blood® Review Article

The evolving landscape of hereditary stomatocytosis

Immacolata Andolfo,[1,2] Achille Iolascon,[1,2] and Roberta Russo[1,2]

[1]Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli Federico II, Naples, Italy; and [2]CEINGE Biotecnologie Avanzate Franco Salvatore, Naples, Italy

Hereditary stomatocytosis represents a heterogeneous group of inherited erythrocyte membrane defects characterized by hemolytic anemia of variable degree, with alterations in cellular salt and water, ranging from dehydration to overhydration, and the presence of stomatocytes on peripheral blood smear. This condition encompasses various subtypes, each with distinct clinical and genetic features. The pathophysiology underlying these conditions involves altered red blood cell membrane properties, leading to impaired deformability and alterations in cation permeability and volume, causing increased susceptibility to hemolysis. Advancements in genetic testing have enabled the identification of some causative genes in the last years, such as *PIEZO1*, *KCNN4*, and *ABCB6*. These genetic discoveries have facilitated a deeper understanding of the molecular mechanisms underlying the pathogenesis and have paved the way for improved diagnostic accuracy and genetic counseling. This review provides an overview of the clinical presentation, pathophysiology, molecular genetics, diagnosis, and management strategies of hereditary stomatocytosis, highlighting recent advancements in the field of dehydrated hereditary stomatocytosis (DHS), or hereditary xerocytosis, and hepatic iron overload. This latter is directly associated with the physiological role of *PIEZO1*, the causative gene of DHS, at hepatic and macrophagic levels. Particularly, gain-of-function mutations in *PIEZO1* account for a pleiotropic syndrome characterized by different phenotypes depending on the expression of PIEZO1 in multiple cells and tissues.

Introduction

Hereditary stomatocytosis (HSt) is a group of inherited human hemolytic anemias characterized by increased membrane permeability (leak) of red blood cells (RBCs) to sodium and potassium.[1-9] The regulation of cation permeability is essential in RBCs for preventing uncontrolled water movement, which stabilizes cellular volume. The volume homeostasis of RBCs results from the combined activity of the Na^+/K^+ ATPase pump and the sodium and potassium leaks through ion channels, maintaining low intracellular sodium and high potassium levels.[4-6] The leak through yet unidentified ion channels is extremely low in human RBCs; hence, the pump activity is also very faint. The increase in cation leak in dehydrated HSt (DHS) disrupts the balance between pump and leak activities, leading to altered erythrocyte water content and the formation of stomatocytes (abnormally shaped RBCs with slit-like areas) visible in peripheral blood (PB) smears.[7-10]

Patients with HSt may experience mild to severe hemolytic anemia, with symptoms including fatigue, jaundice, splenomegaly, and gallstones. The severity of symptoms varies among affected individuals, and most disorders are inherited in an autosomal dominant pattern. Treatment focuses on managing symptoms and complications. Folic acid supplementation may be recommended to support RBC production. In severe cases, transfusion might be considered.[1]

This review highlights the clinical presentation, pathophysiology, genetics, diagnosis, and management strategies of HSt, emphasizing recent advancements in DHS, also known as hereditary xerocytosis, and its association with hepatic iron overload.

Classification, clinical, and molecular characteristics of HSt

HSt can be classified into syndromic forms, which exhibit extrahematologic symptoms, and nonsyndromic forms, characterized by selective involvement of the erythroid system. Within the syndromic forms, we included (1) stomatin-deficient cryohydrocytosis (CHC) with neurodevelopmental disability, seizures, and hepatosplenomegaly and (2) DHS with perinatal edema with or without pseudohyperkalemia. The nonsyndromic forms comprise (1) overhydrated HSt (OHS), (2) isolated CHC, (3) DHS, and (4) familial pseudohyperkalemia (FP).[1-9] A detailed overview of the genetic, clinical, and laboratory features of these conditions is presented in Table 1.

Recent advancements have particularly focused on DHS and FP. In particular, we will discuss DHS caused by PIEZO1 mutations; DHS owing to KCNN4 mutations, also known as Gardos channelopathy; and FP caused by ABCB6 mutations. We herein propose a new classification based on the mutated gene.

Table 1. Key phenotypes associated with genes causing HSt

Gene Location	Phenotype symbol*	Phenotype(s)†	Inheritance	Clinical and laboratory features
PIEZO1 16q24.3	DHS1	DHS with or without pseudohyperkalemia and/or preperinatal edema	AD	Hemolytic anemia of varying severity, typically moderate to mild, characterized by elevated hemolytic markers. Severe hepatic iron overload is often present with hyperferritinemia, increased LIC, and TSAT. Pseudohyperkalemia, a transient increase in potassium levels at temperatures <37°C, may be present. Preperinatal edema, of the chylous type, can lead to life-threatening hydrops fetalis, which may require therapeutic drainage. This edema usually resolves spontaneously before birth or within several months postnatally and does not reoccur. In some cases, the edema is limited to the prenatal period, presenting as clinically silent ascites detectable only via ultrasound.
	—	DHS	AD	The isolated form only shows the alterations of the hematologic parameters and severe hepatic iron overload with hyperferritinemia, increased LIC, and TSAT.
	ER	[ER blood group system]	AR	Piezo1 is the carrier molecule for the Er blood group antigens: Era, Erb, Er3, Er4, and Er5. Er alloimmunization is rare, with limited clinical data. In some cases, patients experienced pregnancies complicated by hydrops fetalis and intrauterine death, along with a history of transfusion and a positive DAT. However, the evidence remains insufficient to definitively confirm or rule out the role of these antibodies in HTR or HDFN.
	—	Hereditary erythrocytosis	AD	Increased red cell mass and Hb levels; alteration of hemolytic indices; in some cases, hyperferritinemia and increased TSAT.
	—	Hyperferritinemia with or without hepatic iron overload	AD	Increased ferritin levels and TSAT without anemia, and LIC
KCNN4 19q13.31	DHS2	DHS2	AD	Anemia of variable degree, ranging from transfusion-dependent (mainly in the first year of life) to moderate/mild. Ferritin levels and TSAT can be increased.
ABCB6 2q35	FP	Pseudohyperkalemia, familial, type 2, owing to red cell leak	AD	Pseudohyperkalemia; in some cases, increased MCV and absolute reticulocyte count.
	—	DHS	AD	The same clinical phenotype as in DHS1 with mild hemolytic anemia
	LAN	[Blood group, Lan system]	AR	ABCB6 is the carrier molecule for the Lan blood group. Individuals carrying LoF variants may have anti-Lan antibodies in their serum, which can cause transfusion reactions or hemolytic disease of the fetus or newborn.
SLC4A1 17q21.31	CHC	CHC	AD	Mild hemolytic anemia is sometimes associated with pseudohyperkalemia.
	DRTA4	Distal renal tubular acidosis 4 with hemolytic anemia	AR	Mild acidosis typically begins during adolescence or adulthood. If left untreated, patients may experience metabolic acidosis, which can be associated with hypercalciuria, nephrolithiasis, nephrocalcinosis, osteomalacia, and erythrocytosis. Hypokalemia, urine pH >6.4; hypercalciuria and hypocitraturia.
	SAO	Ovalocytosis, Southeast Asian type	AD	Infants may present with anemia and elevated bilirubin levels. In older children or adults, SAO is often discovered incidentally on a blood smear, typically without any signs of anemia.
	HS4	Spherocytosis, type 4	AD	Mild hemolytic anemia or compensated hemolysis with hyperbilirubinemia, jaundice, and cholelithiasis; presence of spherocytes at PB smear.

