, FP) of mutations in the *SLC4A1*, *PIEZO1*, and *ABCB6* genes. This paradigmatic clinical case underlines the dual importance of correct clinical assessment and genetic diagnosis to guide personalized clinical management of the patient. Earlier diagnosis of DHS would certainly have prevented the splenectomy and ensuing thrombotic complications. This case confirms the importance of NGS-based testing in the diagnostic workflow of hereditary anemias for complete differential diagnosis in both research and clinical settings.

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Contributions

IA, AI, and RR designed and conducted the study and prepared the manuscript; RR and IA performed *in silico* design of the NGS panels and interpretation of the genetic variants; BER prepared the initial draft of the manuscript; BER prepared the library enrichment, performed the Sanger sequencing analysis and western blotting analysis; AI cared for the patient; GT performed the ektacytometry analysis; SLA carried out a critical revision of the manuscript.

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Data-sharing statement

The original data and protocols are available to other investigators upon request.

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