The Hereditary Stomatocytoses: Genetic Disorders of the Red Cell Membrane Permeability to Monovalent Cations

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The hereditary stomatocytoses are mostly accounted for by genetic disorders of red cell membrane permeability to monovalent cations. These conditions, all very rare, are comprised of a hemolytic anemia, frequently macrocytosis, and the presence of abnormally shaped red blood cells. The key test for diagnosis is osmotic gradient ektacytometry, which measures the osmotic resistance and hydration of the red blood cell; the curve depicting the temperature dependence of the cation leak is also important. Syndromes include familial pseudohyperkalemia (FP), which is devoid of hematological features, dehydrated hereditary stomatocytosis (DHS), and overhydrated hereditary stomatocytosis (OHS). Some forms of DHS may be a pleiotropic, showing pseudohyperkalemia and/or perinatal edema. Perinatal edema, if not properly treated, may be lethal but may also resolve spontaneously prior to or shortly after birth and never reappear. Hereditary cryohydrocytosis, type 1 (CHC 1) is characterized by a dramatic resumption of the leak in vitro as the temperature approaches 0°C ; cell hydration seems unaltered. In OHS, stomatin, a membrane protein, is sharply reduced; however, this is a secondary event and the primarily mutated protein remains unknown. Hereditary cryohydrocytosis, type 2 (CHC 2) presents similar to OHS, except that the leak dramatically increases close to 0°C. In addition, hematological manifestations are associated with neurological disorders. Of critical practical importance is that splenectomy in DHS or OHS causes thromboembolic events that may be fatal. The genes involved in hereditary stomatocytoses have yet to be identified. Apart from the 16q24-qter locus, related to subsets of DHS and FP, and a chromosome 2 locus assigned to a single case of FP, gene mapping has been difficult. The eventual discovery of individual genes will clarify complicated classification of the stomatocytoses, now based solely on phenotype.

THE PASSIVE, monovalent cation leak, which reflects red cell membrane permeability to cations and will be referred to as the leak, is all that remains of the cation movements through the red cell membrane when the sodium pump and the $Na^+,K^+,2Cl^-$-cotransporter are inhibited in vitro by ouabain and bumetanide, respectively. An increase of the leak underlies the hereditary stomatocytoses, a group of rare hemolytic anemias, and familial pseudohyperkalemia (FP).

The first example of stomatocytosis was discovered more than 40 years ago by Lock et al[1] and is now called overhydrated hereditary stomatocytosis (OHS). Since then, other entities have been delineated. However, diagnostic criteria are not consistent and for present genotype data are too scarce to resolve an inflation of phenotype-based descriptions.

We here consider the different classes of the genetic disorders of the red cell membrane, as currently based on phenotypes, aware that overlapping entities are inevitable. We will review (1) FP; (2) dehydrated hereditary stomatocytosis (DHS), alone or combined with other manifestations; (3) hereditary cryohydrocytosis, type 1 (CHC 1), with normal stomatin; (4) OHS; and (5) hereditary cryohydrocytosis, type 2 (CHC 2). OHS and CHC 2 are associated with reduced amounts of the membrane protein stomatin. All these conditions are very rare and display a dominant inheritance pattern. In OHS, de novo mutations are common but, once present, the mutations are dominantly transmitted.

The Phenotypic Approach of the Genetic Diseases of the Red Cell Membrane Permeability

Except for FP, the genetic disorders of the red cell membrane are primarily associated with a hemolytic anemia of variable severity.

Red Cell Indices and Other Routine Tests

The red cell indices are used to assess the anemia; moderate to conspicuous, macrocytosis is present. The blood smears reveal stomatocytes (Fig 1),