AD, autosomal dominant; AR, autosomal recessive; DAT, direct antiglobulin test; DI, digenic inheritance; HDFN, hemolytic disease of the fetus and newborn; HTR, hemolytic transfusion reactions; LIC, liver iron concentration; MCV, mean corpuscular volume; TSAT, transferrin saturation; XLD, X-linked dominant; XLR, X-linked recessive.

*Phenotype symbols as retrieved from the OMIM database (https://www.omim.org/).

†Only hematologic phenotypes and those associated with iron metabolism were reported. Square brackets indicate a blood group.

Table 1 (continued)

Gene Location	Phenotype symbol*	Phenotype(s)†	Inheritance	Clinical and laboratory features
	DI	[Blood group, Diego]	—	The Diego blood group system currently includes 22 antigens. Antibodies to Diego system antigens, except anti-Dia, anti-Dib, anti-Wra, anti-ELO, and anti-DISK, are generally not considered clinically significant for transfusion or relevant in hemolytic disease of the fetus and newborn.
	FROESE	[Blood group, Froese]		Low-incidence blood antigen group
	SW	[Blood group, Swann]		Low-incidence blood antigen group
	WD	[Blood group, Waldner]		Low-incidence blood antigen group
	WR	[Blood group, Wright]		Low-incidence blood antigen group
SLC2A1 1p34.2	SDCHCN	Stomatin-deficient CHC with neurologic defects (*608885*)	AD	Neurodevelopmental disability, seizures, hepatosplenomegaly, and CHC. CHC with cation leak <20°C, leading to cold-induced swelling and hemolysis.
RHAG 6p12.3	OHS	OHS	AD	Hemolytic anemia characterized by anemia of a variable degree with macrocytosis
	RHNR	Anemia, hemolytic, Rh null, regulator type	AR	A rare hemolytic anemia characterized by a lack or severe reduction of Rh blood group antigens and increased erythrocyte osmotic fragility, with stomatocytosis and spherocytosis at PB smear.

AD, autosomal dominant; AR, autosomal recessive; DAT, direct antiglobulin test; DI, digenic inheritance; HDFN, hemolytic disease of the fetus and newborn; HTR, hemolytic transfusion reactions; LIC, liver iron concentration; MCV, mean corpuscular volume; TSAT, transferrin saturation; XLD, X-linked dominant; XLR, X-linked recessive.

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PIEZO1-related disorders

Recent years have seen the identification of the eukaryotic mechanical channels PIEZO1 and PIEZO2.[11] In 2021, David Julius and Ardem Patapoutian were awarded the Nobel Prize in Physiology or Medicine for their discovery of receptors for temperature and touch, including the ion channels PIEZO1 and PIEZO2, first reported by Patapoutian et al in 2010.[12]

PIEZO proteins are pore-forming subunits of ion channels that open in response to mechanical stimuli, allowing positively charged ions, including calcium and sodium, to flow into the cell.[13-17] Although the 2 mammalian isoforms are abundantly expressed in a wide range of mechanically sensitive cells, PIEZO1 is primarily expressed in nonsensory tissues exposed to fluid pressure and flow (eg, kidneys, erythrocytes, liver, and heart). It forms a trimeric propeller-like structure of ~900 kDa in the plasma membrane of cells[14-17] and has roles in a broad and varied set of mechanotransduction processes: lymphatic valve and heart valve development, angiogenesis and stem cell differentiation, bone formation, cell migration, axon regeneration, inflammatory response of innate immune cells, and RBC deformability.[18-24] Owing to its widespread expression across different tissues, variants in *PIEZO1* gene may have pleiotropic effects (Figure 1).

PIEZO1 LoF

In humans, loss-of-function (LoF) pathogenic variants in PIEZO1 lead to autosomal recessive generalized lymphatic dysplasia with nonimmune fetal hydrops, characterized by widespread lymphedema and a fully compensated hemolytic state (Figure 1).[25,26] A case with biallelic LoF mutations in PIEZO1 and lymphatic dysplasia showed similarities to hereditary spherocytosis (HS), including spherocytes at PB smear and normal mean corpuscular volume (MCV), and features of OHS, such as stomatocytes, decreased mean corpuscular hemoglobin concentration (MCHC), rightward shift of the osmolarity curve, and decreased intracellular potassium.[27]

PIEZO1 animal models shared some characteristics with the human disease. Piezo1-deficient mice die in utero owing to defective vasculogenesis,[28] prompting the development of Vav1-P1cKO mice with a specific deletion in the hematopoietic system. These mice showed elevated MCV and MCH, reduced MCHC, and increased osmotic fragility, indicating overhydrated RBCs.[28] Zebrafish models have also been created with morpholino-knockdown of Piezo1 expression in *Danio rerio* resulting in severe anemia.[29,30] However, this phenotype was absent in another zebrafish model carrying a predicted truncated Piezo1 form.[31,32] The differences in phenotypes between these models remain a topic of debate.

PIEZO1 GoF

Gain-of-function (GoF) mutations in PIEZO1 cause DHS1 or hereditary xerocytosis, inherited as an autosomal dominant condition (Figure 1; Table 1). Of note, autosomal dominant mutations are also associated with a predisposition to colorectal adenomatous polyposis syndrome,[23] but this aspect will not be discussed over in this review.

