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## Advanced methods in diagnosis and translational research of red blood cell disorders

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Diagnostics and research on benign disorders of the red blood cell have always been an important focus of the Department of Clinical Chemistry and Haematology of the University Medical Center Utrecht (UMCU). Both diagnostic and research activities are performed in close collaboration with the Department of (pediatric) Hematology/Oncology of the UMCU and other (inter)national hospitals. In this brief review, an update will be provided with regard to the current status of diagnostic developments, as well as to approaches aimed at a better understanding of the pathophysiology of red blood cell disorders, in particular those related to hereditary hemolytic anemia.

### The red blood cell and hereditary hemolytic anemia

The moment the mature red blood cell (RBC) leaves the bone marrow, it is optimally adapted to perform the binding and transport of oxygen, and its delivery to all tissues. This is the most important task of the erythrocyte during its estimated 120-day journey in the blood stream. The membrane, hemoglobin, and proteins involved in metabolic pathways of the RBC interact to modulate oxygen transport, protect hemoglobin from oxidant-induced damage, and maintain

the osmotic environment of the cell. The biconcave shape of the RBC provides an optimal area for respiratory exchange. The latter requires passage through microcapillaries, which is achieved by a drastic modification of its biconcave shape, made possible only by the loss of the nucleus and cytoplasmic organelles and, consequently, the ability to synthesize proteins. Hence, disturbances of proteins that constitute the RBC membrane, the red cell's major protein (hemoglobin), or of proteins involved in red cell metabolism may ultimately result in decreased RBC survival: hemolysis.

### Advanced diagnostics for hereditary hemolytic anemia – enzymopathies and membrane disorders

RBC enzyme and membrane disorders constitute 2 of the major causes of hereditary hemolytic anemia. Nevertheless, they are rare diseases. The diagnosis of a RBC enzymopathy due to disturbed metabolism basically relies on enzyme activity measurements of a variety of enzymes, deficiencies of which are known to have a clear causal relationship with a hematological phenotype (1). We are currently equipped to perform extensive biochemical analysis of these clinically relevant enzymes in adults and, notably, in children (as children's RBCs are quite different from adult). In most cases of deficient enzymatic function we perform DNA sequence analysis of the respective gene. In this way, the diagnosis of a red blood cell enzymopathy is confirmed on both the biochemical and molecular level, thereby strengthening the correct diagnosis of

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this phenotypically diverse disorder. In addition, molecular analysis enables the establishment of a genotype-to-phenotype correlation, which is in particular poorly understood for red cell enzymopathies (see below).

Diagnostic possibilities to confirm a suspected RBC membrane disorder are in general quite limited. Measuring the RBC's deformability and potential to withstand osmotic stress under shear conditions are important techniques by which RBC disorders can be recognized and potentially differentiated from one another (2). We therefore recently set up such analyses by using the Laser-assisted Optical Rotational Cell Analyzer (LORRCA). Also in case of RBC membrane disorders molecular analysis is often warranted, in particular when (genetic) counseling is involved. The genes encoding red blood cell (trans)membrane and cytoskeletal proteins however are large and complex, thereby hampering efficient mutation detection by conventional DNA sequencing methods. Therefore we are currently setting up mutation detection analysis of the major RBC membrane and cytoskeletal genes using chip-based DNA technologies. This approach is expected to render substantial value to the diagnostic repertoire of RBC membrane disorders, as recently shown for the RBC transmembrane protein band 3 (3). As an Expert Center, and member of the Executive Committee of the European Network for Rare and Congenital Anaemias (ENERCA) the UMC Utrecht is closely involved in harmonization of procedures involved in diagnosis and treatment, education and training, developing of research activities, and epidemiological surveillance of rare and very rare anemias (4).