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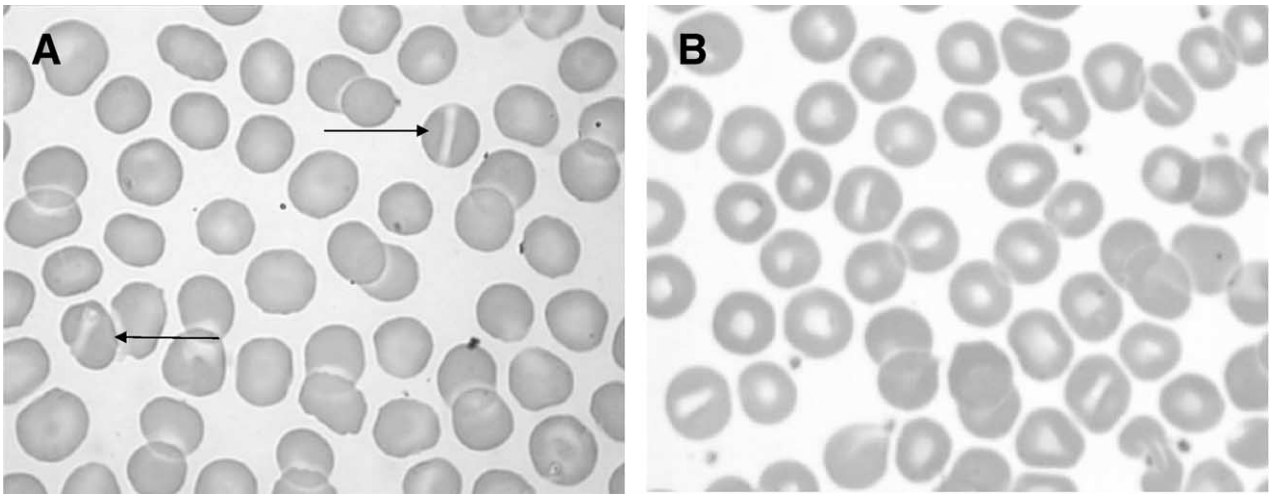

Figure 1. A mini-gallery of stomatocytes. (Courtesy of Dr Thérèse Cynober.) (A) Stomatocytes in dehydrated hereditary stomatocytosis; the condition was mildly expressed in this patient and only two full-fledged stomatocytes are visible (arrows). (B). Stomatocytes in overhydrated hereditary stomatocytosis. Stomatocytes are more numerous.

". . . erythrocytes showing a well-demarcated linear unstained area across their center, instead of the normal circular area of pallor . . ."[1] Stomatocytes may be overlooked due to their infrequency and/or their ill-formed aspect. Bone marrow aspiration is unnecessary for diagnosis, but it would show erythroid hyperplasia with no qualitative abnormalities.

Other routine diagnostic tests may show hyperkalemia, hyperbilirubinemia, and hyperferritinemia. Pseudohyperkalemia should be sought according to a strict protocol for time and temperature, and the contribution of hemolysis, which readily occurs with fragile cells and results in a loss of intracellular K^+, must be subtracted. Gilbert syndrome (OMIM #143500)[2] is assessed through the mutation in the promoter [$A(TA)_7TAA$ instead of $A(TA)_6TAA$] of the *UGT1A1* gene (encoding the bilirubin uridine diphosphate–glucuronosyltransferase 1) (2q37.1).[3]. Gilbert syndrome is likely to enhance icterus, especially in the neonatal period. Genetic hemochromatocytosis (OMIM *235200) is established by C282Y and H63D mutations[4] in the *HFE* gene (6p22.2). The impact of these latter mutations on the course of iron overload in stomatocytoses has not been determined.

Osmotic Gradient Ektacytometry

Osmotic gradient ektacytometry is the key test in the diagnosis of genetic disorders of the red cell membrane permeability. Stretching of the cells is followed by diffraction of a laser beam that strikes erythrocytes in a solution of increasing osmolarity flowing inside a viscosimeter.[5] Variations in the bell-shaped curve (Fig 2) explore (1) deformability, generally unchanged, of the abnormal red blood cells, generally little diminished, if not elevated, (2) osmotic resistance (replacing the tedious assay of hemolysis in solutions of varying osmolarity), and (3) cell hydration. Osmotic gradient ektacytometry is the only direct and reliable test to evaluate cell hydration; unfortunately the required equipment is available only in a handful of laboratories.