DHS prevalence is not yet well established; it is conceivable that several cases are undiagnosed or misdiagnosed.[3] Recent studies estimated the prevalence of DHS1 to be ~1 in 7000 of 8000 adults.[33,34] The widespread PIEZO1 GoF mutations are also linked to malaria resistance and worsening of the anemia in

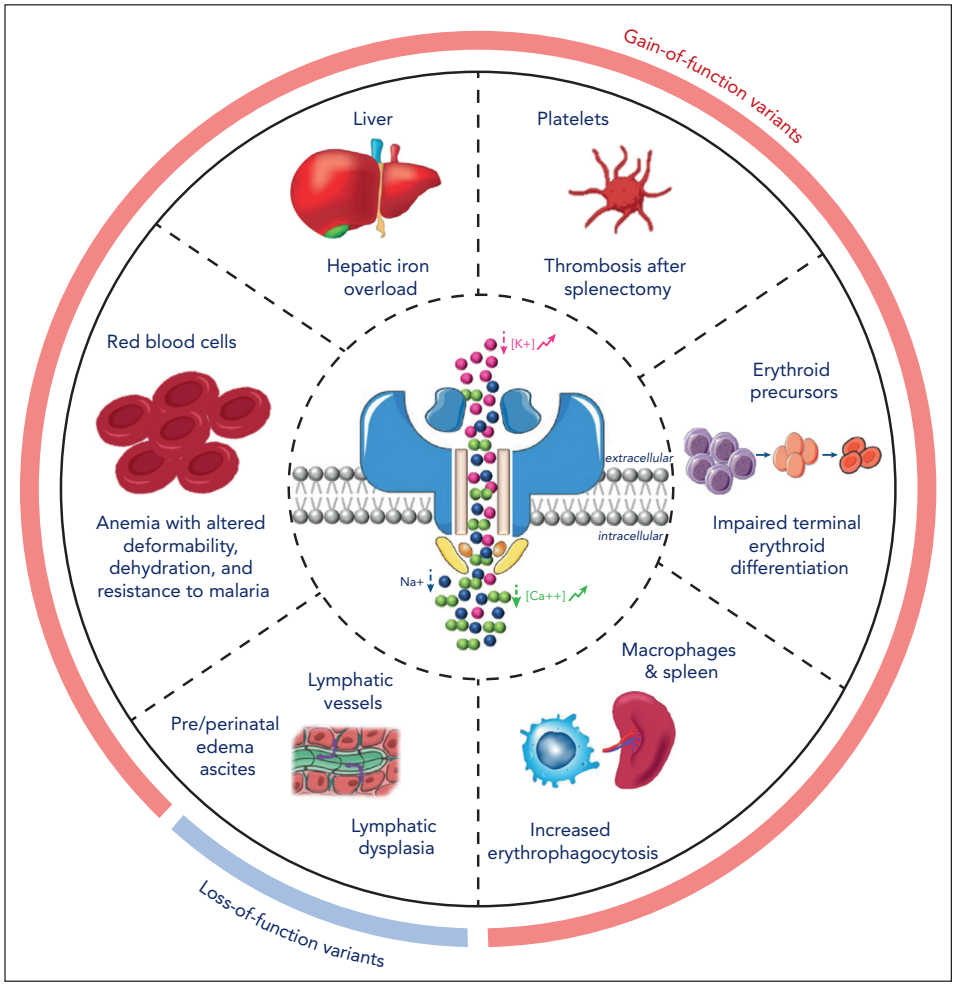

Figure 1. PIEZO1-related phenotypes. PIEZO1 GoF variants (red circle), by delaying the inactivation of the channel, cause DHS, a pleiotropic syndrome characterized by several phenotypes: anemia with dehydration conferring malaria resistance for its expression at the plasma membrane of RBCs, liver iron overload for its expression at hepatic level, thrombosis after splenectomy for its hypothesizing role in the platelets, alterations of erythroid differentiation for its expression in the erythroid precursors during erythroid differentiation, increased erythrophagocytosis accounting for iron overload for its expression in the macrophages, and pre-/perinatal edema and ascites for its expression in the lymphatic vessel. All these phenotypes are mutation dependent. PIEZO1 LoF variants (light blue circle) cause lymphatic dysplasia. The dashed arrows indicate the direction of ion movement, whereas the solid arrows represent changes in concentration.

sickle cell disease.[35-37] Remarkably, the PIEZO1 GoF allele E756del is present in a third of the African population. Erythrocytes from individuals carrying this allele are dehydrated and display reduced *Plasmodium* infection in vitro, and heterozygous subjects for this polymorphism exhibit mild to moderate hemolytic anemia and hepatic iron overload.[35,38] Only 1 genetic association study in children from Ghana, West Africa, showed no association of this variant with protection against severe malaria.[39]

Clinical presentation and pathogenetic mechanism DHS/xerocytosis can manifest as isolated hemolytic anemia or as pleiotropic syndrome characterized by hemolytic anemia owing to altered erythrocyte membrane permeability to cations, leading to erythrocyte dehydration. This condition may also present with preperinatal edema, pseudohyperkalemia, hepatic iron overload, and other phenotypes (Figure 1).[1-4,40,41] Typically, the anemia is mild, with positive hemolytic markers, such as elevated indirect bilirubin and lactate dehydrogenase levels, alongside decreased haptoglobin. The MCV and reticulocyte count are highly variable, ranging from macrocytosis to normal MCV and from an elevated reticulocyte count to reticulocytopenia. PB smears often reveal a variable number of stomatocytes, usually <20%, with some cases showing an absence of stomatocytes.

