



# Abnormal red cell features associated with hereditary neurodegenerative disorders: the neuroacanthocytosis syndromes

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## Purpose of review

This review discusses the mechanisms involved in the generation of thorny red blood cells (RBCs), known as acanthocytes, in patients with neuroacanthocytosis, a heterogeneous group of neurodegenerative hereditary disorders that include chorea-acanthocytosis (ChAc) and McLeod syndrome (MLS).

## Recent findings

Although molecular defects associated with neuroacanthocytosis have been identified recently, their pathophysiology and the related RBC abnormalities are largely unknown. Studies in ChAc RBCs have shown an altered association between the cytoskeleton and the integral membrane protein compartment in the absence of major changes in RBC membrane composition. In ChAc RBCs, abnormal Lyn kinase activation in a Syk-independent fashion has been reported recently, resulting in increased band 3 tyrosine phosphorylation and perturbation of the stability of the multiprotein band 3-based complexes bridging the membrane to the spectrin-based membrane skeleton. Similarly, in MLS, the absence of XK-protein, which is associated with the spectrin-actin-4.1 junctional complex, is associated with an abnormal membrane protein phosphorylation state, with destabilization of the membrane skeletal network resulting in generation of acanthocytes.

## Summary

A novel mechanism in generation of acanthocytes involving abnormal Lyn activation, identified in ChAc, expands the acanthocytosis phenomenon toward protein-protein interactions, controlled by phosphorylation-related abnormal signaling.

## Keywords

chorea-acanthocytosis, McLeod syndrome, membrane, phosphorylation, signal transduction

## INTRODUCTION

In the 1960s Irving M. Levine and Critchley separately reported a new hereditary neurological disorder characterized by hyporeflexia, mild muscle weakness and atrophy associated with thorny red cells, acanthocytes (Fig. 1) [1–4]. This disorder was described as neuroacanthocytosis based upon the clinical phenotype [3,4]. Today, neuroacanthocytosis syndromes consist of chorea-acanthocytosis (ChAc), McLeod syndrome (MLS), Huntington's disease like-2 (HDL2) and pantothenate-kinase-associated neurodegeneration (PKAN) [5]. Acanthocytes are one of the biological hallmarks of neuroacanthocytosis. The analysis of the abnormal red blood cells (RBCs) from neuroacanthocytosis patients, to define the mechanistic basis for the red cell phenotype, will further help our understanding of the pathogenesis of neuroacanthocytosis.

## NORMAL RED CELL MEMBRANE ORGANIZATION AND RED CELL SHAPE

The study of human RBCs has led to the development of a general model of RBC membrane organization based on the functional cross-talk between the membrane lipid bilayer, the integral membrane

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**Curr Opin Hematol** 2014, 21:000–000