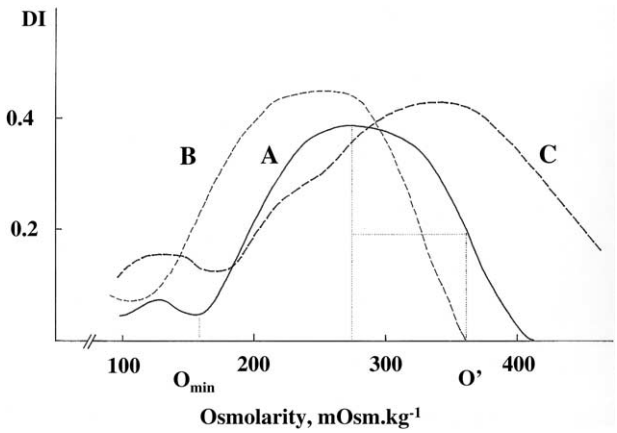

Figure 2. Osmotic gradient ektacytometry curves. (Courtesy of Dr Thérèse Cynober.) The deformability index (DI) is measured as a function of a gradual variation of the osmolarity of the medium within the ektacytometer. X-axis: osmolarity of the medium within the ektacytometer (mOsm · kg^{-1}).Y-axis: DI (arbitrary units). O_{min}: osmolarity at which the cells are maximally swollen (O_{min} corresponds to the hemolysis of 50% of the red blood cells in vitro). O′: osmolarity at which the DI has half its maximal value on the right leg of the bell-shaped curve (O′ is an indication of the red cell hydration). Curves obtained with normal (A), DHS (B), and OHS red cells (C).

Table 1. Main Parameters in Various Genetic Disorders of the Membrane Permeability to Monovalent Cations

	Reference	Reticulocytes (%)	MCV† (fL)	$[Na^+]_i$ (mmol/L cells)	$[K^+]_i$ (mmol/L cells)
Controls				5.9-11.3	88-105
FP Cardiff	Gore et al, 2002[13]	0.71-0.76	83.8-85.7	28-41	73-83
FP Chiswick	Haines et al, 2001[12]	0.66	101.2	22.8-24.9	100.8-101.3
FP Falkirk	Haines et al, 2001[12]	—	81.6	16.5	81.5
DHS	Coles et al, 1999[22]	5.26-6.86	97-115	42.6-77	28-58
CHC 1*	Coles et al, 1999[34]	5.60	91.3	17.8	64.9
		8.99	87.7	18.2	60
CHC 1*	Haines et al, 2001[35]	5.4-24.0	99-104	14.6-23.5	60.4-71.1
		3.5-5.1	89-94		
OHS†	Unpublished data	13.9	135.6	111	32.6
CHC 2†	Unpublished data	13	120.5	106	31.7

*Two distinct kindreds are shown, one on each line.

†MCV, mean corpuscular volume, is highly dependent on the storage conditions (time, temperature), which makes it likely to increase in OHS and CHC 2.

Cation Cytoplasmic Concentrations and Temperature Dependence of the Leak

These tests also are carried out in highly specialized laboratories. Na^+ and K^+ intracellular concentrations, $[K^+]_i$ and $[Na^+]_i$, respectively, are determined: $[K^+]_i$ decreases and $[Na^+]_i$ increases in the genetic diseases of the leak. The temperature-dependence of the leak is measured as the influx of rubidium 86 in the presence of ouabain and bumetanide, which inhibit the sodium pump and the $Na^+,K^+,2Cl^-$ cotransporter, respectively.

Familial Pseudohyperkalemia

FP is a symptomless genetic trait, discovered by Stewart et al in 1979.[6] An elevated potassium level is recorded when freshly drawn blood is allowed to stand at room temperature for some hours; hyperkalemia may reach values as high as 10 mmol · L^{-1} whereas it is normal at the time of blood collection—hence the name *pseudo*hyperkalemia. There are no associated signs of acidosis, renal failure, Addison disease, or hypertension. The chance occurrence of hypertension associated with FP should not result in confusion with Gordon syndrome or hyporeninemic hypoaldosteronism, combining hypertension and genuine hyperkalemia[7] (OMIM #145260), due to mutations in the *WNK1* gene (1q34-q42) or *WNK4* gene (17p11-q21).[8,9] In FP, there are no hematological signs. The osmotic gradient ektacytometric curve is normal. $[Na^+]_i$ is increased and $[K^+]_i$ is decreased. The inheritance pattern is dominant. FP is extremely rare and thus may easily be overlooked. Six kindreds have been reported in the United Kingdom[6,10-13] and two in France.[14,15]