Hemolysis can lead to splenomegaly and gallstones. However, splenectomy is contraindicated owing to the increased risk of severe thromboembolic complications. The pathophysiology behind arterial and venous thrombotic events remains unclear but is likely multifactorial, involving increased platelet aggregation, nitric oxide consumption, thrombophilic circulating microparticles, phosphatidylserine exposure, and increased adherence of erythrocytes to endothelial monolayers in a micropipette assay.[42-44] Recent studies have linked the activation of PIEZO1 to an increase in immature megakaryocytes in mice, with Piezo1 knockout mice exhibiting larger megakaryocytes and higher platelet counts.[45] Proteomics analysis of

patients with PIEZO1 mutations revealed alterations in vesicle-mediated transport, which may explain the elevated thrombotic risk in these individuals.[46]

Concerning the pathophysiology of PIEZO1 GoF variants, changes in energy metabolism and glycolysis have been observed, leading to increased hemoglobin (Hb) oxygen affinity in mature RBCs from patients with DHS1. Accordingly, some patients with PIEZO1 mutations showed unexpected erythrocytosis.[41,47-49] Moreover, proteomic studies on mature RBCs from these patients revealed impaired biological processes, particularly in ion homeostasis, transmembrane transport, regulation of vesicle-mediated transport, and the proteasomal catabolic process.[46,50]

The photogenetic mechanism underlying this condition has been examined using a mouse model of DHS, which exhibits certain similarities to its human counterpart.[35] Heterozygous Piezo1 GoF mice display mild anemia with slight increases in MCV and MCHC, whereas homozygous mice show moderate anemia with elevated MCV and unchanged MCHC levels.[35] Notably, homozygous mutations have been documented in humans, especially in regions with a high consanguinity prevalence.[3,41,46] Recent research emphasized the role of PIEZO1 not only in mature erythrocytes but also during erythropoiesis, where its activation disrupts the maturation of reticulocytes, potentially leading to reticulocytopenia in certain patients with specific mutations.[51,52]

The role of PIEZO1 GoF in iron metabolism Two large cohorts of patients with HSt (126 and 123, respectively) have been described until now demonstrating the high variability in the clinical expression among PIEZO1-related cases.[3,41] Notably, these studies suggested that hepatic iron overload is independent of the degree of anemia and transfusion regimen, with several cases of hepatosiderosis. Most patients with a severe phenotype, particularly those with impaired iron balance, carry mutations mainly in the pore domain, whereas patients with a milder phenotype tend to have variants in the nonpore domain of the channel.[3] In agreement with these findings, a specific role of mutated PIEZO1 at the hepatic level has been hypothesized (Figure 2). Hepatic cellular models expressing PIEZO1 GoF mutants, such as R2456H and R2488Q, show decreased *HAMP* gene (encoding hepcidin) expression compared with wild-type (WT) and increased intracellular calcium concentration. This latter causes alterations of several intracellular pathways such as BMP/SMAD and ERK1/2.[40] Recently, the pathway of RAS has also been involved in the intricate intracellular signaling that starts from PIEZO1 activation and leads to hepcidin suppression. Hep3B cells engineered for the PIEZO1-R2456H variant revealed alterations in the actin cytoskeleton regulation, MAPK cascade, and RAS signaling, primarily through the novel key regulator RRAS, whose protein and messenger RNA (mRNA) levels are regulated by PIEZO1 activation and inhibition. This was further validated in C57BL/6 mouse primary hepatocytes treated with Yoda-1 (PIEZO1 activator) and/or GsMTx-4 (PIEZO1 inhibitor). Indeed, PIEZO1-KI cells exhibited hyperactivated RAS-GTPase activity, which was rescued by PIEZO1 inhibition, restoring *HAMP* gene expression. A negative correlation between RAS signaling and *HAMP* regulation was confirmed by inhibiting RAS-GTPase and MEK1-2 activity. Thus, PIEZO1-GoF variants affect actin cytoskeleton

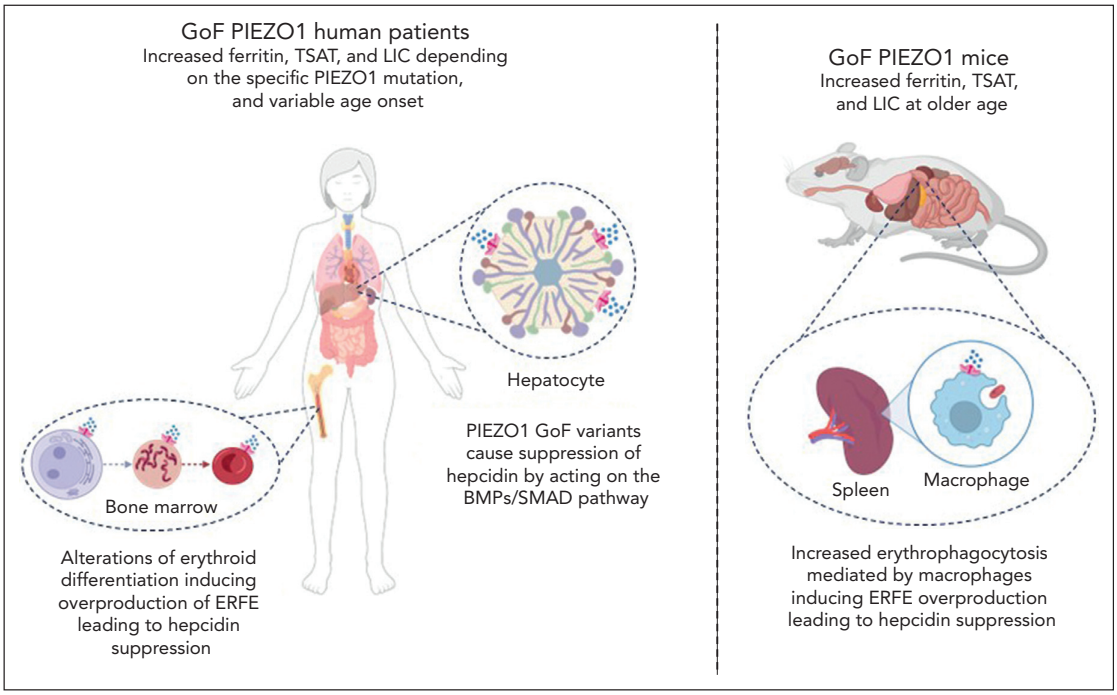

Figure 2. Pathogenetic mechanisms of iron overload in patients with DHS. Schematic representation of the pathogenetic mechanisms of iron overload in DHS involving the role of GoF mutations in PIEZO1 in (1) the regulation of iron metabolism at hepatic levels by interfering with BMP/SMAD pathway and hepcidin expression, (2) the alterations of late stage of erythroid differentiation accounting for overproduction of ERFE and hemolysis of RBCs, and (3) increased erythrophagocytosis mediated by the expression of PIEZO1 in the macrophages. LIC, liver iron concentration.