DOI:10.1097/MOH.0000000000000035

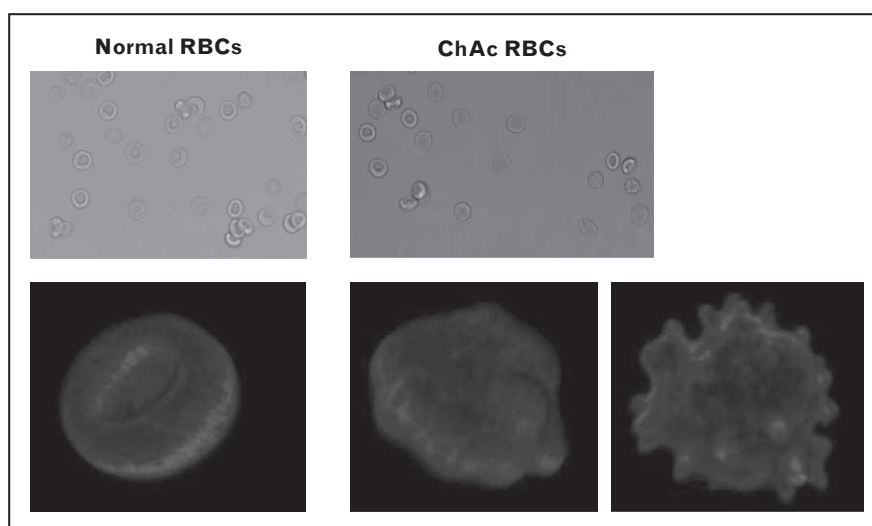
## KEY POINTS

- Acanthocytes are a marker of neuroacanthocytosis, a set of hereditary neurodegenerative diseases affecting the basal ganglia.
- In ChAc, acanthocytosis has been related to increased Lyn activation and alteration of band 3-based protein–protein interactions involved in stability of the RBC membrane skeletal network.
- In MLS, the absence or reduction of XK protein, part of the 4.1 multiprotein complex anchoring the membrane to the cytoskeleton, is associated with a perturbation of the membrane protein phosphorylation state.
- In other neuroacanthocytosis diseases, breakdown products of band 3 and alterations in the interaction of band 3 with neighboring partner proteins have been observed.

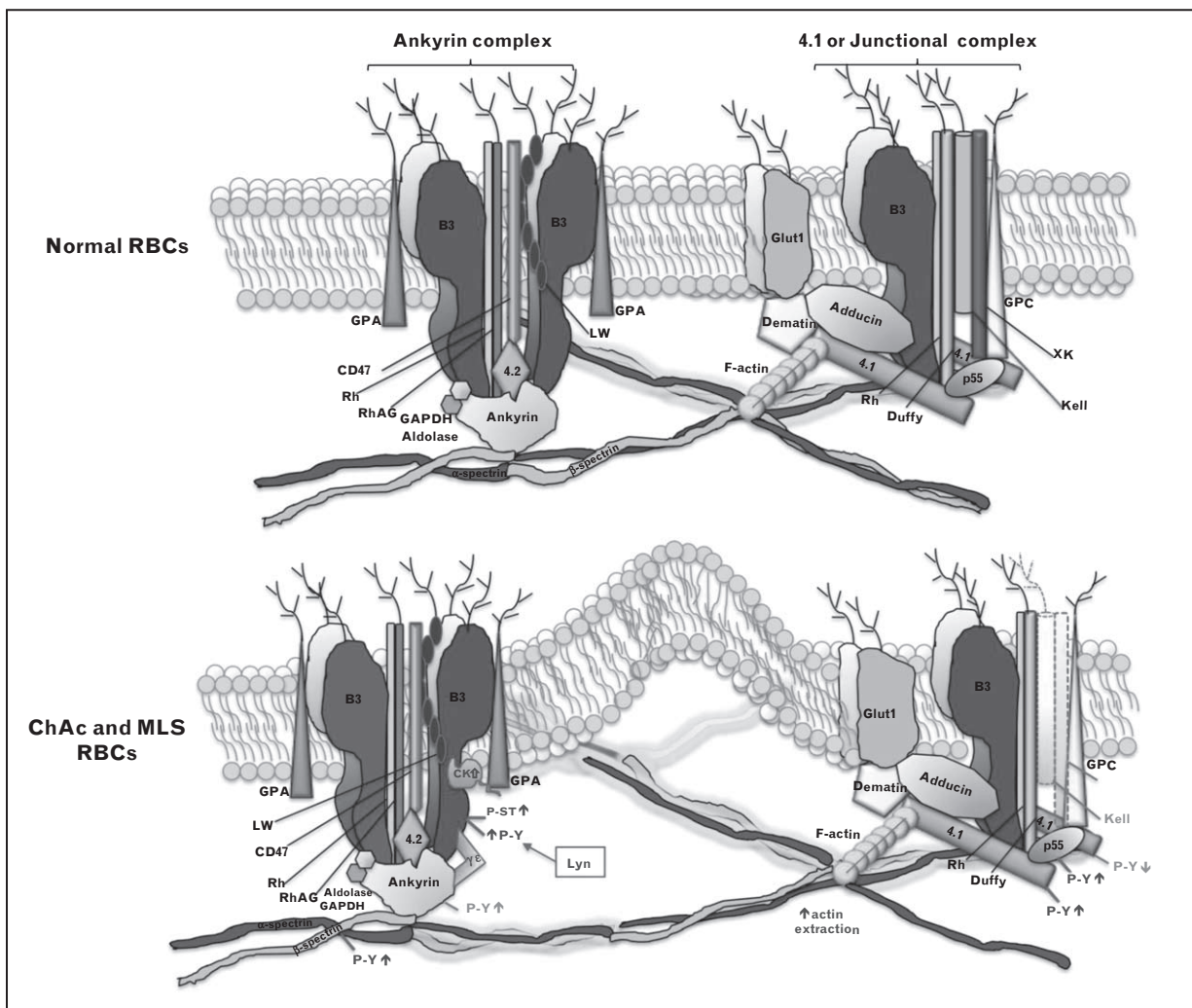
proteins such as band 3 and the peripheral proteins such as spectrins and actin (Fig. 2). The RBC membrane is anchored to the membrane skeleton network through the band 3-based bridges formed by multiprotein complexes involving ankrin and protein 4.1 (or junctional complex) (Fig. 2) [6–14]. The study of hereditary RBC disorders has provided insight into the RBC membrane organization and function. Mutations in proteins involved in the vertical interactions such as band 3 or ankyrin with membrane skeleton result in loss of membrane

