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Diagnostic tool for red blood cell membrane disorders: Assessment of a new generation ektacytometer☆

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ABSTRACT

Inherited red blood cell (RBC) membrane disorders, such as hereditary spherocytosis, elliptocytosis and hereditary ovalocytosis, result from mutations in genes encoding various RBC membrane and skeletal proteins. The RBC membrane, a composite structure composed of a lipid bilayer linked to a spectrin/actin-based membrane skeleton, confers upon the RBC unique features of deformability and mechanical stability. The disease severity is primarily dependent on the extent of membrane surface area loss. RBC membrane disorders can be readily diagnosed by various laboratory approaches that include RBC cytology, flow cytometry, ektacytometry, electrophoresis of RBC membrane proteins and genetics. The reference technique for diagnosis of RBC membrane disorders is the osmotic gradient ektacytometry. However, in spite of its recognition as the reference technique, this technique is rarely used as a routine diagnosis tool for RBC membrane disorders due to its limited availability. This may soon change as a new generation of ektacytometer has been recently engineered. In this review, we describe the workflow of the samples shipped to our Hematology laboratory for RBC membrane disorder analysis and the data obtained for a large cohort of French patients presenting with RBC membrane disorders using a newly available version of the ektacytomer.

Abbreviations: AIHA, auto-immune hemolytic anemia; CBC, complete blood count; CDA, congenital dyserythropoietic anemia; CHCM, Cell Hemoglobin Concentration Mean; CV, coefficient of variation; DAT, direct agglutination test; DHSt, dehydrated hereditary stomatocytosis (xerocytosis); DI, deformability index; EDTA, ethylenediamine tetraacetic acid; EI, elongation index; EMA, eosin-5′ maleimide; G6PD, glucose-6 phosphate dehydrogenase; GPI, glucose 6 phosphate isomerase; Hb, hemoglobin; HE, hereditary elliptocytosis; HPP, hereditary pyropoikilocytosis; HS, hereditary spherocytosis; Hst, hereditary stomatocytosis; LDH, lactate dehydrogenase; MCV, mean corpuscular volume; MFI, mean fluorescence intensity; MGG, May Grümwald Grünwald; MHC, mean hemoglobin content; OHS, overhydrated hereditary stomatocytosis; PBS, phosphate-buffered saline; PK, pyruvate kinase; PVP, polyvinylpyrrolidone; RBC, red blood cell; RDW, red blood cell distribution width; SAO, South-east Asian Ovalocytosis; SD, standard deviation.

☆ Author contributions: LS and TP collected the data. LS, JG, LDC performed the statistical tests of the ROC analysis. JG performed the ektacytometry analysis of the samples, the validation of the ektacytometry method, and analyzed the ektacytometry data. OF and LDC analyzed RBC morphology in blood smears. AB performed the flow cytometry analysis of EMA-labeled RBCs. NC carried out the Hemoglobin electrophoresis, enzymatic activity measurements and G6PD gene mutation screening. LDC designed the study, analyzed RBC morphology in blood smears with OF, analyzed the EMA-tests and the ektacytometry results, validated the final diagnosis with OF, LS, NC and pediatricians and hematologists from the SFH and the SHIP, who sent us patient samples. LDC and NM wrote the article.

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1. Introduction

The red blood cell (RBC) membrane is not a static structure but is highly dynamic, enabling it to undergo the extensive deformations necessary for traversing the vascular bed to perform its main function of oxygen delivery. The complex structural organization of the various RBC membrane components is responsible for its unique features of extensive deformability and mechanical stability [1–4]. The RBC membrane is composed of two important complexes, the ankyrin and the 4.1R complexes, which link various proteins embedded in the phospholipid bilayer to the α/β spectrin heterodimers underlying the lipid bilayer [1,3,5]. Alterations in the structural membrane organization due to various protein defects are responsible for a large panel of human disorders either constitutional or acquired. Inherited RBC membrane disorders, such as hereditary spherocytosis (HS), hereditary elliptocytosis (HE) and its severe form known as hereditary pyropoikilocytosis (HPP), hereditary ovalocytosis (SAO), and hereditary stomatocytosis (HSt) are commonly responsible for hemolytic anemia. The severity of the disease is variable and depends on the extent of surface area loss, ranging from asymptomatic forms to severe neonatal or prenatal forms responsible for rare hydrops fetalis cases requiring transfusion in utero.

HS and HE [1,6–10] are the most common RBC membrane disorders worldwide with a prevalence of 1 out of 2000 cases in North America and Northern European countries. HS has been linked to defects in the ankyrin (*ANK1*), α-spectrin (*SPTA1*), β-spectrin (*SPTB*), band 3 (*SLC4A1*) or protein *4.2* (*EPB42*) genes [11–15] and HE to defects in the *SPTA1*, *SPTB* or protein *4.1R* genes [9,16–21]. In both HS and HE, RBC life span is shortened as a result of splenic sequestration of RBCs with increased sphericity. The abnormal RBCs with decreased membrane surface area and increased sphericity are trapped in the billroth canals in the spleen and phagocytozed by the splenic reticulo-endothelial system [22,23], resulting in regenerative hemolytic anemia, splenomegaly, and ictera with increased free bilirubin level. Hereditary ovalocytosis, also designated as South-east Asian Ovalocytosis (SAO), has a geographical distribution mostly in Indonesia, the Philippines, Melanesia and Southern Thailand [24–26]. The molecular defect responsible for SAO has been identified as a deletion of the 27 nucleotides encoding amino acids 400 to 408 in the *SLC4A1* gene [27,28]. Hereditary stomatocytosis is a rare RBC disorder divided into two different entities: xerocytosis or dehydrated hereditary stomatocytosis (DHSt) and overhydrated hereditary stomatocytosis (OHS) [6,7,9,29,30]. In both cases, RBCs exhibit a leak to the univalent cations Na^+ and K^+, resulting in altered intracellular cation content and cell volume alterations. Although *PIEZO1* and *RhAG* gene mutations have been identified in xerocytosis [31–33] and the overhydrated form [34], respectively, the molecular basis for both forms of hereditary stomatocytosis is still under investigation.