organization by activating the hepatic RAS signaling system in the hepatic cells.[53] Notably, the constitutive GoF Piezo1 mice, over 1 year, develop iron overload, as evidenced by increased liver iron concentration, serum iron, transferrin saturation (TSAT), and ferritin levels compared with the WT ones.[54] Moreover, iron deposition was more severe in homozygous GoF Piezo1 mice than in heterozygous ones in both hepatocytes and Kupffer cells. Owing to the iron accumulation in the Kupffer cells (liver resident macrophages), macrophage-specific GoF Piezo1 mice were generated. The mouse model showed dramatically reduced hepcidin mRNA and serum levels in aging mice compared with WT ones. Erythroferrone (ERFE), the only known negative erythroid regulator of hepcidin, showed significantly increased levels in adult mice compared with WT (Figure 2).[54] Moreover, the in vivo erythrocyte turnover analysis indicates that macrophages with overactive PIEZO1 recycle more RBCs over a given time. Therefore, macrophages with overactive PIEZO1 enhance erythropoiesis and increase ERFE to reduce hepcidin expression (Figure 2). Thus, PIEZO1 is a key regulator of macrophage phagocytic activity and subsequent erythrocyte turnover. These studies indicate that macrophage is a key agent of PIEZO1 function in iron metabolism but cannot explain the phenotype of patients with iron overload, low levels of hepcidin, and normal levels of ERFE.[40] Therefore, additional cell types, such as hepatocytes, can also be involved in the regulation of iron metabolism in patients with PIEZO1 mutations. Indeed, mechanotransduction is a mechanism by which cells convert mechanical stimuli into electrochemical activity. The liver is located in a complicated mechanical microenvironment that is crucial for maintaining physiological homeostasis.[55] Liver resident cells, especially hepatocytes, liver sinusoidal endothelial cells, and hepatic stellate cells, are all sensitive to mechanical forces and able to alter their behaviors and functions through mechanotransduction pathways. Piezo1 is highly expressed in the different cell types of the liver in both humans and mice. The studies on the role of PIEZO1 in the different liver cell types will add other pieces to the complicated puzzle of PIEZO1 and iron metabolism.

Additional data on the role of PIEZO1 in iron metabolism derived from the population of patients carrying the GoF PIEZO1 allele E756del.[38,54] E756del heterozygous individuals older than 40 years had a statistically significant increase in TSAT and ferritin concentrations compared with noncarriers within the same age group.[54] Therefore, PIEZO1 is a genetic risk factor associated with alterations in iron metabolism.

PIEZO1 and Er red cell antigens PIEZO1 is also the carrier molecule for Er red cell antigens identified nearly 40 years ago, establishing a new blood group system. Validation of PIEZO1 as the carrier molecule for the Er blood group antigens was demonstrated using immunoprecipitation, CRISPR/Cas9-mediated gene knockout, and expression studies in an erythroblast cell line. Er alloimmunization is rare. In some cases, patients experienced pregnancies complicated by hydrops fetalis and intrauterine death, along with a history of transfusion and a positive direct antiglobulin test (Table 1).[56]

ABCB6-related disorders

Adenosine triphosphate–binding cassette (ABC) subfamily B member 6 (ABCB6) is part of the ABC transporter family, one of the most abundant families of integral membrane proteins.[57] These transporters use adenosine triphosphate binding and hydrolysis to move endogenous and xenobiotic substrates across cellular membranes.[58]

In 2006, ABCB6 was shown to facilitate mitochondrial uptake of coproporphyrin III, an important step in cellular porphyrin biosynthesis.[58,59] However, later studies indicated that ABCB6 is localized in the endoplasmic reticulum and Golgi membranes, not mitochondria.[60-62] This extramitochondrial localization was further supported by studies on dominantly inherited ABCB6 mutations in patients with ocular coloboma and dyschromatosis universalis hereditaria.[63,64] Gene expression profiling identified *ABCB6* among erythroid cluster genes in zebrafish blood formation mutants.[65]

Clinical presentation and pathogenetic mechanism

Mutations in *ABCB6* gene are associated with FP, a dominant red cell trait (Figure 3).[1,66] Erythrocytes from patients with FP exhibit potassium loss at low temperatures (<37°C, mostly 8-10°C), but not at 37°C. Stomatocytes are rarely observed on PB smears. Most of the *ABCB6* variants found in patients with FP are missense that do not affect mRNA or protein levels or subcellular localization in mature erythrocytes or erythroid precursors.[66] ABCB6 is upregulated during erythroid differentiation and localized at the plasma membrane in mature RBCs and $CD34^+$ cells. In vitro functional characterization of ABCB6 mutants demonstrated that mutations lead to increased cation flux compared with the WT protein.[67] Biallelic ABCB6 mutations have been described. The prevalence of FP may be underestimated, given that several mutations are present in the general population. Of note, screening of ABCB6 mutations in UK and Italian blood donors found a recurrent FP variant R276W.[67,68] This finding has significant implications for transfusion medicine, given that storage of blood from ABCB6-mutated RBCs leads to significant potassium loss, posing serious risks for neonates and infants receiving large volumes of transfused whole hyperkalemic blood, particularly in regions where whole blood is used in transfusion therapy.[67,68] Additional cases were described suggesting that, if a blood donor has FP,[69] reducing the RBCs concentration shelf life to 5 days may be insufficient to reduce the risk of hyperkalemia in neonatal large volume transfusion.[70]

Interestingly, ABCB6 variants can also be associated with a conclamant DHS1 (Figure 3). In particular, this overlapping phenotype has been described in an Irish family carrying the R276W variant with an autosomal dominant DHS with FP. In particular, the propositus showed an increased passive K^+ leak and experienced several thrombotic episodes following splenectomy.[66]

ABCB6 and Lan group

ABCB6 is also known to carry the Lan (Langereis) blood group antigen system.[71] Individuals lacking the Lan antigen (Lan null group) are asymptomatic but carry various recessive null mutations and can experience hemolytic transfusion reactions and hemolytic disease of the newborn if there is a blood group mismatch (Figure 3). In addition, ABCB6 is a host factor for *Plasmodium falciparum* during erythrocyte invasion, Lan null erythrocytes being highly resistant to invasion by *Plasmodium*.[72]