#### **Genotype-to-phenotype correlation in red blood cell enzymopathies – translational research**

The clinical hallmark of patients with red cell enzymopathies is chronic hemolysis. The underlying mechanisms by which these deficiencies cause hemolysis, however, are quite distinct (1). Therefore, the identification of the molecular mechanisms by which inherited enzymopathies lead to impaired enzyme function constitutes the primary basis of the associated hemolytic disease. In specific cases we therefore study the molecular defect underlying the above-mentioned enzyme deficiencies as this provides insight into the diverse clinical expression of RBC enzymopathies. Such studies have contributed to our knowledge regarding erythroid-specific transcriptional regulation of pyruvate kinase (PK) (5) and hexokinase (6, 7), the key role of hexokinase in glycolysis (8), as well as the molecular background of severe and unusual clinical presentations of glucose-6-phosphate dehydrogenase deficiency (9, 10). The most important part of this particular line of research however has always been aimed at elucidating the complex genotype-to-phenotype correlation in PK deficiency. For this a public available mutation database has been constructed ([www.pk1mutationdatabase.com](http://www.pk1mutationdatabase.com)). Many mutations have been studied at the molecular level and their effect has been correlated to the associated hemolytic phenotype (5, 11-17).

#### **Vascular complications of hemolytic anemia**

Apart from the hemolytic phenotype, hypercoagulability is a hallmark of various forms of hereditary hemolytic anemia, in particular sickle cell anemia and thalassemia (18). However, vascular complications have also been reported to occur in other, more rare causes of hemolytic anemia such as spherocytosis and pyruvate kinase deficiency (19) especially after splenectomy (20). The pathophysiology involved may be directly related to our recently described observations that vascular endothelial cells are capable of lactadherin-dependent phagocytosis of RBCs from the circulation via the phosphatidylserine-lactadherin- $\alpha_v$ -integrin pathway (21-23). Research activities to investigate this further are currently in process.

#### **Identification of yet-unknown causes of hereditary hemolytic anemia by proteomics and whole exome sequencing**

Many cases of hereditary hemolytic anemia remain undiagnosed, even after extensive biochemical and genetic analysis. This implies that many causes of hereditary hemolytic anemia remain to be identified. Therefore, we have recently initiated the analysis of a selected number of undiagnosed families on both the protein and molecular level by high-throughput techniques such as proteomics (24) and whole exome sequencing. The latter approach has recently proved to be able to identify the specific genetic defect in a number of disorders (e.g. (25)), including one of our cases of idiopathic hemolytic anemia (26). Similarly, we have validated the use of proteomics in the identification of RBC membrane disorders by quantitatively analyzing the RBC's membrane proteome (27). This work has expanded into the analysis of the RBC's cytoplasmic pool of proteins. In collaboration with the Netherlands Proteomics Center we have developed a highly efficient depletion strategy for the two most abundant RBC proteins (hemoglobin and carbonic anhydrase). This dramatically improves proteome coverage of the cytoplasmic pool of proteins (28) and, thus, strongly enhances the identification of new causes of hereditary hemolytic anemia.

#### **Increased red blood cell production by disturbed oxygen sensing**

The idiopathic erythrocytosis group of disorders is defined by an absolute increase in red cell mass and hematocrit without elevation of the megakaryocytic or granulocytic lineages. This rare hereditary disorder is associated with a wide range of serum erythropoietin (epo) levels and broadly falls into groups of raised/inappropriately normal or low/undetectable Epo levels. Recently, a spectrum of molecular defects has been described in association with idiopathic erythrocytosis, which reflects the heterogeneity of this disorder. These defects often result in aberrant oxygen sensing and dysregulated Epo production (29). Our work has recently expanded into this area which is of strong interest by both the clinical and scientific community. In three patients with unexplained erythrocytosis, novel mutations were identified in the genes encoding VHL, PHD2, and HIF2a (30, 31). The novel mutants

were functionally characterized by binding assays, hydroxylase assays, real-time PCR, and Western blotting of recombinant wild-type and mutant proteins. Our results contribute to the current understanding of proteins implicated in the pathway that senses oxygen and transmits it to signals that eventually propagate erythroid progenitors.

### Dissemination

Many of our diagnostic and research activities and results are communicated to colleagues and to the public through a yearly UMC Utrecht Red Blood Cell Seminar. The next edition will be organised on april 5, 2012.

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