surface area and generation of spherocytes as in patients with hereditary spherocytoses. However, mutations in proteins affecting lateral interactions such as spectrin dimer–dimer or spectrin–actin–protein 4.1 interactions result in loss of RBC membrane mechanical stability and generation of elliptocytes, as in patients with hereditary elliptocytosis (Fig. 2) [8].

Although these RBC shape abnormalities have been related to genes encoding for membrane and skeletal proteins, abnormal RBC morphology has been also described in diseases primarily affecting the lipid bilayer such as abetalipoproteinemia and spur cell anemia. Hypercholesterolemic mice generated by inactivation of *SR-BI* (*SR-BI*<sup>−/−</sup>), a gene encoding the high-density lipoprotein receptor, have macrocytic anemia with abnormal circulating RBCs and large membrane-enclosed intracellular inclusions [15]. The severity of the *SR-BI*<sup>−/−</sup> hematological phenotype was related to the cellular cholesterol content, suggesting that cholesterol is important in erythroid maturation and in generation of normal RBCs [15]. In patients with abetalipoproteinemia or hypobetalipoproteinemia, the deficiency of apolipoprotein B is associated with acanthocytosis and neurological manifestation but without movement disorders such as those seen in the neuroacanthocytosis syndromes [5]. Changes in the composition of RBC membrane lipids have been also associated with the generation of acanthocytes in spur cell anemia, which occurs in patients with advanced



**FIGURE 1.** Upper panel. The brightfield images of fixed and permeabilized red blood cells (RBCs) from a normal control and from a patient with chorea-acanthocytosis (ChAc). Lower panel. The corresponding 3D-confocal immunofluorescence staining with the anti-band 3 antibody of fixed and permeabilized red cells from a normal control and from a patient with ChAc. The images were captured by a confocal laser-scanning microscope (LSM 510, Carl Zeiss) equipped with a 63×, 1.4 numerical aperture water immersion lens equipped with LAS AF software. The 3D reconstruction was performed by ImageJ software (<http://rsb.info.nih.gov/ij/>).