The first recorded case of FP, now called FP Edinburgh, shows a "shallow" and regular descending curve for the temperature-dependence of the leak; the same pattern is seen for a French kindred of Flemish descent, presenting with FP Lille[15] (Carella M, et al, unpublished data). FP Chiswick and FP Falkirk show a different curve, with a minimum at 25°C, a maximum at 12°C, and then a further fall.[12] Patients with FP Chiswick and FP Falkirk have macrocytosis, a hint of a link of these two forms with hematology. In these types of FP and also in FP Cardiff,[13] $[Na^+]_i$ is increased and $[K^+]_i$ decreased moderately (Table 1). The curve accounting for the temperature-dependence of the leak shows a minimum at 23°C, a feature characterizing cryohydrocytosis (see below); again, classification based solely on phenotypes leads to overlaps.

It is odd that so rare a disease as FP produces such a variety of phenotypes, at least as reflected by the temperature-dependence of the leak. This diversity could be compared with the different electrophoretic mobilities of abnormal hemogobins caused by mutations in the β-globin gene. The heterogeneity of the curves of temperature-dependence of the leak could be accounted for by distinct mutations within a single gene, an assumption which would simplify the genotyping of FP. Conversely, and puzzling, FP Edinburgh and FP Lille, which display the same type of temperature dependence of the leak, map to two distinct loci, 16q23-q24[16] and 2q35-36 (Carella M, et al, unpublished data), respectively. FP Edinburgh and Lille might stem from mutations in one of the two chains of a dimer. Gene mapping has not been performed for other forms of FP.

Dehydrated Hereditary Stomatocytosis

DHS (OMIM #194380) was first described by Oski et al.[17] DHS, or more accurately one subset of DHS, may appear alone or with other manifestations, including

pseudohyperkalemia and/or perinatal fluid effusions. DHS alone is associated with a usually well-compensated hemolytic anemia; severe forms are DHS alone are unknown. The spleen is moderately enlarged. Mild if not borderline macrocytosis may be present; blood smears show stomatocytes, but usually less than 10%, many of them being poorly formed and easily overlooked stomatocytes (Fig 1). Osmotic gradient ektacytometry is thus important for the diagnosis of DHS (Fig 2), showing unambiguous abnormalities even when the hematological presentation is minimal. The bell-shaped curve is shifted leftward, indicating an increase in the osmotic resistance (left leg of the curve) and cell dehydration (right leg). There is a more or less pronounced increase of $[K^+]_i$ and decrease of $[Na^+]_i$. The temperature-dependence of the leak, in one subset of DHS,[18] shows a smooth, shallow slope, reminiscent of FP Edinburgh and FP Lille; the curve is only shifted upwards along the Y-axis (not shown). Resemblance between these curves suggests that FP Edinburgh and FP Lille and this subset of DHS are related entities. Indeed, linkage analysis showed that one subset of DHS mapped to 16q23-q24,[19] as does FP Edinburgh. The DHS inheritance pattern is autosomal dominant. The incidence of DHS is low but DHS is the most frequent of the genetic disorders of red cell membrane permeability to cations: we recruited over 20 DHS families in 6 years.

The course of DHS is dominated by iron overload, even in the absence of transfusions, for unknown reasons. Iron overload should be treated before ferritinemia reaches the threshold of 1,000 $ng \cdot mL^{-1}$.

Splenectomy is strongly contraindicated in DHS because venous thromboembolic complications occur with near certainty.[20] In one patient with DHS and heterozygous hemoglobin S, repeated embolisms led to pulmonary hypertension and cor pulmonale; eventually, heart-lung transplantation was required.[21] In DHS of the Blackburn type, the splenectomized patient died from pulmonary hypertension[22]; another splenectomized patient with a leak pattern comparable to the Blackburn type DHS developed pulmonary embolism, and she died after bone marrow transplantation from broncholiotis obliterans.[23] Among factors likely to predispose to these accidents are mutation G1691A (CGA→CAA; R506Q); mutation Leiden,[24] within the *F5* gene (1q21-q25) encoding coagulation factor V, which causes resistance to activation by activated protein C; and mutation G20210A,[25] within the the 3′-UTR of the *F2* gene (11p11-q12), encoding coagulation factor II, in which prothrombinemia is increased. There are no studies, however, on the precise impact of these factors in splenectomized DHS patients.

