Recent progress in the diagnosis of RBC membrane and enzyme defects

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Hereditary red cell membrane disorders and defects of RBC metabolism are heterogeneous groups of diseases characterized by hemolytic anemia of variable degree, jaundice and splenomegaly. The diagnosis of these disorders is the final step of a diagnostic workout based not only on laboratory tests but also on clinical examination, personal family history, and the exclusion of other causes of hemolysis. However, given the rarity and the wide clinical heterogeneity, the diagnosis of these defects can be difficult, in particular in mild and atypical forms.

Red cell membrane disorders. The red cell cytoskeleton is a very complex system consisting of multiple integrated proteins that provides the erythrocyte with its shape and deformability. As a consequence, a defect of a single protein may impair the structural and functional integrity of the whole system and result in an alteration of red cell shape. In general, abnormalities of spectrin, ankyrin, protein 4.2 and protein band 3, weaken the cohesion between cytoskeleton and the lipid bilayer, leading to the release of microvesicles and progressive transformation of the discocyte into a spherocyte. This typically occurs in hereditary spherocytosis (HS). Abnormalities due either to defective spectrin dimer-dimer interaction or defective spectrin-actin-protein 4.1 complex, results in hereditary elliptocytosis (HE). Mutations responsible for HS are mainly localized in the genes coding for RBC membrane proteins SPTA1 (α-spectrin), ANK1 (ankyrin), SLC4A1 (band 3), EPB42 (protein 4.2); mutations in SPTA1 (α-spectrin), SPTB (β- spectrin), EPB41 (protein 4.1) and GYP C (glycophorin C) genes are responsible for HE. If cytoskeleton weakening is excessive, red blood cells can undergo severe deformations, mimicking red cell fragmentation due to exposure to heat resulting in hereditary pyropoichilocytosis (HPP) (Mohandas and Gallagher 2008, Perrotta et al 2008).

The laboratory hallmarks are the presence of specific red cell abnormalities at blood smear examination, although not specific, such as spherocytes and ovalo/elliptocytes.

In HS, spherocytes are detectable in 97% of HS patients; however, they may be very few in some patients (Mariani et al. 2009). The laboratory diagnosis of HS therefore commonly relies on indirect tests that exploit the surface area-to-volume ratio, typically reduced in spherocytes i.e. NaCl osmotic fragility tests, Glycerol Lysis and Acidified Glycerol Lysis tests, and Pink test. These methods miss a variable portion of HS cases, particularly the mildest ones, and do not differentiate HS from secondary spherocytosis. The direct flow cytometric EMA-binding test have been proposed (King et al, 2000), showing high sensitivity and specificity. By the analysis of a series of 150 HS patients it has been observed that the association of EMA-binding and AGLT allowed to identify all the examined patients and therefore may represent an effective diagnostic tool for HS also in mild/compensated cases (Bianchi et al., 2012).
In severe/atypical HS, the diagnostic workout may be more complex requiring SDS-PAGE analysis of RBC membrane proteins; spectrin functional analysis or tryptic digestion of spectrin may be helpful in HE cases. SDS-PAGE analysis is also required in differential diagnosis of CDAII. Recently, the use of ektacytometry and Laser-assisted Optical Rotational Cell Analyzer (LoRRca), useful in the diagnosis of hereditary stomatocytosis, have been proposed as a laboratory method to detect red cell membrane abnormalities (Da Costa, 2013). Finally, the recent advent of Next Generation Sequencing (NGS) will make more feasible molecular analysis in these disorders, particularly in atypical cases.

A recent survey by the European Network for Rare Red Cell Anemia (ENERCA, www.enerca.org) aimed at understanding which laboratory tests were used for the diagnosis of RBCs membrane defects in 26 European reference Centres showed that the most frequently adopted tests were EMA binding (60% of centres), followed by NaCl curve on fresh blood and AGLT (50%); cryohemolysis test was used in less than 20% of Centres, whereas ektacytometry, SDS-PAGE and molecular analysis were performed only in selected atypical cases. It is worth of noting that there was a wide heterogeneity of opinions about the methods with the best specificity and sensitivity, and that the majority of Centres admitted to rely on a combination of tests, greatly variable form Centre to Centre, rather than on a single method.

**Defect of red cell metabolism.** Hereditary hemolytic anemia also occurs as a consequence of defect of erythocyte metabolism. Three main pathways are active in RBCs: the glycolysis, involved in the conversion of glucose into pyruvate and lactate with production of ATP, NADH and 2,3 DPG; the nucleotide metabolism that act in the final part of glycolysis, and the exose-monophosphate shunt that generates NADPH to keep glutathione in the reduced state and to protect red cells from oxidative damage. Metabolic energy is used to maintain red cell shape, to keep the iron of hemoglobin in the divalent form, to pump ions against electrochemical gradients and to keep sulhydryl groups of red cell enzymes, hemoglobin, and membranes in the active, reduced forms.

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common erythrocyte enzyme defect usually associated with acute hemolysis occurring during oxidative stress (as infections or with administration of “oxidative” drugs and foods).

The most common cause of chronic hemolytic anemia is pyruvate kinase (PK) deficiency followed by glucosephosphate isomerase (GPI), pyrimidine 5’-nucleotidase (P5’N) deficiency and triosephosphate isomerase (TPI). When the gene involved in the enzyme defects is expressed not only in the hemopoietic tissue, the deficiency may lead to non-hematological signs such as myopathy and neuromuscular abnormalities (as in the case of triosephosphate isomerase, aldolase, and phosphoglycerate kinase deficiency).

The diagnosis relies on the determination of red cell enzyme activity by quantitative assays or screening tests. Except for the basophilic stippling of erythrocytes which is characteristic for pyrimidine 5’ nucleotidase deficiency, red cell morphology is unusually unremarkable. Molecular testing for RBC enzyme defects is helpful to confirm the diagnosis.
A recent survey by ENERCA showed that, with the exception of PK and G6PD assays, only a limited number of laboratories in the EU perform biochemical and genetic tests required to diagnose rarer red cell enzyme disorders. The number is even much smaller considering the laboratories which offer a complete panel of tests.