**FIGURE 2.** Schematic diagram of membrane organization in normal red blood cells (RBCs) and acanthocytes from patients with chorea-acanthocytosis (ChAc, changes are indicated in blue) and McLeod Syndrome (MLS, changes are indicated in light brown). B3, band 3; 4.2, protein 4.2; 4.1, protein 4.1; Kell, Kell glycoprotein; XK, XK protein; GPA, glycoprotein A; GPC, glycoprotein C; LW: Landsteiner-Weiner blood group glycoprotein; RhAG: Rh-associated glycoprotein; p55: protein 55; Glut1: glucose transporter-1; P-ST, phospho-serine-threonine; P-Y, phospho-tyrosine membrane associated proteins;  $\gamma\epsilon$ , N<sup>c</sup> ( $\gamma$ -glutamyl) lysine isopeptide; CK, casein kinase. The absence of XK protein and the reduction in Kell protein as observed in MLS red cells is represented by the dashed lines of lighter forms.

liver failure and resolves after liver transplantation [16].

In the present review, we focus on acanthocytosis in neuroacanthocytosis, summarizing the recent advances in our knowledge on the mechanisms underlying the generation of acanthocytes in neuroacanthocytosis syndromes.

## CHOREA-ACANTHOCYTOSIS

Chorein is the 360-kDa protein product of the *VPS13A* gene (chromosome 9), mutations of which are associated with the autosomal recessive disorder of ChAc [5,17,18]. Chorein is present in mature RBCs and is partially or completely absent in ChAc

RBCs [19]. At the present, the lack of information regarding the biochemical structure and interactions of chorein with other proteins makes it difficult to formulate a hypothesis on its role in RBC homeostasis (Table 1) [20–24,25<sup>□□</sup>,26–31,32<sup>□</sup>, 33–39].

In ChAc RBCs, electron microscopic studies have revealed a heterogeneous distribution of the membrane skeleton characterized by condensed cytoskeletal structures around protrusions and less filamentous structure in some large patches of the membrane (Table 1) [21]. Although no major abnormalities in RBC membrane protein composition and abundance have been observed in ChAc RBCs, an increased amount of N<sup>c</sup> ( $\gamma$ -glutamyl) lysine

**Table 1.** Summary of the genetic and hematological features of neuroacanthocytosis syndromes

Disease	Mode of inheritance	Gene (location)	Protein product	Acanthocytes/anemia	Red cell alterations	Refs.
ChAc	AR	<i>VPS13A</i> (9q21)	Chorein	+++/No anemia	Low RBC K <sup>+</sup> content and subpopulation of dense RBCs Heterogenous distribution of RBC membrane skeleton Increased band 3 Ser-Threo-phosphorylation Increased casein kinase (CK) membrane activity Increased band 3, p55, protein 4.1, actin Tyr-phosphorylation Increased Lyn activity Increased N <sup>ε</sup> (γ-glutamyl)lysine isopeptide RBC membrane association Reduced response to drug induced endovesicle formation	[20] [21] [22] [22] [22,23] [23] [24] [25 <sup>■</sup> ]
MLS	X-linked	<i>XK</i> (Xp21)	XK protein	+++/Mild compensated hemolytic anemia	Absent or truncated XK protein Reduced Kell glycoprotein Low RBC K <sup>+</sup> content and subpopulation of dense RBCs Decreased deformability of MLS RBCs by ektacytometry Heterogenous distribution of RBC membrane skeleton Increased Tyr-phosphorylation pattern of RBC membrane proteins	[26] [27–29 <sup>■</sup> ] [30] [31] [21] [32 <sup>■</sup> ]
HDL2	AD	<i>JPH3</i> (16q24.3)	Junctophilin-3	++/No anemia	Proteolytic band 3 products Increased small G proteins RBC membrane associated	[33] [34]
PKAN	AR	<i>PANK2</i> (20p.13)	Pantothenate kinase 2	±/No anemia	Reduced response to drug induced endovesicle formation in acanthocytic but not in normally shaped RBCs	[25 <sup>■</sup> ,35–39]

++, acanthocytes present between 30 and 35%; +++, acanthocytes present between 25 and 60% of the whole red cell population; ±, acanthocytes ~3%; AD, autosomal dominant; AR, autosomal recessive; ChAc, chorea-acanthocytosis; HDL2, Huntington's disease-like 2; JPH3, junctophilin-3; MLS, McLeod syndrome; PKAN, pantothenate kinase-associated neurodegeneration; PS, phosphatidylserine; RBC, red blood cell; Ser-Threo, serine-threonine; Tyr, tyrosine.