Some DHS can be associated with pseudohyperkalemia and/or perinatal fluid effusions[18,26-30]; a new syndrome was thus delineated (OMIM #603528), consistent with a continuum between at least a subset of pseudohyperkalemia and a subset of DHS. Perinatal fluid effusions are remarkable in two ways. First, they are widely variable: some cases are detectable only using sonography; others are life-threatening and require that the effusions (usually ascites) be removed in order to ease a possibly fatal mechanical effect. Second, fluid effusions resolve spontaneously weeks or (at most) a few months following birth, and they do not reappear. Transient effusions contrast with the life-long character of DHS and pseudohyperkalemia.

Grootenboer et al,[18,29] using a limited number of markers, found that the DHS cases they had studied were compatible with a 16q23-q24 location in 10 different kindreds. Further saturation of the region of interest confirmed this location, but not in all instances (unpublished data). In other families, DHS failed to map to 16q23-q24. In DHS Blackburn, (22,31),. The involved gene was tentatively mapped to chromosome 17 (32). This finding, however, need be substantiated. In DHS Blackburn, $[K^+]_i$ was decreased and $[Na^+]_i$ was much increased (Table 1). The temperature dependence of the leak was still shallow, but the curve was higher relative to the Y-axis (Fig 3).[22] In general, the shallow character of the curve is associated with the occurrence of thromboembolic accidents postsplenectomy.

Hereditary Cryohydrocytosis, Type 1, With Normal Stomatin

CHC (OMIM #185020) is a variant of stomatocytosis: salient is the dramatic resumption of the leak in vitro when the temperature nears 0°C. There are two forms of CHC, depending on whether stomatin, or protein 7.2b, is present (CHC, type 1, or CHC 1) in normal amount or nearly absent (CHC, type 2, or CHC 2). Stomatin and its gene, *EPB72,* will be described below. In brief, stomatin is assessed by Western blotting following polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and immunofluorescence of red cell membrane proteins.

CHC was first described by Miller et al[33] and named later based on the study of four families from the United Kingdom.[34,35] Anemia was well compensated in these cases, with reticulocyte counts moderate to much elevated. Stomatin quantity was normal. The increase of $[Na^+]_i$ and decrease of $[K^+]_i$ were mild (Table 1). Temperature-dependence of the leak showed a minimum at about 20°C; at lower temperatures, the curve increased again and values at 0°C were higher than those at 37°C, hence the use of the prefix "cryo." Eventually, the red blood cells lysed and lost their entire K^+ content into the plasma. Osmotic gradient ektacytometry, performed in two