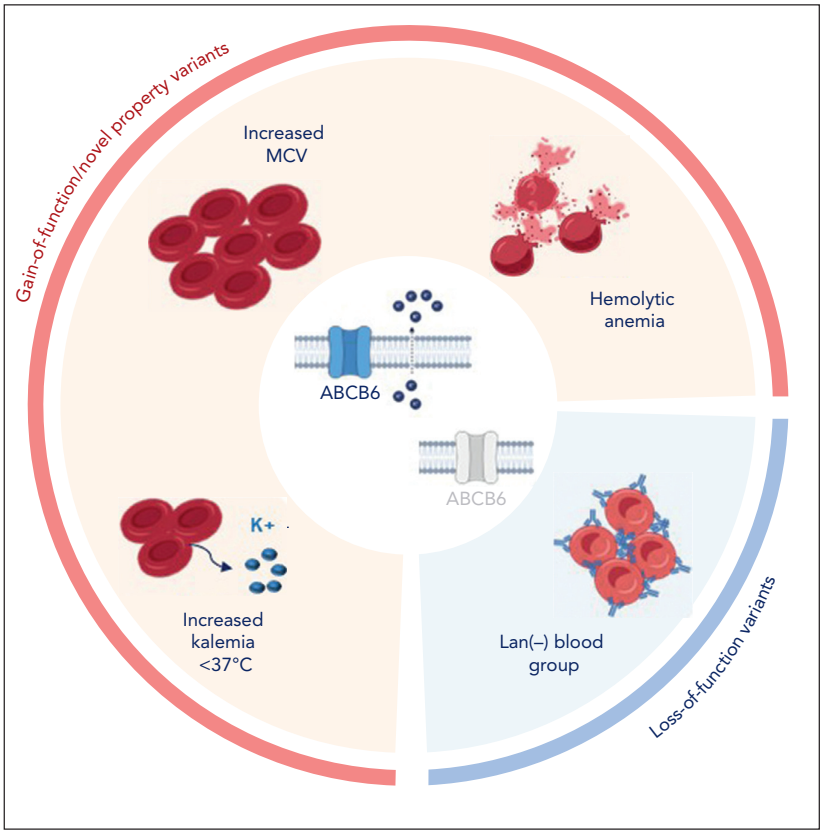

Figure 3. ABCB6-related phenotypes. GoF mutations in ABCB6 cause FP with increased MCV, increased kalemia on storage of blood at temperatures <37°C, and hemolytic anemia similar to DHS in some cases. LoF mutations in ABCB6 cause Lan$^-$ blood group.

KCNN4 disease or Gardos channelopathy

The *KCNN4* gene encodes the Gardos channel, a calcium-dependent potassium channel of intermediate conductance that plays a crucial role in mediating the primary potassium conductance in RBCs. The potassium channel domain (amino acids 231-289) and the calmodulin-binding domain (amino acids 304-377) of KCNN4 are highly conserved, emphasizing their functional importance.[9,73-75]

Clinical presentation and pathogenetic mechanism

Despite KCNN4 being implicated in various conditions, such as inflammatory bowel disease, Crohn disease, and Alzheimer disease, its germ line pathogenic variants predominantly associate with DHS2 (Table 1).[76-86] To date, 10 KCNN4 variants have been identified in 13 unrelated families, with the R352H missense variant being particularly recurrent (53.8% of described families).[73-87] KCNN4 variants lead to increased channel activity owing to altered open probability, trafficking, and unitary conductance.[73-84] This pathological mechanism suggests potential functional overlap with mutations in PIEZO1, implicating both in a shared cation pathway and in volume alterations. It has been suggested that chronic dehydration and ionic imbalance in RBCs, driven by either KCNN4 or PIEZO1 mutations, may lead to functional inactivation of the Gardos channel in patients with DHS2.[73] Of note, the GoF mutations in KCCN4 are not associated with RBC dehydration. Standard hematologic tests often fail to diagnose DHS2 in these cases, raising questions about the classification of DHS2 as a true dehydrated stomatocytosis disorder. Gardos channelopathy, as suggested for KCNN4 variant carriers, represents a distinct condition within the DHS spectrum.

Indeed, although DHS1 and DHS2 share overlapping features, important distinctions exist (Table 1). PIEZO1-related patients may present with pseudohyperkalemia and perinatal edema, whereas these symptoms are absent in DHS2. Moreover, DHS1 is more frequently associated with severe thrombosis after splenectomy. Ektacytometry in DHS1 generally shows a left-shifted curve, whereas Gardos channelopathy demonstrates more variability (Figure 4), with some mutations, such as V282M, showing a left shift and others, such as R352H, exhibiting normal curves. KCNN4-related cases tend to be more severe, showing lower Hb levels and, in some cases, mild reticulocytosis. Notably, no syndromic forms of DHS2 have been documented.[3]

Iron metabolism in KCNN4-mutated patients also varies, presenting a milder iron overload than PIEZO1-related cases, although this difference is still debated. Indeed, the significant variability and rarity of KCNN4 cases hinder comprehensive genotype-phenotype correlations, leaving many questions unresolved.