isopeptide associated with RBC membrane was reported in a small number of ChAc patients (Table 1) [24,40]. Transglutaminase 2 catalyzes the organization of multicomplex protein cross-linked by the N<sup>ε</sup> (γ-glutamyl) lysine isopeptide. The main protein involved is band 3 through its Gln-30 residues, together with bridging proteins such as ankyrin or protein 4.1 and skeletal proteins such as spectrins [40–42]. A recently described enrichment of actin in the Triton-soluble fraction of ChAc RBCs supports earlier data showing a perturbation of the skeletal network and possible instability in bridging band 3-based multiprotein complexes in the absence of chorein [43]. This finding is also supported by the observation of a reduced response of ChAc red cells to drugs inducing endovesicles [25<sup>■</sup>] In addition, a reduced RBC K<sup>+</sup> content has been described in ChAc patients [20]. These data suggest a more complex scenario in ChAc that might

involve posttranslational modifications such as phosphorylation affecting protein-protein interactions between membrane proteins and skeletal network. Indeed, recent in-vitro studies have shown that an imbalance between phosphatase-kinase activities might result in an altered band 3 phosphorylation state [11,12,44]. This might affect the stability of the complexes involving band 3, bridging the membrane to the skeletal network [22,23,45] (Fig. 2). In RBCs, Syk, a Src-related kinase, and Lyn, a Src kinase, are part of the signaling pathway targeting band 3 and affecting its protein interactions [46]. In ChAc RBCs, we recently studied the tyrosine (Tyr)-phosphoproteome and found an increased Tyr-phosphorylation state of several membrane proteins, including band 3, compared with controls [23]. We observed an increased phosphorylation of the Tyr-904 residue on band 3, which is a functional target of Lyn, but not of the Tyr-8 residue that is a

target of Syk (Fig. 2). We demonstrated an abnormal Lyn activation independent from its canonical pathway through the primary Syk activation. We then postulated that the ChAc-associated alterations in RBC membrane protein organization are the result of increased Tyr-phosphorylation state leading to an altered linkage of band 3 to the junctional complexes, and generation of acanthocytes (Fig. 2). This conclusion is also supported by the recent observations that the blood of mice with constitutively active Lyn contains acanthocytes [47<sup>\*\*\*</sup>]. To date, there are not consistent data implicating an altered membrane lipid composition in acanthocytosis in ChAc [20].

ChAc presents clinically in young adulthood [5,48]. An increased serum creatine kinase may precede the neurological symptoms, and sometimes is associated with hepatosplenomegaly [5,48]. At the present, 120 mutations in the *VPS13A* gene of various types have been described, resulting in low or absent synthesis of chorein or normal expression of a functionally defective protein [17,49].

VSP13A is a member of the VPS13 family (VPS13A–D) [50,51]. A comparison of the human gene sequence with those of other organisms has shown large similarities of the human protein VPS13A with the yeast Vps13p protein and the *Dictyostelium discoideum* TipC proteins, whereas the other *VSP13* genes seem to result from more recent evolutionary events [52,53]. Vps13p protein is involved in trafficking of various transmembrane proteins, in particular in the recruitment of three membrane proteins to the trans-Golgi network: Kex2p, a Golgi-endoprotease; Ste13p, a dipeptidyl aminopeptidase; and Vps10p, a cargo-sorting receptor, which delivers the cargo enzymes to the prevacuolar compartment similar to human late endosome [54–56].

Human chorein has been recently cloned and expressed in various cell lines [50]. Confocal microscopy showed a cytoplasmic localization of chorein in a vesicular-like pattern [50].