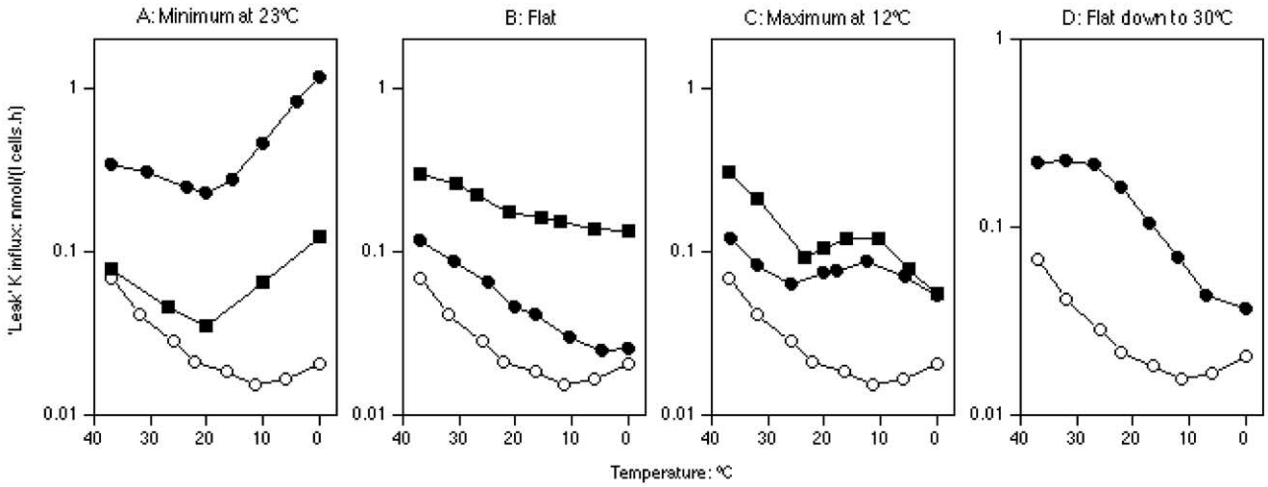

Figure 3. A gallery of curves accounting for the temperature dependence of the leak. (Courtesy of Dr Gordon W. Stewart.) K^+ influx (^{86}Rb tracer) was measured in a 5 mmol/L K^+, 145 mmol/L Na^+, 150 mmol/L chloride, 15 mmol/L morphilinopropanesulfonic acid (MOPS) (pH 7.4 at 20°C), 5 mmol/L glucose solution with ouabain and bumetanide, 0.1 mmol/L each. X-axis: temperature. Y-axis: leak. Open circles: controls. (A) U-shaped with minimum at 23°C, that is seen in CHC 1.[34,35] (B) Shallow slope pattern, often associated with thrombosis after splenectomy.[22,23] (C) Maximum at 12°C, associated with minimal hemolysis and marked pseudohyperkalemia.[35] (D) Flat down to 30°C, below which temperature the rate of flux falls sharply (unpublished data).

patients, showed neither cellular overhydration nor dehydration (Cynober T, unpublished data). There is doubt as to whether the prefix "hydro" is warranted in the designation of CHC 1. In CHC 1 the inheritance pattern is autosomal dominant; the incidence is extremely low.

Stomatin and Its Gene

Stomatin is a 29-kd integral membrane protein. Its cDNA was cloned by Hiebl-Dirschmied et al[36] and Stewart et al.[37] Stomatin has 287 amino acid residues excluding the start methionine, which is removed; it is comprised of a 25–amino acid extracellular segment, a 28–amino acid transmembrane segment, and a 235–amino acid cytoplamic domain. There are potential sites for *N*-glycosylation, *N*-palmitoylation, and cyclic adenosine monophosphate (cAMP)-dependent and protein kinase C phosphorylation. Cys 29 is the major palmitoylation site.[38] Ser 9 is the only phosphorylation site.[39] Thus the possibility exists that both extramembranous segments of stomatin are intracytoplasmic, the transmembrane segment bending like a hairpin within the lipid bilayer. Stomatin, as well as flotillins-1 and -2, is preferentially located in lipid rafts,[40] which are sphingolipid- and cholesterol-rich membrane microdomains.[41]

The *EPB72* gene, encoding stomatin, maps to 9q33-q34[42,43] and is centromeric to the *ABL* proto-oncogene. The *EPB72* gene spans over 30 to 40 kb and has seven exons.[44,45] Exon 1 contains the 5′-UTR (61 nt) and the sequence encoding the *N*-terminal region; exon 2 encodes the transmembrane domain. The corresponding cDNA is 3,047 bp in length. The potential promoter is TATA-less, (G+C)-rich, and there are several potential sequences for binding of ubiquitous transcription factors (Sp1, AP1, AP2, CP1/2, NFκB, CREB, Est-1), consistent with the wide distribution of stomatin mRNA. *Alu* repeats were found within introns and the 3′-UTR. There is a single transcription initiation site; alternative polyadenylation takes place. Stomatin homologues are widely encountered in species as remote from man as *Caenorabdtitis elegans*. In this nematode, the gene *mec-2* encodes the MEC-2 protein, whose central part is homologous to stomatin. Mutations in *mec-2* cause a defect in neuronal mechanosensory function.[46]