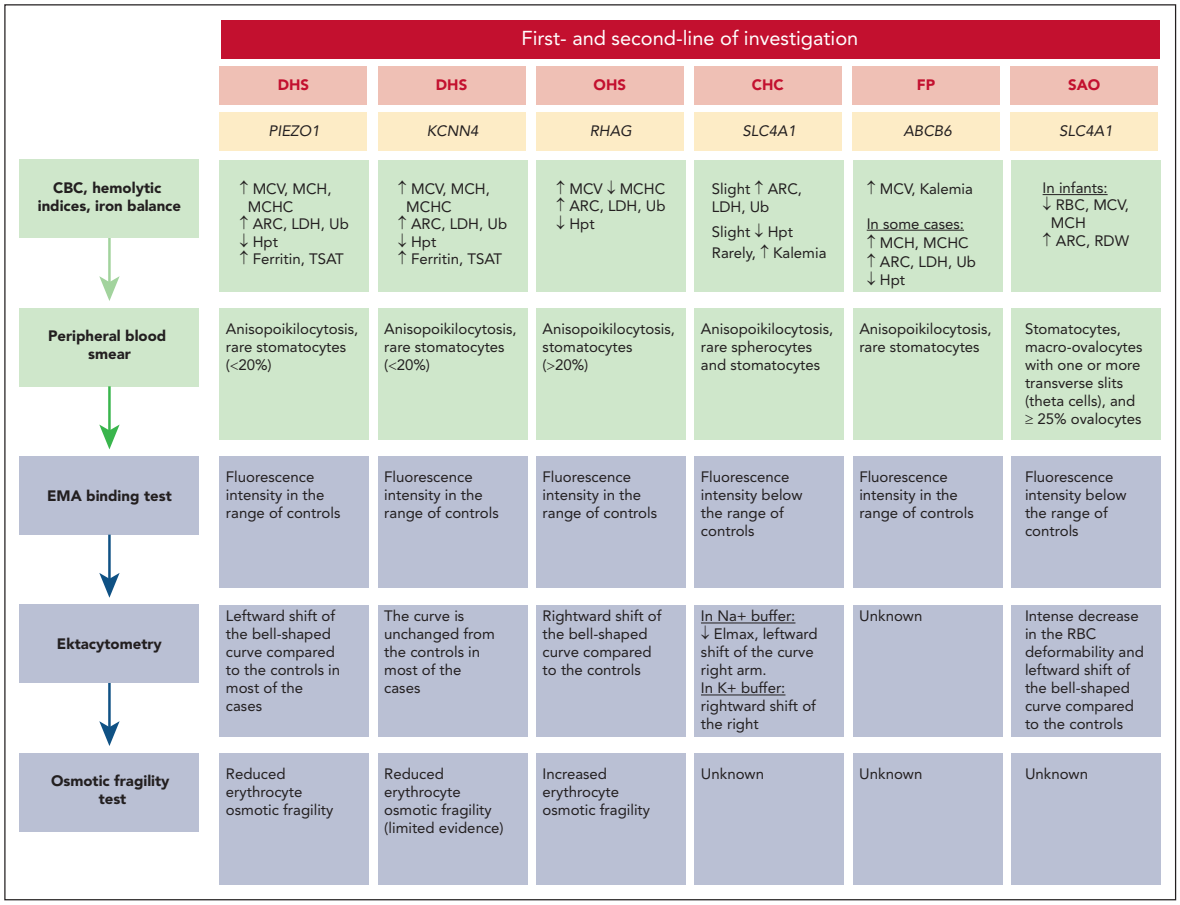

Figure 4. Diagnostic workflow of HSt. Schematic representation of the first and second lines of investigations for different subtypes of HSt, categorized according to mutated genes. ARC, absolute reticulocyte count; CBC, complete blood count; Elmax, maximum erythrocyte deformability; Hpt, haptoglobin; LDH, lactate dehydrogenase; RDW, red cell distribution width; SAO, Southeast Asian ovalocytosis; Ub, unconjugated bilirubin.

Diagnostic workflow and differential diagnosis

Although the diagnostic process for HSt is well established, it remains challenging owing to significant phenotypic variability. Diagnosis relies on 3 lines of investigation. The first includes evaluation of complete blood count, hemolytic indices, iron balance, PB smear analysis, and family history. Pseudohyperkalemia can be an indicator in diagnosing FP and DHS1 (Figure 4). Many patients are diagnosed in early adulthood, often without anemia, but may experience complications such as gallstones, iron overload, and splenomegaly.[2] Vaso-occlusion and thrombosis appear to be related to interactions between stomatocytes and the vasculature, particularly after splenectomy. The PB smear may show stomatocytes, but the simple morphological analysis poses challenges, given that stomatocytes are predominantly found in OHS (>20%) and less in dehydrated forms (<20%). Notably, many patients do not show stomatocytes at PB smear.[1] In addition, spherocytes and ovalocytes can indicate other conditions, such as South Asian ovalocytosis (Figure 4). Phytosterolemia, characterized by non-leaky stomatocytosis and macrothrombocytopenia, should be considered in the differential diagnosis with DHS.[1,2] This is a lipid metabolic disorder associated with stomatocytosis, splenomegaly, macrothrombocytopenia, and abnormal bleeding, with elevated plant sterol levels and accelerated atherosclerosis. Severe hypercholesterolemia has also been reported. It has autosomal recessive or digenic inheritance linked to *ABCG5* and *ABCG8* gene variants. Patients exhibit normal erythrocyte cation content and osmotic fragility, differentiating it from DHS.[1,2]

The second line of investigation includes biochemical tests that assess osmotic resistance through assays such as osmotic fragility test, AGLT50, Pink test, and eosin-5′-maleimide (EMA) binding test. The EMA test, in which EMA binds to RBC membrane proteins, mainly to band 3 protein, typically shows normal results in all patients with HSt (Figure 4), unlike in patients with HS. Osmotic gradient ektacytometry is another valuable tool for diagnosing RBC membrane defects (Figure 4). It is a viscometer that evaluates the deformability and hydration status of RBCs at different osmolarities. Ektacytometry shows a leftward shift of the minimum deformability index at low osmolarities (indicating dehydration) and a decrease in the maximum deformability index (indicating low deformability) in most of the DHS caused by PIEZO1 mutations and normal for most of KCNN4 mutations (Figure 4).[88] The osmotic fragility test assesses the stability of erythrocytes in saline solutions of

decreasing tonicity. This test can aid in the differential diagnosis of DHS and HS, given that RBCs in DHS typically show reduced osmotic fragility, whereas in HS the osmotic fragility is increased, such as OHS. Data on osmotic fragility findings in Southeast Asian ovalocytosis, CHC, and FP are currently limited.[89] The third line of investigation focuses on molecular analysis. Identifying genetic defects is crucial for accurate diagnosis, genetic counseling, and treatment. This is fundamental in cases of nonspecific symptomatic treatments for hemolytic anemias, such as splenectomy, which could be ineffective or even harmful in patients carrying PIEZO1 mutations.[90] Delayed diagnosis can lead to complications such as multiorgan damage from iron overload, thrombosis, or chronic transfusion regimens.[3-6] The genetic diagnosis of DHS is complicated by the high allelic heterogeneity of PIEZO1, which has a high mutation frequency and low genic intolerance, resulting in numerous variants being classified as variants of uncertain significance or likely benign, based on current genetic data analysis pipelines. Consequently, this poses challenges in the molecular diagnosis of affected patients, given that standard American College of Medical Genetics and Genomics rules often fail to accurately classify PIEZO1 variants. Notably, a reevaluation of well-established PIEZO1 pathogenic variants using American College of Medical Genetics and Genomics rules revealed that a considerable proportion were misclassified as variants of uncertain significance.[38,91] Additional criteria, such as ektacytometry results and variant segregation in families, can aid in classifying variants as pathogenic.