A mouse model with a *VPS13A* deletion showed reduced expression of chorein and production of truncated protein (Table 2 [57–61,62<sup>\*</sup>,63–66]). These ChAc mice developed a mild, late-onset motor disturbance with late adult onset and acanthocytes with increased osmotic fragility; however, no functional studies have been reported [57,58].

## McLEOD SYNDROME

The XK protein (444 amino acid residues, containing the Kx antigen) is the membrane protein associated with MLS, which is an X-linked form of neuroacanthocytosis [26–28,67]. The XK protein

is predicted to have 10 transmembrane domains with structural characteristics of a membrane transporter protein, but its function is yet to be defined. In the RBC membrane, the XK protein (50 kDa) is covalently linked to the Kell glycoprotein (93 kDa) by a single disulfide bond (XK Cys<sup>347</sup>–Kell Cys<sup>72</sup>) and is part of the multiprotein 4.1 junctional complex (Fig. 2) [29,68,69]. In MLS RBCs, truncation of the XK protein is associated with reduced expression of the Kell blood group antigen and a compensated hemolytic anemia [70–73].

Functional studies of MLS RBCs have shown a reduced resistance to mechanical stress and increased RBC density associated with reduced RBC K<sup>+</sup> content (Table 1) [21,30,31,74–76]. Electron microscopic studies demonstrate a heterogeneous distribution of the RBC membrane skeleton, mainly present in the denser RBC fraction containing acanthocytes [20,21,26].

The abnormal Tyr-phosphorylation state of some membrane proteins in MLS RBCs indicates once more an involvement of posttranslational modifications and/or disturbed intracellular signaling in acanthocyte formation (Table 1) [34,76]. Preliminary investigations of the RBC membrane phosphoproteome showed that the RBC membrane Tyr-phosphorylation pattern was significantly different in MLS RBCs compared with controls. More specifically, an increased Tyr-phosphorylation state of ankyrin and protein 4.1 was found in MLS RBCs [32<sup>\*</sup>]. In this regard, it may be relevant that changes in protein 4.1 phosphorylation state affect RBC membrane mechanical stability [77]. Furthermore, RBCs of protein 4.1 knockout mice (4.1<sup>-/-</sup>) show a perturbation of the RBC membrane multiprotein complexes involving glycophorin C, XK, Duffy and Rh proteins [78]. In MLS, it is likely that besides a reduction of XK–Kell complex, multiple factors contribute to an alteration of RBC membrane stability, such as changes in protein phosphorylation state, resulting in a destabilization of the skeletal network with generation of acanthocytes (Fig. 2).

MLS is found worldwide, and has a variable clinical presentation, with a mean age of onset between 30 and 40 years. It is mainly characterized by chorea, generalized seizures, neuropsychiatric abnormalities, cardiac myopathy, mild hemolytic anemia with acanthocytosis and hepatosplenomegaly [5,71]. The majority of the 28 identified XK mutations comprise small and large (5 Mb) deletions, frameshift and nonsense mutations, resulting in an absent or truncated XK protein [5,71]. Two missense mutations in the XK gene and one single nucleotide mutation in an intron near the splice junction have been reported as

**Table 2.** Animal models developed to study neuroacanthocytosis syndromes

Disease	Animal models	Phenotype	Refs.
ChAc	Engineered mouse strain carrying with deletion of <i>VPS13A</i> gene	Reduced chorein protein expression in whole brain homogenate Male infertility Abnormal RBC morphology and increased osmotic fragility in aged mice (>70 weeks of age) Behavioral abnormalities in aged mice (>70 weeks of age) Reduced striatum/whole brain ratio	[57,58]
HDL2	Mice <i>JPH3</i> <sup>-/-</sup>	No or mild neurological phenotype	[59]
	Mice <i>JPH3</i> <sup>-/-</sup> <i>JPH4</i> <sup>-/-</sup>	Cognitive and motor deficiencies No data on RBC features	[60]
	Mice BAC-HDL2	Age dependent motor deficiency Neuronal phenotype of HDL2 No data on RBC features	
PKAN	Mice <i>PANK2</i> <sup>-/-</sup>	Retinal degeneration, male infertility, no dystonia and little alteration in basal ganglia, no brain iron accumulation No data on RBC features	[61,62]
	Normal mice treated with pantothenate kinase inhibitor or with diet absent in pantothenic acid	Movement disorders and azoospermia, no brain iron accumulation in basal ganglia No data on RBC features	[63,64]
	<i>Drosophila melanogaster</i> with insertion of human <i>PANK2</i> mutation on <i>flb</i> gene	Shortened lifespan Male infertility Progressive locomotion defect Degeneration in central nervous system and retina, but without neuronal iron accumulation	[65,66]