The RBC membrane disorders described above can be usually diagnosed easily, sometimes even without any specialized laboratory tests besides a meticulous cytologic microscopic examination and measurement of RBC and reticulocyte indices. In 2011, Bolton-Maggs et al. [35] defined guidelines for the diagnosis and the management of hereditary spherocytosis. Indeed, patients with a family history of HS and typical HS biological manifestations (hemolytic anemia with high Cell Hemoglobin Concentration Mean (CHCM) >36 g/dl on Siemens/Advia hematological analyzers, high percentage (>4%) of hyperdense cells (gated RBCs with a CHCM >41 g/dL) and the presence of spherocytic cells in blood smears) do not require any additional test (grade 1 recommendation, grade A evidence). However, more specific biological tests may be required in cases where the HS diagnosis is not readily evident including: lack of HS family history, lack of typical biological manifestation (in particular normal osmotic fragility and iron deficiency, which may mask the regeneration, the increased CHCM, and the increased reticulocyte count) or severe forms of elliptocytosis and stomatocytosis, in which the diagnosis may be difficult in particular in the dehydrated form. It is critical to accurately diagnose hereditary stomatocytosis in order to exclude splenectomy as part of patient treatment because, for reasons that are still unclear, splenectomy in the case of stomatocytosis leads to lethal thrombosis. Various specialized laboratory tests are available to clinicians to help them to select for the most pertinent ones based upon sensitivity and specificity. Flow cytometry measurement of the mean RBC fluorescence, after labeling of RBCs with the dye eosin-5′ maleimide (EMA), is often used to document surface area loss and is a test of choice for screening of HS [35–45]. This test is able to detect HS with a sensitivity of 92.7% and a specificity of 99.1%, with a positive predictive value of 97.8% and a negative predictive value of 96.9%. However, this EMA-based test fails to identify some HS cases associated with ankyrin defects and is not as reliable for other RBC membrane disorders such as elliptocytosis, pyropoikilocytosis, stomatocytosis and SAO. Osmotic gradient ektacytometry fills this gap and has therefore been considered a reference technique and the diagnostic test for HS and the other red cell membrane disorders. However, until now, the use of ektacytometry has not been widespread in the routine hematology laboratory due to the limited availability of the ektacytometer, originally designed in the seventies. Ektacytometry has not even been evaluated in the guidelines published in 2011. Recently, a new generation ektacytometer, the Osmoscan LoRRca MaxSis, has been engineered by Mechatronics Instruments BV® (Zwaag, The Netherlands), which measures RBC deformability under a defined shear stress as a function of suspending medium osmolality. The differences between this new generation laser diffraction viscometer and the previous ektacytometer (Technicon®) reside mostly in the analysis of the diffraction images acquired: while the Technicon model measures the extent of light scattering by a photodiode to define ellipticity of the diffraction images, the LoRRca MaxSis scans images of acquired diffraction patterns using a digital camera and analyzes the images with the gray nuance from 0 to 128 of 256,000 pixels. In both systems, a value for ellipticity of the diffraction images, a measure of cellular deformability defined as the deformability index (or IE) is generated. The 3 key features of the osmotic gradient ektacytometer profile (Omin, DImax and Hyper or O′) can be calculated and analyzed. In this review, we describe the results of the analysis of RBC deformability features in patients presenting with various RBC membrane disorders and other diseases obtained over a period of 20 months in our hematology laboratory using this new generation ektacytometer and usual diagnostic tests.

2. Methods

2.1. Characteristics of the population studied for red blood cell membrane disorders

A total of 321 patients have been referred to our hematology clinical diagnosis laboratory, at the Robert Debré hospital in Paris (France), for RBC membrane disorder diagnosis during this 20-month study. Some of them have been diagnosed before and have been sent to us for the purpose of the study. The vast majority of the patients have been studied in the regular work-flow of the laboratory. The population studied included 166 male patients and 155 female patients. As expected, the population pyramid was in favor of infants with 220 out of 321 samples obtained from patients under 16 years of age, 185 of which were from individuals less than 10 years old (Fig. 1A–B). In most cases, the underlying pathologies were diagnosed between birth and 10 years of age, with only 35 patients being diagnosed between 10 and 16 years of age. Of particular interest, 3 blood cord samples have been successfully studied leading to an early diagnosis of HS in one of these cases. This study was carried out in accordance with the Declaration of Helsinki.

2.2. Work-flow of a laboratory specialized in diagnosis of red blood cell membrane disorders

2.2.1. RBC indices, reticulocyte count and biochemical analysis

RBC indices including hemoglobin concentration, hematocrit, mean cell volume (MCV), mean corpuscular hemoglobin concentration