The lack of clear genotype-phenotype correlations complicates genetic counseling and gene-specific patient management or treatment. Genetic testing has evolved from single-gene analysis to broader gene panels using next-generation sequencing, achieving a diagnostic yield exceeding 70%.[91]

Next-generation sequencing data reveal that DHS is often misdiagnosed as HS owing to similar clinical and biochemical features, or as congenital dyserythropoietic anemias (CDAs), particularly in patients with specific PIEZO1 mutations who exhibit reduced reticulocyte counts. The bone marrow responsiveness index can help distinguish DHS from CDA and HS, showing intermediate values in patients with DHS compared with CDA, which is characterized by ineffective erythropoiesis, and HS, which is marked by well-compensated hemolytic anemia.[3,92] This aligns with the observed delay in erythroid differentiation of progenitor cells from PIEZO1 patients and the alterations in reticulocyte deformability and vesicle content, indicating a maturation defect in DHS.[51,52]

In addition, PIEZO1-related conditions may be misdiagnosed as myelodysplastic syndrome (MDS) owing to a history of iron overload and hypercellular bone marrow findings.[93] Genetic analysis of a cohort with early-onset MDS revealed that 24% carried germ line pathogenic variants in PIEZO1.[94] When MDS is suspected, it is essential to explore inherited RBC defects, especially if MDS presents unusually early. Anemia with unilineage bone marrow erythroid dysplasia can mimic MDS, leading to potential misdiagnoses.

Finally, DHS, often associated with hepatic iron overload, can be misdiagnosed as hereditary hemochromatosis, particularly in cases of iron overload and compensated hemolytic anemia.

Multilocus inheritance

Similar to other erythrocyte membrane defects, the significant phenotypic variability in HSt is influenced by high genetic heterogeneity. Many Mendelian disorders arise from combinations of multiple disease-causing alleles or their interaction with polymorphic variants. In a study of 155 patients suspected of hereditary erythrocyte defects, monogenic inheritance was identified in 69% of cases, whereas 15% exhibited multilocus inheritance.[91] Notably, 70% of patients with multilocus inheritance exhibited a dual molecular diagnosis of DHS and HS, primarily involving variants in *PIEZO1* and *SPTA1*. These cases were clinically indistinguishable from isolated HS, although ektacytometry curves showed some distinct alterations.[91]

Potential coinheritance of β-thalassemia trait (BT) with RBC membrane defects should also be considered, as evidenced by a case series where 75% of symptomatic BT cases were also affected by DHS.[95] Notably, genetic analysis of this cohort revealed a more severe phenotype in patients with both BT and DHS, including lower erythrocyte count, reduced Hb levels, increased splenomegaly, disrupted iron balance, and increased hemolytic indices. Similarly, the coinheritance of variants in *HFE* or other iron overload genes may account for a more severe iron overload.[96] The presence of complex genotypes complicates genotype-phenotype correlations, making clinical interpretation challenging. Although the interpretation of dual inheritance or genetic modifiers is highly challenging, clinicians should assess risk alleles to better define the phenotype, prognosis, and treatment for these patients.

Management and therapy

Limited data are available on the management of HSt, which is individualized based on the severity of hemolytic anemia. Most patients experience mild anemia, typically requiring routine supplements such as folic acid. Occasionally, transfusions may be necessary for intermittent hemolysis or transient aplastic crises, often triggered by parvovirus B19 infection. Newborns may need phototherapy owing to immature glucuronyl transferase activity. Despite low transfusion rates, HSt can lead to hemosiderosis, a major complication.[3,40,41,97] Key indicators of iron overload include ferritin levels, TSAT, and liver iron concentration, with T2* magnetic resonance imaging recommended for its high sensitivity. Iron chelation therapy, using drugs such as deferoxamine, deferasirox, and deferiprone, is crucial.

After the diagnosis of HSt, follow-up evaluations are suggested every 6 to 12 months, depending on the patient's age and severity of the phenotype. These evaluations should include a complete blood count, hemolytic indices, iron balance assessment, and abdominal ultrasound to monitor for cholelithiasis and splenomegaly. Splenectomy is generally contraindicated in DHS and OHS owing to the increased thromboembolic risk, including portal thrombosis, and limited effectiveness,[90] although it has been performed in individuals with severe hemolysis. However, evidence of therapeutic benefit is limited, and the procedure has risks, including surgical complications, infection with encapsulated organisms, and thromboembolism. Thus, splenectomy is indicated only as a last resort for patients who are severely symptomatic from splenomegaly.[90]

Bone marrow transplantation could be a potential treatment, even if data on its risks are limited. Perinatal edema in patients with syndromic DHS should be evaluated during pregnancy and at birth. Although this feature often regresses spontaneously, drainage may be necessary to prevent or treat pulmonary hypoplasia.[98] Pseudohyperkalemia in syndromic DHS does not require treatment, because it is artifactual and observable only at low temperature.[66]

Future therapies are being developed, including mitapivat, a pyruvate kinase activator that has shown efficacy in increasing Hb levels and reducing hemolysis in patients with pyruvate kinase deficiency, thalassemia, and sickle cell disease, as well as in a mouse model of HS. In the phase 2 clinical trial Satisfy, initial improvements in Hb levels and hemolytic markers were observed in 4 treated patients with DHS.[99]

Gene therapy and genome editing are also promising avenues for future treatments, using techniques such as CRISPR/Cas9 to modify human stem progenitor cells $CD34^+$ or reprogram fibroblasts into induced pluripotent stem cells for erythroid lineage differentiation.

Conclusions

HSt includes various inherited disorders affecting erythrocyte membranes, marked by clinical and genetic diversity. Diagnostic challenges arise from similarities with other RBC membrane disorders and dyserythropoietic anemias. Molecular diagnosis is particularly difficult owing to variant classification issues, especially with the *PIEZO1* gene. Treatment options are limited, given that splenectomy is contraindicated in patients with PIEZO1 mutations owing to thrombosis risk. Iron overload is a common severe complication, prompting advancements in diagnostic algorithms to emphasize genetic testing.

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ORCID profiles: I.A., 0000-0003-0493-812X; A.I., 0000-0002-9558-0356; R.R., 0000-0002-3624-7721.

Correspondence: Achille Iolascon, Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli Federico II, CEINGE Biotecnologie Avanzate Franco Salvatore, Via G. Salvatore, 486, 80145 Naples, Italy; email: achille.iolascon@unina.it.

Footnote

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