ChAc, chorea-acanthocytosis; HDL2, Huntington's disease like-2; mice BAC-HDL2, bacterial artificial chromosome transgenic mice; mice *JPH3*<sup>-/-</sup> *JPH4*<sup>-/-</sup>, mice genetically lacking junctophilin-3 and 4; mice *JPH3*<sup>-/-</sup>, mice genetically lacking junctophilin-3; mice *PANK2*<sup>-/-</sup>, mice genetically lacking pantothenate kinase 2; NA, neuroacanthocytosis; PKAN, pantothenate kinase 2 associated neurodegeneration; RBC, red blood cell.

causing MLS with a milder clinical phenotype [5,26]. The XK protein is ubiquitously expressed [5]. Phylogenetic analysis showed that XK protein is a member of the XK family of proteins, containing XPLAC and XTEST proteins that share 37% and 31% homology, respectively, with XK. These proteins have a common domain with a consensus sequence that shares homologies with the ced-8 protein from the nematode *Caenorhabditis elegans* [29,79]. Ced-8 is involved in regulation of apoptosis in *C. elegans*, but it is not known whether XK plays a similar role in RBCs, erythroid precursors or in neuronal cells.

## HUNTINGTON DISEASE-LIKE 2

Acanthocytes have been observed in patients with Huntington's disease-like 2 (HDL2), an autosomal dominant neurodegenerative disorder resembling Huntington's disease. In HDL2, acanthocytosis-specific changes in membrane organization are suggested by the presence of band 3 breakdown products [80] and by the in-vitro production of vesicles that are different from the vesicles of control RBCs [34]. Preliminary RBC membrane proteomic analysis suggested that proteasome components and small G proteins were increased in HDL2 patients compared with controls [34]. Recently, it has been reported that RBCs from mice genetically

lacking the small G protein Rac GTPase have a perturbation of dynamic regulation of the RBC membrane skeletal network [81]. This suggests a possible involvement of small G proteins in membrane skeletal rearrangement in acanthocytosis in HDL2 (Table 2).

HDL2 is caused by a CAG/CTG expansion mutation in exon 2A of the junctophilin-3 gene (*JPH3*) on chromosome 16q24.3 [82]. HDL2 has only been identified in individuals of African descent, in mid-life, with involuntary movements, neuropsychiatric symptoms and dementia [82,83]. Post-mortem studies of HDL2 patients have shown intranuclear aggregates that were labeled with anti-ubiquitin antibodies and with anti1C2 antibody against long polyglutamine tracts in striatum and cortex, similar to those observed in Huntington's disease [80,84]. To date, it is not known how these aggregates might contribute to the neurological damage of HDL2. Mice genetically lacking the *JPH3* protein present a very mild phenotype, whereas cognitive and motor deficiencies were only present when both *JPH3* and *JPH4* genes were knocked out simultaneously, suggesting a more complex pathogenesis of HDL2 disease than the loss of *JPH3* function (Table 2) [59]. A recently developed bacterial artificial chromosome-transgenic mouse model for HDL2 [60] shows age-dependent motor deficiency

and a pathological HDL2 neuronal phenotype, but no data are reported on RBC features (Table 2).