Overhydrated Hereditary Stomatocytosis

The first case of OHS (OMIM #185000) was described by Lock et al[1] as an uncompensated hemolytic anemia with splenomegaly, frank macrocytosis, and reticulocytosis (Table 1). Stomatocytes were usually plentiful and well formed (in contrast with DHS). It is not known whether the stomatocyte morphology increases during storage, when red blood cells could potentially become overhydrated. The osmotic gradient ektacytometric curve is highly typical, with no decrease of the deformability index (DI), but a dramatic rightward shift of the bell-shaped curve involving both of its legs (Fig 2), indicating a reduction in osmotic fragility and cell overhydration. As with stomatocyte shape, blood storage prior to

ektacytometry is likely to accentuate the abnormal aspect of the curve. The temperature-dependence of the leak is accounted for by a steep monotonic curve (not shown). There is a pronounced imbalance in $[Na^+]_i$ and $[K^+]_i$ (Table 1). Cholelithiasis and iron loading are common complications of OHS; as in DHS, splenectomy is contraindicated.[20]

The OHS inheritance pattern is autosomal dominant. Sporadic mutations in a number of cases may incorrectly suggest recessive transmission. The incidence of OHS is low; we recruited only one family and one individual in 6 years, and in total 12 individual or familial OHS cases have been collected[1,47-51] (Fricke B, unpublished data).

There is near total absence of membrane protein stomatin in OHS. However, the condition in which stomatin deficiency was first described[52] was not OHS, but CHC 2 (see below). Indeed, the reduction of stomatin is misleading, and mutations in the *EPB72* gene are not primary in OHS. Mutations in stomatin cDNA have not been found in OHS patients,[53,54] and targeting of the stomatin gene in the mouse left red blood cell shape unchanged.[55] Stomatin is puzzlingly lacking by more than 50 % in heterozygotes, but stomatin naturally tends to be lost in senescent red blood cells.[54] The mutated gene in OHS remains undiscovered. Flotillin-1 and -2 are normal in OHS.[40]

Hereditaty Cryohydrocytosis, Type 2, With Reduced Stomatin

To date, it is unclear whether CHC 2 is a different nosological entity or a variant of OHS; the first clinical occurrence of stomatin deficiency was actually a case of CHC 2.[52] Hematologically, CHC 2 is indistinguishable from OHS (Table 1), except for neurological features, which are missing in OHS (Fricke B, unpublished data). The curve accounting for the temperature dependence of the leak is sharply descending, shows a minimum at 20°C, and then increases again to reach values higher at 0°C than at 37°C (not shown), reminiscent of the pattern in CHC 1. CHC 1 and CHC 2 are phenotypically quite different and, in all likelihood, stem from mutations in distinct genes that nonetheless produce a similar temperature-dependence of the leak.

A Unique Multi-system Syndrome Associated With Abnormal Splicing of Stomatin Pre-messenger

In a child born of Tunisian consanguineous parents, Argent et al[56] described a recessively inherited syndrome, associating the following features: dyserythropoiesis, sideroblastic anemia, delayed neurological development with hypotonia and convulsions, salt-loosing nephropathy, chronic watery diarrhea, lactic acidosis with mitochondrial dysfunction, fatty liver, and terminal pulmonary fibrosis. There was a deficiency within "band 7." Stomatin mRNA sequencing showed a complex series of aberrant spliceoforms centered around exon 3. However, no mutations were found at the gene level. The molecular etiology of this unusual condition, which lethally affected four other members of the sibship, remains elusive.

Stomatocytosis, Abnormal Platelets, and Pseudo-homozygous Hypercholesterolaemia

In a 13-year-old girl with congenital hemolytic anemia associated with pseudo-homozygous hypercholesterolemia, erythrocyte morphology showed 50% to 80% stomatocytes, but no abnormalities of membrane lipid or protein composition or of cation transport.[57] Platelets were reduced in number, abnormally large, and showed reduced adhesion. Successful treatment of the hypercholesterolemia did not affect the red blood cell morphology.

Hypertrophic Gastritis Associated With Stomatocytosis in the Dog

The *Drentse patrijshond* dog shows a recessively inherited syndrome combining stomatocytosis (cup- or bowl-shaped red blood cells) and hypertrophic gastritis, the latter comparable to the Ménétrier syndrome in man.[58] The condition might stem from an abnormality of lipid metabolism.[59]

Conclusions

The rarity and heterogeneity of the genetic disorders of red membrane permeability to monovalent cations have hindered the localization and identification of the responsible genes and understanding of the underlying pathophysiology. Once the genetics are understood, the disturbed mechanisms, when elucicated, likely will open new perspectives extending beyond the red blood cell.

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