## PANTOTHENATE KINASE-ASSOCIATED NEURODEGENERATION

Acanthocytes are also found in approximately 10% of the pantothenate kinase-associated neurodegeneration (PKAN) patients [35]. Data from preliminary proteomic experiments indicate a PKAN RBC membrane protein composition different from both control and other neuroacanthocytosis RBCs (Bosman *et al.*, unpublished data). A recent study has shown reduced response of acanthocytic PKAN RBCs but not of normal shaped PKAN RBCs to drug-induced endovesicle formation [25<sup>\*\*\*</sup>]. We know of no other detailed studies on RBCs from PKAN patients. PKAN is an autosomal recessive disease caused by mutations in the human *PANK2* gene on chromosome 20p13 encoding for the pantothenate kinase 2 protein isoforms, which are localized in mitochondria [36–38,85]. *PANK2* is a key enzyme in the biosynthesis of coenzyme A (CoA), which is important for energy metabolism, and fatty acid and neurotransmitter metabolism. A founder mutation effect has been described in the Netherlands [39]. The typical onset of PKAN is in childhood. A specific pattern of brain iron accumulation in the globus pallidus results in the eye-of-the-tiger pattern on magnetic resonance imaging [35,36].

Down-regulation of *PANK2* expression *in vitro* in different cell lines resulted in reduced cell growth related to iron deficiency without mitochondrial iron deposition, associated with a significant increase in ferroportin [86]. As ferroportin is involved in iron homeostasis through the hepcidin–ferroportin pathway and might be also important in regulation of brain iron levels, ferroportin has been suggested to play a role in the altered iron transport to brain observed in PKAN patients [86]. Mice genetically lacking *PANK2* (*pank2*<sup>-/-</sup>) show retinal degeneration, mitochondrial neuronal dysfunction and male infertility, but no dystonia and only minor alterations in the basal ganglia (Table 2) [61,62<sup>\*</sup>]. Normal mice treated with either pantothenate kinase inhibitor or on a diet without pantothenic acid develop movement disorders and male infertility but show no iron accumulation in the basal ganglia (Table 2) [63,64]. Thus, to date, mouse models seem to be of limited used in the study of PANK. However, three human mutations of *PANK2* have been expressed in the *fb1* gene in *Drosophila melanogaster*, corresponding to the human *PANK2* gene. In these flies, the decrease in pantothenate kinase activity correlated with the severity of the phenotype (Table 2). This represents an interesting model to study PKAN, even if the

observed neurodegeneration was not associated with neuronal iron accumulation [65,66].

## CONCLUSION

The significance of the combination of neuronal degeneration in the basal ganglia with the formation of acanthocytes in patients with various neuroacanthocytosis syndromes is still far from being understood. Recent proteomic and functional data on RBC from ChAc and MLS patients confirm previous evidence on the central role of band 3 in acanthocyte formation, and expand the acanthocytosis phenomenon toward protein–protein interactions, controlled by phosphorylation-related signaling [34]. This opens new diagnostic possibilities, and suggests that, in principle, signaling-based intervention is possible. At present, the clinical approach to the neuroacanthocytosis syndromes is essentially symptomatic with a combination of medical therapy to reduce involuntary movements and/or neurosurgery with deep brain stimulation or ablative procedures, although the latter remains to be validated in a large cohort of patients [87]. In view of the specific and characteristic association of acanthocytosis with neurodegeneration, RBCs constitute a promising target for future mechanistic and functional studies.

## Acknowledgements

*The work of L.D.F. and G.B. described in this manuscript is supported by the Advocacy for Neuroacanthocytosis Patients (John Grooms Working with Disabled people), Carl H. and Elizabeth S. Pforzheimer III (New York), Ginger and Glenn Irvine (London) and Telethon grant (GPP07007 and GPP13005, LDF). We thank Dr Ruth H. Walker and Prof. Adrian Danek for revision of the manuscript on neurological aspects of NA and Dr Carlo Tomelleri for the images on normal and ChAc RBCs.*

## Conflicts of interest

*There are no conflicts of interest.*

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Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

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